

(51) **International Patent Classification:**
C07K 14/82 (2006.01) A61K 39/395 (2006.01)
C07K 16/32 (2006.01) A61P 35/00 (2006.01)

(21) **International Application Number:**
PCT/US2020/065617

(22) **International Filing Date:**
17 December 2020 (17.12.2020)

(25) **Filing Language:** English

(26) **Publication Language:** English

(30) **Priority Data:**
62/949,220 17 December 2019 (17.12.2019) US
63/059,638 31 July 2020 (31.07.2020) US

(71) **Applicant: THE JOHNS HOPKINS UNIVERSITY**
[US/US]; 3400 North Charles Street, Baltimore, Maryland 21218 (US).

NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— with international search report (Art. 21(3))

(72) **Inventors; and**

(71) **Applicants: VOGELSTEIN, Bert** [US/US]; 3700 Breton Way, Baltimore, Maryland 21208 (US). **KINZLER, Kenneth W.** [US/US]; 616 Ponte Villas North, Baltimore, Maryland 21230 (US). **HSIUE, Emily Han-Chung** [US/US]; 929 N Wolfe St, Apt 1101, Baltimore, Maryland 21205 (US). **DOUGLASS, Jacqueline** [US/US]; 3400 N. Charles Street, Baltimore, Maryland 21218 (US). **HWANG, Michael S.** [US/US]; 2542 39th Avenue East, Seattle, Washington 98112 (US). **PEARLMAN, Alexander** [US/US]; 3400 N. Charles Street, Baltimore, Maryland 21218 (US). **PAPADOPOULOS, Nickolas** [US/US]; 606 Horncrest, Towson, Maryland 21204 (US). **ZHOU, Shibin** [US/US]; 820 Crystal Palace Court, Owings Mills, Maryland 21117 (US). **MOG, Brian** [US/US]; 3400 North Charles Street, Baltimore, Maryland 21218 (US). **WRIGHT, Katharine M.** [US/US]; 611 South Charles Street, Unit 112, Baltimore, Maryland 21230 (US). **GABELLI, Sandra B.** [US/US]; 2933 Excelsior Springs Court, Ellicott City, Maryland 21042 (US).

(74) **Agent: WILLIS, Margaret S. et al.**; Fish & Richardson P.C., P.O. Box 1022, Minneapolis, Minnesota 55440-1022 (US).

(81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,

(54) **Title:** MANAbodies TARGETING TUMOR ANTIGENS AND METHODS OF USING

(57) **Abstract:** This document provides methods and materials for assessing a mammal having or suspected of having cancer and/or for treating a mammal having cancer. For example, molecules including one or more antigen-binding domains (e.g., a single-chain variable fragment (scFv)) that can bind to a modified peptide (e.g., a tumor antigen), as well as method for using such molecules, are provided.

WO 2021/127184 A1

MANAbodies TARGETING TUMOR ANTIGENS AND METHODS OF USING**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims the benefit of U.S. Patent Application Serial No. 62/949,220, filed on December 17, 2019 and U.S. Patent Application Serial No. 63/059,638, filed on July 31, 2020. The disclosure of the prior applications are considered part of (and are incorporated by reference in) the disclosure of this application.

STATEMENT REGARDING FEDERAL FUNDING

This invention was made with government support under grant CA062924 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND*1. Technical Field*

This document relates to methods and materials for assessing a mammal having or suspected of having cancer and/or for treating a mammal having cancer. For example, this document provides methods and materials for using a molecule including one or more antigen-binding domains (*e.g.*, a single-chain variable fragment (scFv)) that can bind to a modified peptide (*e.g.*, a tumor antigen) to treat a mammal having a cancer.

2. Background Information

Somatic mutations in cancer are ideal targets for cancer therapy as they are expressed only in tumor cells and not in normal cells. Targeting driver gene proteins (broadly subdivided into oncogene proteins and tumor suppressor proteins) have added benefits. First, these mutations typically occur early during the development of the tumor, thus essentially all daughter cancer cells will contain the mutation. Second, the tumor's dependence on their oncogenic-endowing capacity makes resistance less likely. Finally, driver gene proteins tend to have hotspot mutations shared among many patients, thus a therapy targeting a single mutation could be applied to a broad patient population.

Most mutant proteins, including most mutant driver gene proteins, are intracellular. While small molecules can target intracellular proteins, developing small molecules that can specifically inhibit the activity of a mutant driver gene and not its wild-type (WT)

counterpart has remained out of reach for the majority of such driver gene proteins. Antibodies, which can have the capacity to distinguish a single amino acid mutation, can typically only target extracellular epitopes.

The immune system samples the intracellular contents of cells through antigen
5 processing and presentation. Following protein proteolysis, a fraction of the resulting peptides are loaded onto a human leukocyte antigen (HLA) and sent to the cell surface where they serve as a way for T cells, via their T cell receptor (TCR), to distinguish self from non-self peptides. For example, a virally-infected cell will present viral peptides in its HLA, triggering T cells to kill that cell. Similarly, in cancer, mutant peptides can be presented in an
10 HLA on the cancer cell surface, referred to as MANAs, for **M**utation-**A**ssociated **N**eo-**A**ntigens. In some cases, and to varying degrees, patients may mount an anti-cancer T cell response against these mutant-peptide-HLA neoantigens, and checkpoint blockade antibodies can further augment this response. However, many patients, particularly those with a low mutational burden, cannot mount a sufficient anti-cancer T cell response. A therapy or
15 diagnostic specifically targeting MANAs could therefore provide a truly tumor-specific method to diagnose or treat cancer.

HLA class I proteins are present on all nucleated cells. There are three classical HLA class I genes, A, B, and C, each of which are highly polymorphic. Each HLA allele has a particular peptide-binding motif, and as a result, only certain peptides will bind to certain
20 HLA alleles.

There is a continuing need in the art to develop new methods to diagnose, monitor, and effectively treat cancers.

SUMMARY

Identification of therapeutic targets highly specific to cancer cells is one of the
25 greatest challenges for developing an effective cancer therapy.

This document provides methods and materials for treating a mammal having cancer. For example, this document provides methods and materials for using one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide (*e.g.*, a modified peptide present in a peptide-HLA- beta-2 microglobulin (b2M or
30 β 2M) complex) to treat a mammal having a cancer (*e.g.*, a cancer expressing the modified peptide). In some cases, one or more molecules including one or more antigen-binding

domains (*e.g.*, scFvs) that can bind to a modified peptide (*e.g.*, a modified peptide present in a peptide-HLA- β 2M complex) can be administered to a mammal having a cancer (*e.g.*, a cancer expressing the modified peptide) to treat the mammal.

As demonstrated herein, scFvs were identified that target (*e.g.*, bind to) numerous
5 MANAs present in HLA-restricted MANAs derived from common cancer driver mutations, including RAS Q61H/L/R and p53 R175H. Also as demonstrated herein, the scFvs were used to design bispecific antibodies capable of inducing MANA-dependent T cell activation that can lead to recognition and killing of cells (*e.g.*, cancer cells) expressing MANAs.

MANAs can be used as highly specific cancer targets because they are not present in
10 normal tissue(s). The ability to specifically target MANAs provides a tumor-specific method to diagnose and/or treat cancer. For example, scFvs specifically targeting MANAs can be used in full-length antibodies or fragments thereof, antibody drug conjugates (ADCs), antibody radionuclide conjugates, T cells expressing a chimeric antigen receptor (CARTs), or bispecific antibodies to diagnose and/or treat a mammal having cancer. Further, an antibody
15 that can bind to a MANA (a MANAbody), or a fragment thereof capable of binding to a MANA, have the potential of becoming widely applicable and genetically predictable off-the-shelf targeted cancer immunotherapy.

In general, one aspect of this document features molecules having an antigen-binding domain that can bind to a peptide-HLA- β 2M complex, where the peptide can be derived
20 from a modified p53 polypeptide. The modified p53 polypeptide can include from 7 amino acids to 25 amino acids (*e.g.*, the modified p53 polypeptide can include 9 amino acids). The modified p53 polypeptide can include the amino acid sequence set forth in SEQ ID NO:1. The antigen binding domain can include an amino acid sequence set forth in any one of SEQ ID NO:137, SEQ ID NO:138, SEQ ID NO:139, SEQ ID NO:140, and SEQ ID NO:141. The
25 molecule can be any one of an antibody, an antibody fragment, a single chain variable fragment (scFv), a chimeric antigen receptor (CAR), a T cell receptor (TCR), a TCR mimic, a tandem scFv, a bispecific T cell engager, a diabody, a single-chain diabody (scDb), an scFv-Fc, a bispecific antibody, and a dual-affinity re-targeting antibody (DART). The molecule also can include an antigen-binding domain that can bind to an effector cell receptor selected
30 from any one of CD3, CD28, CD4, CD8, CD16a, NKG2D, PD-1, CTLA-4, 4-1BB, OX40, ICOS, CD27, and an Fc receptor. In some cases, the antigen-binding domain that can bind to an effector cell can bind to CD3, and the antigen-binding domain can include an amino acid

sequence set forth in any one of SEQ ID NO:170, SEQ ID NO:171, SEQ ID NO:172, SEQ ID NO:173, SEQ ID NO:174, SEQ ID NO:175, SEQ ID NO:176, SEQ ID NO:177, SEQ ID NO:178, SEQ ID NO:179, SEQ ID NO:180, SEQ ID NO:181, SEQ ID NO:182, and SEQ ID NO:183.

5 In another aspect, this document features molecules that have an antigen-binding domain that can bind to a peptide-HLA- β 2M complex, where the peptide can be derived from a modified RAS polypeptide. The modified RAS peptide can include from 7 amino acids to 25 amino acids (*e.g.*, the modified RAS peptide can include 10 amino acids). The modified RAS peptide can include an amino acid sequence set forth in any one of SEQ ID
10 NO:2, SEQ ID NO:3, and SEQ ID NO:4. In some cases, the modified RAS peptide can include the amino acid set forth in SEQ ID NO:2, and the antigen binding domain can include an amino acid sequence set forth in any one of SEQ ID NO:142, SEQ ID NO:143, SEQ ID NO:144, SEQ ID NO:145, SEQ ID NO:146, SEQ ID NO:147, SEQ ID NO:148, and SEQ ID NO:149. In some cases, the modified RAS peptide can include the amino acid
15 sequence set forth in SEQ ID NO:3, and the antigen binding domain can include an amino acid sequence set forth in any one of SEQ ID NO:150, SEQ ID NO:151, SEQ ID NO:152, SEQ ID NO:153, SEQ ID NO:154, SEQ ID NO:155, SEQ ID NO:156, SEQ ID NO:157, SEQ ID NO:158, SEQ ID NO:159, and SEQ ID NO:160. In some cases, the modified RAS peptide can include the amino acid sequence set forth in SEQ ID NO:4, and the antigen
20 binding domain can include an amino acid sequence set forth in any one of SEQ ID NO:161, SEQ ID NO:162, SEQ ID NO:163, SEQ ID NO:164, SEQ ID NO:165, SEQ ID NO:166, SEQ ID NO:167, SEQ ID NO:168, and SEQ ID NO:169. The molecule can be any one of an antibody, an antibody fragment, a scFv, a CAR, a TCR, a TCR mimic, a tandem scFv, a bispecific T cell engager, a diabody, a scDb, an scFv-Fc, a bispecific antibody, and a DART.
25 The molecule also can include an antigen-binding domain that can bind to an effector cell receptor selected from any one of CD3, CD28, CD4, CD8, CD16a, NKG2D, PD-1, CTLA-4, 4-1BB, OX40, ICOS, CD27, and an Fc receptor. In some cases, the antigen-binding domain that can bind to an effector cell can bind to CD3, and the antigen-binding domain can include an amino acid sequence set forth in any one of SEQ ID NO:170, SEQ ID NO:171, SEQ ID
30 NO:172, SEQ ID NO:173, SEQ ID NO:174, SEQ ID NO:175, SEQ ID NO:176, SEQ ID NO:177, SEQ ID NO:178, SEQ ID NO:179, SEQ ID NO:180, SEQ ID NO:181, SEQ ID NO:182, and SEQ ID NO:183.

In another aspect, this document features methods for treating a mammal having a cancer. The methods can include, or consist essentially of, administering to a mammal having cancer one or more molecules described herein (*e.g.*, a molecule having an antigen-binding domain that can bind to a peptide-HLA- β 2M complex, where the peptide can be derived from a modified p53 polypeptide or a modified RAS polypeptide), where the cancer includes cancer cells expressing the modified peptide. The mammal can be a human. The cancer can be any one of Hodgkin's lymphoma, non-Hodgkin's lymphoma, acute myeloid leukemia, acute lymphoblastic leukemia, multiple myeloma, a myelodysplastic syndrome (MDS), a myeloproliferative disease, lung cancer, pancreatic cancer, gastric cancer, colorectal cancer, ovarian cancer, endometrial cancer, biliary tract cancer, liver cancer, breast cancer, prostate cancer, esophageal cancer, stomach cancer, kidney cancer, bone cancer, soft tissue cancer, head and neck cancer, glioblastoma multiforme, astrocytoma, thyroid cancer, germ cell tumor, and melanoma.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF THE DRAWINGS

Figure 1 contains a graph showing flow cytometry on peptide-pulsed A2+ cells. T2 cells were peptide-pulsed overnight at 37°C in serum-free media with beta-2 microglobulin (β 2M) protein only, or β 2M with a p53 WT(168-176) peptide (HMTEVVRRC; SEQ ID NO:135), or p53 R175H(168-176) peptide (HMTEVVRHC; SEQ ID NO:1). Cells were stained with 50 μ L of phage supernatant per 50 μ L of cells, washed and stained with rabbit anti-M13 antibody, then washed and stained with anti-Rabbit-PE antibody. Cells were

stained with a live/dead Near-infrared (IR) dye, washed, and analyzed by an iQue Screener (IntelliCyt, Albuquerque, NM).

Figure 2 contains a graph showing flow cytometry on peptide-pulsed A1+ cells. SigM5 cells were peptide-pulsed overnight at 37°C in serum-free media with β 2M only, β 2M with the H/K/N RAS WT(55-64) peptide (ILDTAGQEEY; SEQ ID NO:136), β 2M with a H/K/N RAS mutant Q61H(55-64) peptide (ILDTAGHEEY; SEQ ID NO:2). Cells were stained with 50 μ L of phage supernatant per 50 μ L of cells, washed and stained with rabbit anti-M13 antibody, then washed and stained with anti-Rabbit-PE antibody. Cells were stained with a live/dead Near-IR dye, washed, and analyzed by an iQue Screener (IntelliCyt, Albuquerque, NM).

Figure 3 contains a graph showing flow cytometry on peptide-pulsed A1+ cells. SigM5 cells were peptide-pulsed overnight at 37°C in serum-free media with β 2M only, β 2M with the H/K/N RAS WT(55-64) peptide (ILDTAGQEEY; SEQ ID NO:136), β 2M with a H/K/N RAS mutant Q61L(55-64) peptide (ILDTAGLEEY; SEQ ID NO:3). Cells were stained with 50 μ L of phage supernatant per 50 μ L of cells, washed and stained with rabbit anti-M13 antibody, then washed and stained with anti-Rabbit-PE antibody. Cells were stained with a live/dead Near-IR dye, washed, and analyzed by an iQue Screener (IntelliCyt, Albuquerque, NM).

Figure 4 contains a graph showing flow cytometry on peptide-pulsed A1+ cells. SigM5 cells were peptide-pulsed overnight at 37°C in serum-free media with β 2M only, β 2M with the H/K/N RAS WT(55-64) peptide (ILDTAGQEEY; SEQ ID NO:136), β 2M with a H/K/N RAS mutant Q61R(55-64) peptide (ILDTAGREEY; SEQ ID NO:4). Cells were stained with 50 μ L of phage supernatant per 50 μ L of cells, washed and stained with rabbit anti-M13 antibody, then washed and stained with anti-Rabbit-PE antibody. Cells were stained with a live/dead Near-IR dye, washed, and analyzed by an iQue Screener (IntelliCyt, Albuquerque, NM).

Figure 5 contains graphs showing that scDBs can induce mutation-specific T-cell cytokine release. p53 R175H(168-176)-A2 cl.2, cl.6, cl.15, cl.16, and cl.20 scDBs, containing the anti-CD3 clone UCHT1, were incubated at the specified concentrations with T cells and COS-7 cells co-transfected with plasmids encoding various combinations of HLA-A2, p53(WT), p53(R175H), and GFP for 20 hours at 37°C. Following co-culture, conditioned media was collected and assayed for secreted IFN γ by ELISA.

Figure 6 contains graphs showing that scDBs can induce mutation-specific T-cell cytokine release. H/K/N RAS Q61H(55-64)-A1 cl.1, cl.2, and cl.4 scDBs, containing the anti-CD3 clone hUCHT1v9, were incubated at the specified concentrations with T cells and COS-7 cells co-transfected with plasmids encoding various combinations of HLA-A1,
 5 KRAS(WT), KRAS(Q61H), and GFP for 20 hours at 37°C. Following co-culture, conditioned media was collected and assayed for secreted IFN γ by ELISA.

Figure 7 contains graphs showing that scDBs can induce mutation-specific T-cell cytokine release. H/K/N RAS Q61L(55-64)-A1 cl.1, cl.2, cl.9, and cl.13 scDBs, containing the anti-CD3 clone hUCHT1v9, were incubated at the specified concentrations with T cells
 10 and COS-7 cells co-transfected with plasmids encoding various combinations of HLA-A1, KRAS(WT), KRAS(Q61L), and GFP for 20 hours at 37°C. Following co-culture, conditioned media was collected and assayed for secreted IFN γ by ELISA.

Figure 8 contains a graph showing scDb can induce mutation-specific T-cell cytokine release. H/K/N RAS Q61R(55-64)-A1 cl.6 scDb, containing the anti-CD3 clone hUCHT1v9,
 15 was incubated at the specified concentrations with T cells and COS-7 cells co-transfected with plasmids encoding various combinations of HLA-A1, KRAS(WT), KRAS(Q61R), and GFP for 20 hours at 37°C. Following co-culture, conditioned media was collected and assayed for secreted IFN γ by ELISA.

Figures 9A-9C contain graphs showing scDBs can react against Q61H, Q61L, and
 20 Q61R mutations in all 3 RAS isoforms, namely HRAS, KRAS, and NRAS. Fig. 9A H/K/N RAS mutant Q61H(55-64)-A1 cl.1 scDb was incubated at the specified concentrations with T cells and COS-7 cells co-transfected with plasmids encoding various combinations of HLA-A1, H/K/N RAS(WT), H/K/N RAS(Q61H), H/K/N RAS(Q61K), H/K/N RAS(Q61L), and H/K/N RAS(Q61R) and for 20 hours at 37°C. Following co-culture, conditioned media was
 25 collected and assayed for secreted IFN γ by ELISA. Fig. 9B H/K/N RAS mutant Q61L(55-64)-A1 cl.2 scDb was incubated at the specified concentrations with T cells and COS-7 cells co-transfected with plasmids encoding various combinations of HLA-A1, H/K/N RAS(WT), H/K/N RAS(Q61H), H/K/N RAS(Q61K), H/K/N RAS(Q61L), and H/K/N RAS(Q61R) and for 20 hours at 37°C. Following co-culture, conditioned media was collected and assayed for
 30 secreted IFN γ by ELISA. Fig. 9C H/K/N RAS mutant Q61R(55-64)-A1 cl.6 scDb was incubated at the specified concentrations with T cells and COS-7 cells co-transfected with plasmids encoding various combinations of HLA-A1, H/K/N RAS(WT), H/K/N

RAS(Q61H), H/K/N RAS(Q61K), H/K/N RAS(Q61L), and H/K/N RAS(Q61R) and for 20 hours at 37°C. Following co-culture, conditioned media was collected and assayed for secreted IFN γ by ELISA.

Figure 10 contains graphs showing scDb can induce mutation-specific T-cell cytokine release against tumor cell line. p53 R175H(168-176)-A2 cl.2 UCHT1 scDb was incubated at the specified concentrations with T cells and either with parental TYKnu or p53 knockout (KO) TYKnu for 20 hours at 37°C. Following co-culture, conditioned media was collected and assayed for secreted IFN γ by ELISA.

Figure 11 contains graphs showing scDb can induce mutation-specific T-cell cytokine release against tumor cell line. H/K/N RAS Q61L(55-64)-A1 cl.2 UCHT1 scDb was incubated at the specified concentrations with T cells and either with parental HL-60 or HLA-A1 KO HL-60 for 20 hours at 37°C. Following co-culture, conditioned media was collected and assayed for secreted IFN γ by ELISA.

Figure 12 shows that a MANAbody clone converted into a scDb can specifically kill tumor cells. p53 R175H(168-176)-A2 cl.2 UCHT1 scDb was incubated at the specified concentrations with T cells and either with parental TYKnu or p53 KO TYKnu for 20 hours at 37°C. Following co-culture, CellTiter-Glo was used to assay viable cells in each well. Percent target cell viability was calculated by subtracting the value from T cell only wells and normalizing to the value from target cell only wells.

Figure 13 shows that a MANAbody clone converted into a scDb can specifically kill tumor cells. H/K/N RAS Q61L(55-64)-A1 cl.2 UCHT1 scDb was incubated at the specified concentrations with T cells and either with parental HL-60 or HLA-A1 KO HL-60 for 20 hours at 37°C. Following co-culture, CellTiter-Glo was used to assay viable cells in each well. Percent target cell viability was calculated by subtracting the value from T cell only wells and normalizing to the value from target cell only wells.

Figures 14A-14C show biological and biophysical characteristics of scFv clone H2. (A) H2-scDb binding to immobilized p53^{R175H}/HLA-A*02:01 (red) or p53^{WT}/HLA-A*02:01 (gray) pHLA was assessed by ELISA. Data shown represent mean $\pm$ SD of three technical replicates. (B) H2-scDb binding to p53^{R175H}/HLA-A*02:01 was measured by single-cycle kinetics using SPR. H2-scDb was loaded at increasing concentrations, from 3, 12, 50, 200 to 800 nM. The blank- and reference-subtracted binding is shown for p53^{R175H}/HLA-A*02:01 (red) and p53^{WT}/HLA-A*02:01 (gray). H2-scDb binds to the p53^{R175H}/HLA-A*02:01 pHLA

with a one-to-one binding kinetics at a K_D of 86 nM (fitted line in black). There was negligible p53^{WT}/HLA-A*02:01 binding. (C) T2 cells pulsed with p53^{R175H} or p53^{WT} peptide was co-incubated with H2-scDb and T cells at an effector:target (E:T) ratio of 2:1. IFN- γ release was measured by ELISA (left) and cell lysis was evaluated by the CellTiter-Glo assay
 5 (right). Data indicate mean $\pm$ SD of three technical replicates and are representative of three independent experiments.

Figures 15A-15C show that H2-scDb activates T cells in the presence of tumor cells presenting p53^{R175H}. (A) HLA-A*02:01 positive tumor cell lines with different HLA expression levels and p53^{R175H} status were co-incubated with H2-scDb and T cells at an E:T
 10 ratio of 2:1. IFN- γ release was measured by ELISA. Data indicate mean $\pm$ SD of 6 technical replicates and are representative of two independent experiments. The HLA-A*02 median fluorescence intensity (MFI) ratio is defined as MFI (anti-HLA-A*02)/MFI (isotype control). (B) Polyfunctional T-cell activation mediated by H2-scDb in response to KMS26 at an E:T ratio of 2:1 was assessed by luminescence cytotoxicity and antibody-based assays (see
 15 Supplementary Materials). EC₅₀ (M) for each assay is shown in the corresponding graphs. Data indicate mean $\pm$ SD of three technical replicates and are representative of two independent experiments. (C) Real-time live-cell imaging of T cells with CellTracker Green CMFDA-labelled TYK-nu at an E:T ratio of 5:1 with or without H2-scDb. Representative phase contrast and green fluorescence images taken at 24 hours (left)
 20 and 60 hours (right) after mixing cells are shown.

Figures 16A-16D show a determination of H2-scDb specificity using isogenic target cell lines. (A) HEK293FT and Saos-2 cell lines that were transfected with full-length p53^{WT}, full-length p53^{R175H}, or were not transfected were co-incubated with T cells at an E:T ratio of 2:1 in the presence of increasing amounts of H2-scDb. IFN- γ release was measured by
 25 ELISA. Data indicate mean $\pm$ SD of three technical replicates. (B) Cell lines expressing p53^{R175H} and transduced or not transduced with HLA-A*02:01 were co-incubated with T-cells and H2-scDb. IFN- γ release was measured by ELISA. Experiments were performed at an E:T ratio of 2:1 in duplicate (AU565) or triplicate (other 3 lines). (B) Cell lines expressing p53^{R175H} were transduced with HLA-A*02:01. Recognition of these cell lines by
 30 H2-scDb was assessed by IFN- γ release. Experiments were performed at an E:T ratio of 2:1 in duplicate (AU565) or triplicate (other 3 lines). Data indicate mean $\pm$ SD. (C) IFN- γ release mediated by H2-scDb in response to parental tumor cell lines and their TP53 KO

counterparts at an E:T ratio of 2:1 (KMS26, TYK-nu) or 5:1 (KLE) was measured by ELISA. Data indicate mean $\pm$ SD of two (TYK-nu) or three (KMS26, KLE) technical replicates and are representative of two independent experiments. (D) Growth of parental (left) or *TP53* KO (right) TYK-nu cells co-incubated with H2-scDb and T cells at an E:T ratio of 5:1 was
 5 measured by well confluence using real-time live-cell imaging. Data indicate mean $\pm$ SD of three technical replicates. One-way ANOVA with Tukey's multiple comparison was used to evaluate statistical significance, *denotes $P < 0.0001$.

Figures 17A-17H show that H2 binds to the HLA-A*02:01 and the C-terminus of the p53^{R175H} neoantigen. (A) Overall structure of p53^{R175H}/HLA-A*02:01 bound to the H2-Fab
 10 fragment (PDB: 6W51). The HLA-A*02:01, β 2 microglobulin (β 2M), p53^{R175H} peptide, and the light and heavy chain of the H2-Fab are labeled accordingly. The p53^{R175H} nine amino acid peptide is between helices α 1 and α 2 of the HLA. (B) Structure of H2-Fab--
 p53^{R175H}/HLA-A*02:01 at 90° to that shown in (A). (C) Electron density map (2mFo-DFc) of the p53^{R175H} neoantigen. (D) Electron density map (2mFo-DFc) of a selected area of the
 15 H2-Fab at CDR-L3 from residues 95 to 99. (E) Zoom in of the interaction of H2-Fab to p53^{R175H}/HLA-A*02:01 with CDRs labeled in order from left to right: H2, H1, L3, H3, L1, L2. (F) Bird's-eye view of surface representation of the HLA-A*02:01, p53^{R175H} peptide, and the contacting residues labeled according to CDRs of the H2-Fab. (G) Schematic representation of (F). (H) Diagram of the orientation angle of the H2-Fab. The angle of the
 20 orientation was calculated from two vectors: one from N and C termini of the peptide and the other between the intermolecular disulfide bonds of the V_L and V_H domains. The arrowed lines indicate the direction of the vectors.

Figures 18A-18F show a structural basis of H2 specificity and identification of putative cross-reactive peptides. (A) Detailed interactions of the p53^{R175H} neoantigen with
 25 HLA-A*02:01. The peptide (green) and the side chains (grey) of interacting residues of HLA-A*02:01 are represented as sticks. Hydrogen bonds are shown as dashed lines. (B) Perpendicular view of the p53^{R175H} peptide binding cleft. (C) C-terminus of the p53 peptide (aa Val6-Cys9) with Arg7 and His8 surrounded by the interacting residues of CDR-H1, -H2, -H3, and -L3 shown as sticks. Hydrogen bonds are shown as dashed lines. (D) T2 cells were
 30 loaded with 10 μ M of HMTEVVRHC (SEQ ID NO:1) peptide variants from the positional scanning library and co-incubated with 1 nM H2-scDb and T cells at an E:T ratio of 2:1. IFN- γ release was measured by cytometric bead array and the mean of triplicate wells was

used to plot the heatmap. Black boxes represent the parental p53^{R175H} peptide. (E) Illustration of the binding pattern of H2-scDb as Seq2Logo graph (SEQ ID NO:184), calculated by dividing the IFN- γ value by 10^4 and using the PSSM-Logo algorithm. (F) T2 cells were loaded with 10 μ M of p53^{R175H} (SEQ ID NO:1), p53^{WT} (SEQ ID NO:135), STAT2 (SEQ ID NO:185), VP13A (SEQ ID NO:186), or ZFP3 (SEQ ID NO:187) peptide and co-
 5 incubated with 1 nM H2-scDb and T cells at an E:T ratio of 2:1. IFN- γ secretion was measured by ELISA. Data indicate mean $\pm$ SD of three technical replicates.

Figures 19A-19C show *in vivo* antitumor efficacy of H2-scDb. In the early treatment model, NSG mice were engrafted with 1×10^7 human T cells and either 1×10^6 parental
 10 KMS26 (A) or 1×10^6 TP53 KO KMS26 (B) on day 0. On day 1, intraperitoneal infusion pumps were placed to administer H2-scDb or isotype control scDb. (C) In the established tumor model, mice were engrafted with 1×10^7 human T cells and 3.5×10^5 parental KMS26 on day 0, followed by administration of H2-scDb or isotype scDb at the specified dose on day 6. Tumor growth was monitored by bioluminescent imaging. N = 4 or 5 mice per group. Color
 15 bars denote the radiance (p/sec/cm²/sr) scale at each time point. Plotted data indicate mean $\pm$ SD. * denotes $P < 0.05$ and NS denotes no statistical significance compared to isotype control by multiple t-test with Holm-Šídák correction.

Figure 20 shows the detection and quantification of p53^{R175H} neoantigen peptide in cells. COS-7 cells transfected with constructs expressing HLA-A*02:01 and p53^{R175H}, as
 20 well as cells lines with endogenous HLA-A*02:01 and p53^{R175H} expression, were analyzed for the presentation of p53^{R175H} neoantigen peptide HMTEVVRHC (SEQ ID NO:1; upper panel). Heavy isotope labeled p53^{R175H} neoantigen peptide was spiked into the assay and served as standards for absolute copy number quantification (lower panel).

Figure 21 shows a flow cytometric screening of phage clones enriched by panning.
 25 After 5 rounds of panning, phage clones from the enriched phage pool were isolated by limiting dilution and grown in deep 96-well plates. Supernatants containing individual phage clones were used to assess binding to T2 cells loaded with β 2 macroglobulin (β 2M) only, β 2M plus p53^{WT} peptide, or β 2M plus p53^{R175H} peptide via flow cytometry. The median fluorescence intensity (MFI) ratio was defined as MFI (R175H peptide)/MFI (WT peptide).
 30 NC, no phage control.

Figure 22 contains a schematic representation of the structure of the T cell-engaging bispecific single-chain diabody (scDb) used in our experiments. V_L , variable light domain; V_H , variable heavy domain; pHLA, peptide-HLA complex; LL, long linker; SL, short linker.

Figure 23 shows a screening of scDb clones via IFN- γ stimulation by p53-expressing cells. scDbs generated by linking each anti-p53^{R175H}/HLA-A*02:01 pHLA scFv clone with an anti-CD3 scFv (UCHT1) were coincubated with T cells and COS-7 cells transfected with GFP, HLA-A*02:01 + GFP, HLA-A*02:01 + p53^{WT}, or HLA-A*02:01 + p53^{R175H} plasmids at an effector:target (E:T) ratio of 1:1. After a 20-hour coincubation, the supernatant was harvested for IFN- γ detection by ELISA. Arrows indicate clones H2 and H20. A2, HLA-A*02:01.

Figure 24 shows a characterization of H20-scDb. H20-scDb was incubated with biotinylated p53^{R175H}/HLA-A*02:01 (red) and p53^{WT}/HLA-A*02:01 (gray) pHLA monomers coated on streptavidin microplates at the specified concentrations, then binding detected with protein L and anti-protein L HRP. Data indicate mean $\pm$ SD of three technical replicates.

Figure 25 contains a comparison of scDbs generated with different anti-CD3 scFvs. H2 was linked with different anti-CD3 scFvs in the scDb format and co-incubated with T cells and T2 cells pulsed with titrated concentrations of p53^{R175H} or p53^{WT} peptide at an E:T ratio of 2:1. IFN- γ release was measured by ELISA. Data indicate mean $\pm$ SD of three technical replicates.

Figure 26 shows a H2-scDb-induced polyfunctional T-cell response. T-cell cytokine release mediated by H2-scDb in response to KMS26 cell line at an E:T ratio of 2:1 was assessed by antibody-based assays. EC_{50} (M) for each analyte is shown in the corresponding graphs. Data indicate mean $\pm$ SD of three technical replicates.

Figure 27 shows a flow cytometric evaluation of HLA-A*02 expression on tumor cell lines. Expression of HLA-A*02 on tumor cell lines was evaluated by flow cytometry. The red histogram represents staining with anti-HLA-A*02 (clone BB7.2), and the gray histogram represents staining with an isotype control. The median fluorescence intensity (MFI) ratio is defined as MFI (anti-HLA-A*02)/MFI (isotype control).

Figure 28 shows a flow cytometric evaluation of tumor cell lines transduced with HLA-A*02:01-encoding retrovirus. HLA-A*02:01-encoding retrovirus was transduced into cell lines that weakly (AU565, SK-BR-3) or do not detectably (HuCCT1, CCRF-CEM) express HLA-A*02:01. Expression of HLA-A*02:01 in the parental and transduced and

sorted cell lines was evaluated by flow cytometry. The red histogram represents staining with anti-HLA-A*02 (clone BB7.2) and the gray histogram represents staining with an isotype control.

Figures 29A-29B show a determination of H2-scDb specificity using CRISPR-edited isogenic cell lines. (A) Expression of p53 protein in the parental and *TP53* KO clones of KMS26, KLE, and TYK-nu was assessed by Western blot staining with anti-p53 antibody (clone DO-1). (B) Cytotoxicity mediated by H2-scDb in response to parental tumor cell lines and their *TP53* KO counterparts at an E:T ratio of 2:1 (KMS26, TYK-nu) or 5:1 (KLE) was measured by the Bio-Glo (KMS-26) or CellTiter-Glo (TYK-nu, KLE) assay. Data indicate mean $\pm$ SD of two (TYK-nu) or three (KMS26, KLE) technical replicates and are representative of two independent experiments.

Figures 30A-30B shows a H2-Fab-p53^{R175H}/HLA-A*02:01 complex. (A) Size-exclusion chromatogram of the pHLA-A*02:01 in complex with the H2-Fab. Protein was monitored by A280 nm with a major peak (~100 kDa). (B) Coomassie-stained gradient SDS-PAGE gel of the eluted fractions at 11-17 mL from (A).

Figures 31A-31D show that the neoantigen p53^{R175H} binds to HLA-A*02:01 in a canonical fashion. (A) Bird's-eye view of the p53^{R175H} neoantigen interactions with HLA-A*02:01. The p53 peptide and the side chains of interacting residues of HLA-A*02:01 are represented as sticks. Hydrogen bonds are shown as dashed lines. The N-terminal His1 is anchored by three tyrosine residues of HLA-A*02:01, one at the base of the cleft (Tyr7, not shown) and two on α 2 (Tyr159, 171), while its side chain is within hydrogen bonding distance of Lys66 (α 1) and Thr163 (α 2). Glu63 of HLA-A*02:01 α 1 forms a hydrogen bond with the backbone amino of Met169 at P2, an anchor residue of p53^{R175H} that is situated within the hydrophobic B pocket of the HLA. The main chain of Thr3 is stabilized by a hydrogen bond to Tyr99, located at the base of the cleft while the side chain of Glu4 forms a salt-bridge with the side chain of Arg65. Positions 5-8 (Val172, Val173, Arg174, His175) of the p53^{R175H} neoantigen are stabilized by multiple hydrophobic and aliphatic residues with no direct hydrogen bonding contacts to the HLA-A*02:01. Towards the C-terminus of the neoantigen, the carboxyl group of Cys176 at P9, another anchor residue that lies within the F pocket, is secured by Tyr84 (α 1) and Lys146 (α 2), while the side chain sulfhydryl is near Thr143 on α 2. (B) Surface representation of the HLA-A*02:01 with the p53^{R175H} neoantigen shown as sticks. Anchor pockets B and F are circled in black. (C) Structural alignment of

the following HLA-A*02:01-bound peptides in the binding pocket: p53^{R175H} (PDB ID 6W51), p1049 (PDB ID 2JCC), NY-ESO-1 (PDB ID 3HAE), and WT1 (PDB ID 4WUU). (D) Zoomed in view of (B) with helix α 1 transparent and residues at position 7 (P7, Arg174) and 8 (P8, His175) shown as sticks.

Figure 32 shows a hydrogen bonding pattern of the H2-Fab cage-like configuration. The imidazole ring of His175 at P8 was at the center of the cage-like structure. The guanidinium group of Arg7 was at hydrogen bonding distance to the backbone carbonyl of Ala31 (CDR-H1). Another neoantigen-antibody direct contact involves the backbone carbonyl of Val6 hydrogen bonding with the side chain of Arg93 (CDR-L3).

Figures 33A-33P show a comparison of binding orientations among TCRm-pHLA and TCR-pHLA complexes. (A) Close depiction of binding of the H2-Fab to p53^{R175H}/HLA-A*02:01 with CDRs labeled as in Fig. 17. (B) Binding of a TCR to melanoma-associated antigen 3 (MAGE-A3) and HLA-A*01:01 (PDB 5BRZ). Same orientation as (A). The MAGE-A3 TCR displays the canonical, diagonal binding motif to that of most known TCR topologies. (C) Recognition of the 3M4E4 Fab for the NY-ESO-1₁₅₇₋₁₆₅/HLA-A*02:01 complex (PDB 3HAE). Same orientation as (A). (D) Binding of the ESK1 Fab to Wilms tumor 1 peptide and HLA-A*02:01 (PDB 4WUU). Same orientation as (A). (E, F, G and H) Bird's-eye view of surface representation of the HLA-A*02:01/*01:01 with the contacting residues of H2-Fab, MAGE-A3 TCR, 3M4E4 Fab, and ESK1 Fab, respectively, labeled and indicated with arrows. (I, J, K and L) Schematic representation of E, F, G, and H, respectively. H2-Fab-p53^{R175H}/HLA-A*02:01 shows a different mode of antibody recognition compared with other Fab/TCR-pHLA complexes. (M, N, O and P) Schematic representation of Fab/TCR orientation angle.

Figure 34 shows binding of p53^{R175H} positional scanning library peptides to HLA-A*02:01. A peptide library was generated by systemically substituting the amino acid at each position of the target peptide (HMTEVVRHC; SEQ ID NO:1) with each of the remaining 19 common amino acids. T2 cells were loaded with each of the variant peptides at 100uM in the presence of 10 μ g/ml β 2M and anti-HLA-A*02 antibody (clone BB7.2). Peptide binding was evaluated by flow cytometry. Black boxes represent the parental peptide. MFI, median fluorescence intensity.

Figure 35 shows recognition of the p53^{R175H} positional scanning library peptides by H2-scDb. T2 cells loaded with variant peptides in the positional scanning library was

incubated with 1nM H2-scDb and T cells at an E:T ratio of 2:1. IFN- γ release was measured by ELISA. Dotted lines represent 20% of parental peptide IFN- γ value. Peptides for Positions 1-9 are SEQ ID NOs:188-196, respectively. The binding motif established by the 20% reactivity cutoff, expressed in PROSITE pattern, was x-[AILMVNQTC]-[ST]-[DE]-
 5 [IV]-[IMVST]-R-H-[AILVGHSTYC] (SEQ ID NO:197). Data indicate mean $\pm$ SD of three technical replicates.

Figures 36A-36B contain an assessment of H2-scDb cross-reactivity H2-scDb was co-incubated with T cells and COS-7 cells transfected with HLA-A*02:01 and full-length p53^{R175H}, STAT2, or ZFP3 at an E:T ratio of 5:1. (A) Expression of target proteins by COS-
 10 7 cells was assessed by Western blot staining. (B) IFN- γ secretion was measured by ELISA. The signals for all of the transfectants except for p53^{R175H} were indistinguishable and are clustered near the x-axis. Data indicate mean $\pm$ SD of three technical replicates.

Figure 37 shows the body weight of NSG mice in the established tumor model. NSG mice were engrafted with 1×10^7 human T cells and 3.5×10^5 parental KMS26 on day 0,
 15 followed by administration of the specified scDb on day 6. Body weight of the mice was serially monitored. N = 5 mice per group. Data shown represent mean $\pm$ SD.

Figures 38A-38H. ELISA and flow cytometry characterization of RAS MANA scFvs. scFvs against RAS MANAs were characterized using ELISA, flow cytometry, and SPR. (A,D-F) Biotinylated G12V or G12WT pHLA-A3 (A) or Q61WT, Q61H, Q61L, or
 20 Q61R pHLA-A1 (D-F) were coated on a streptavidin plate at the specified concentrations. Recombinant RAS G12V clone V2 (A), Q61H clone H1 (D), Q61L clone L2 (E), or Q61R clone R6 (F) scFvs were incubated in the wells at 1 μ g/mL, followed by detection with protein L and horseradish peroxidase (HRP)-conjugated anti-protein L. All ELISAs were performed in triplicate. (B,G) T2A3 or SigM5 cells were pulsed with the specified peptides
 25 at 50 μ M, followed by flow cytometric analysis. (B) T2A3 cells were stained with V2 scFv pre-conjugated to anti-FLAG-phycoerythrin (PE), with mean fluorescence intensities (MFI) plotted. (G) SigM5 cells were stained with clone H1, L2, or R6 phage, then detected with rabbit anti-M13 phage and PE-conjugated anti-rabbit antibodies. Phage background-subtracted PE MFI are plotted. (C,H) V2 scFv and L2 single chain diabody (scDb) binding
 30 were evaluated by single-cycle kinetics using SPR. (C) The V2 scFv bound to G12V pHLA-A3 with one-to-one binding kinetics and a K_D of 8.7 nM, with negligible binding to G12WT

pHLA-A3. (H) The L2-U scDb bound to the Q61L pHLA-A1 with one-to-one binding kinetics and a K_D of 65 nM, with negligible binding to Q61WT pHLA-A1.

Figures 39A-39E. Schematic and ELISA characterization of MANA scDbs. (A) Schematic showing the optimal bispecific format, an scDb with variable light (VL) and
 5 variable heavy (VH) domains arranged in the following order: VL_{V2}-VH_{UCHT1}-VL_{UCHT1}-VH_{V2}. SL, short linker (GGGGS; SEQ ID NO:200); LL, long linker (GGGGS)₃ (SEQ ID NO:201). (B-E) anti-MANA/anti-CD3 scDbs were characterized via ELISA. Biotinylated pHLA-A3, pHLA-A1 or recombinant CD3 ϵ/δ protein were coated on a streptavidin plate. Recombinant V2-U (B), H1-U (C), L2-U (D), or R6-U (E) scDbs were incubated at the
 10 specified concentrations then detected with protein L and anti-protein L HRP. All ELISAs were performed in triplicate.

Figures 40A-40D. Co-cultures with peptide-pulsed cells. (A,B) T2A3 cells were pulsed with either the G12V or G12WT peptides at the specified concentrations. (C,D) SigM5 cells were pulsed with either the Q61L or Q61WT peptides at the specified
 15 concentrations. 5×10^4 (T2A3) or 2.5×10^4 (SigM5) peptide-pulsed cells were combined with 5×10^4 human T cells (effector:target ratio or E:T = 1:1 or 2:1) and V2-U (A,B), V2-U2 scDb (A,B), or L2-U scDb (C,D) at 1 nM. Plates were incubated for 24 hours and the supernatants assayed for IFN γ (A,C). Target cell cytotoxicity was assayed using CellTiter-Glo (B,D). Percent cytotoxicity was determined by subtracting the T cell signal and
 20 normalizing to the no peptide condition. All experiments were performed in triplicate.

Figures 41A-41D. Co-culture with transfected COS-7 cells. COS-7 cells were transfected with a 1:1 ratio of plasmids encoding HLA-A3 ("A3") or HLA-A1 ("A1") and RAS variants or other negative controls. 24 hours later, 1×10^4 COS-7 cells were combined with 5×10^4 human T cells (E:T = 5:1) and V2-U (A), H1-U (B), L2-U (C), or R6-U (D)
 25 scDb at the specific concentrations. Plates were incubated for 24 hours and supernatants assayed for IFN γ . All experiments were performed in triplicate.

Figures 42A-42F. Effects of V2-U scDb on co-cultures of human T cells with cancer cells. 2×10^4 target cells from parental NCI-H441 (A,B), NCI-H441 isogenic variants (C,D) or NCI-H358 isogenic variants (E,F) were combined with 6×10^4 human T cells (E:T = 3:1)
 30 and V2-U scDbs at the specified concentrations. Cells were incubated for 24 hours and assayed for IFN γ release (A,C,E) and target cell cytotoxicity using CellTiter-Glo (B,D,F). Cytotoxicity was calculated by subtracting the T cell alone signal and normalizing to the no

scDb condition (considered as 0% cytotoxicity). All experiments were performed in triplicate. For *KRAS* genotypes: V/ Δ , G12V/frameshift (also see Figure 55).

Figures 43A-43E. Effects of L2-U scDb on co-cultures of human T cells with cancer cells. 2.5×10^4 target cells from different cancer cell lines expressing HLA-A1, RAS Q61L, or both (A), parental HL-60 cells (B-C), or HL-60 isogenic variants expressing different *RAS* Q61 mutations or with HLA-A1 knocked out (KO) (D-E) were combined with 5×10^4 human T cells (E:T = 2:1) and L2-U scDb at the specified concentrations. Cells were incubated for 24 hours and assayed for IFN γ release by ELISA (A,B,D) and target cell cytotoxicity (C,E). Target cell cytotoxicity was assessed via CellTiter-Glo. Cytotoxicity was calculated by subtracting the T cell alone signal and normalizing to the no scDb condition (considered as 0% cytotoxicity). All experiments were performed in triplicate. (For HL-60 *NRAS* genotypes, also see Figure 55).

Figures 44A-44D. Peptide scanning to assess V2-U and L2-U scDb cross-reactivity. Each amino acid position of the G12V and Q61L 10-mer peptides were systematically changed to the other 19 amino acids, thereby generating libraries of variant peptides each differing from the original peptide by a single amino acid. T2A3 cells were pulsed with 10 μ M of the G12V peptide scanning library (A,C) and SigM5 cell cells were pulsed with 10 μ M of the Q61L peptide scanning library (B,D). 2.5×10^4 peptide-pulsed target cells were combined with 5×10^4 human T cells (E:T = 2:1) and either the V2-U (A,C) or L2-U scDb (B,D) at 1 nM. (A,B) Plates were incubated for 24 hour and assayed for IFN γ release, with the mean of three technical replicates plotted as a heat map. Black boxes indicate amino acids in the parental peptides. (C,D) Illustration of the binding pattern of V2-U and L2-U scDb as Seq2Logo graph (SEQ ID NOs:683 and 684, respectively), calculated by dividing the IFN γ value by 10^3 and using the PSSM-Logo algorithm.

Figures 45A-45C. *KRAS* neoantigen transitions detected through MANA-SRM. (A-C) COS-7 cells transfected with constructs expressing HLA-A*03:01 and *KRAS* G12V (A), as well as cells lines NCI-H441 (B) and CFPAC-1 (C) with endogenous HLA-A*03:01 and *KRAS* G12V expression, were analyzed for the presentation of G12V[7-16] VVGAVGVGK (SEQ ID NO:205; left) and G12V[8-16] VVGAVGVGK (SEQ ID NO:206; right) peptides (lower panels). The presence or absence of these peptides is denoted with a red arrow or "X," respectively. Heavy isotope labeled RAS G12V peptides were spiked into the assay and served as standards for absolute copy number quantification (upper

panels). Transition details for each peptide in each sample were shown on the right side of each individual plot after zooming in on the retention time (x-axis). Peptide quantification was performed based on the plots as previously described.

Figures 46A-46C. Design and sequencing of CDR-H3 of the phage library. (A) Expected amino acid diversity at variable codons of CDR-H3, using Kabat numbering. (B) Expected percent of clones with a given codon present in CDR-H3. (C) Expected vs. actual amino acid diversity at variable codons in CDR H3. The actual amino acid diversity was determined by MiSeq next-generation sequencing (NGS) of a portion of the library and subsequent analysis of the top 100,000 most frequent reads. T, theoretical distribution; M, MiSeq NGS analysis.

Figures 47A-47E. Characterization of the V2 scFv. (A) ELISA with monoclonal phage. After 4, 5, or 6 rounds of selection, monoclonal phage were amplified in bacteria in a 96-well plate format. Well H12 was not inoculated and thus served as a no phage control. Monoclonal phage were incubated in G12V pHLA-A3 or G12WT pHLA-A3 coated streptavidin ELISA plate. Plates were washed and phage were detected using rabbit anti-M13 and HRP-conjugated anti-rabbit antibodies. Phage clone V2 was identified in the four wells designated by the red arrows. (B) Flow cytometry. T2A3 cells pulsed or not pulsed with the indicated peptides were incubated with the top four candidate phage clones selected for their ability to bind G12V pHLA-A3. The binding was assessed by rabbit anti-phage M13 and PE-conjugated anti-rabbit antibodies. MFI are plotted. (C) ELISA with V2 phage after dilution. Biotinylated pHLA-A3 were coated on a streptavidin plate. V2 phage were incubated at the specified dilutions and detected as in (A). (D) ELISAs with V2 scFv. Similar to (C), except that recombinant V2 scFv was used instead of phage, and detection employed protein L and HRP-conjugated anti-protein L. All ELISAs were performed in triplicate. (E) Peptide pulsing of T2A3 cells. T2A3 cells were pulsed with the specified peptides at 50 μ M. Cells were stained with anti-HLA-A3 monoclonal antibody GAP.A3 conjugated to PE. MFI are plotted.

Figures 48A-48F. Characterization of the RAS Q61H, Q61L, and Q61R scFvs. (A) Flow cytometry. After 5 rounds of selection, monoclonal phage were amplified in bacteria in 96-well plate format. Monoclonal phage were sequenced and clustered to unique phage clones. (A,C,E) SigM5 cells pulsed with the specified peptides were incubated with phage clones from Q61H, Q61L, and Q61R selections, respectively and assessed by rabbit anti-

phage M13 and PE-conjugated anti-rabbit antibodies. Mean fluorescence intensities (MFI) are plotted. The top candidates are indicated with arrows. (B,D,F) COS-7 cells were transfected with a 1:1 ratio of plasmids encoding HLA-A1 ("A1") and KRAS Q61 variants (or other negative controls). 24 hours later, 5×10^4 COS-7 cells were combined with 5×10^4 human T cells (effector:target ratio or E:T = 1:1) and single chain diabody (scDb) proteins generated from the top flow cytometry candidates at the specified concentrations. Plates were incubated for 24 hours and assayed for secreted IFN γ . Black arrows denote the single most specific and reactive clone for each target pHLA complex selected for further investigation.

Figures 49A-49D. Bispecific antibody formats and format comparison. (A) Cartoon depictions of the six bispecific formats tested with the RAS G12V pHLA-A3 targeting V2 scFv. (B) Schematic showing the tested orientations of the variable light (VL) and variable heavy (VH) domains of the V2 scFv with different anti-CD3 scFv clones. SL, short linker (GGGGS; SEQ ID NO:200); ML, medium linker (GGGGS) $_2$ (SEQ ID NO:694); LL, long linker (GGGGS) $_3$ (SEQ ID NO:201). (C) ELISAs of V2-bispecific antibody formats to assess binding to G12V pHLA-A3 and recombinant CD3 ϵ/δ protein. Biotinylated G12V pHLA-A3 or CD3 ϵ/δ protein was coated on a streptavidin plate. Different bispecific antibody formats were incubated in wells at specified concentrations followed by detection with protein L and HRP-conjugated anti-protein L. (D) Testing of V2 bispecific formats in co-cultures of T cells and peptide-pulsed T2A3s. T2A3 cells were pulsed with either the G12V or G12WT peptide at the specified concentrations. 2.5×10^4 peptide-pulsed T2A3 cells were combined with 5×10^4 T cells (E:T = 2:1) and V2 bispecific formats at either 1 nM or 0.2 nM bispecific antibody concentration. Plates were incubated for 24 hours and assayed for IFN γ release. Note that the scFv-Fc format is a heterodimer of an FcKnob and an FcHole protein, with the bivalent scFv-Fc containing one V2 moiety and one anti-CD3 moiety, and the trivalent scFv-Fc containing two V2 moieties and one anti-CD3 moiety.

Figures 50A-50D. V2 scDbs made with various anti-CD3 clones. Twelve different anti-CD3 clones were tested in the VL $_{V2}$ -VH $_{CD3}$ -VL $_{CD3}$ -VH $_{V2}$ format. (A) scDbs (all bearing C-terminal 6xHIS tags) were expressed in 293FT cells and purified identically. See Table 12 for sequences of the anti-CD3 clones. The Western blot shows the purified scDbs detected with anti-6xHIS and HRP-conjugated anti-rabbit antibodies. (B) Biotinylated recombinant CD3 ϵ/δ protein was coated on a streptavidin plate. V2 scDbs were added to the plate at 2

μg/ml and detected with protein L and HRP-conjugated anti-protein L. (C) COS-7 cells were transfected with a 1:1 ratio of plasmids encoding HLA-A3 and KRAS G12V. 24 hours later, 1×10^4 COS-7 cells were combined with 5×10^4 human T cells (E:T = 5:1) and V2 scDb at the indicated concentrations. Cells were incubated for 24 hours and assayed for secreted IFN γ . (D) 2×10^4 NCI-H441 target cells were combined with 6×10^4 human T cells (E:T = 3:1) and V2 scDb proteins at the indicated concentrations. Cells were incubated for 24 hours and assayed for secreted IFN γ .

Figures 51A-51E. Specificity of scDbs. (A) Biotinylated pHLA-A3 or biotinylated recombinant CD3 ϵ / δ protein were coated on a streptavidin plate. V2-U2 scDb was added to the plate at the indicated concentrations and detected with protein L and HRP-conjugated anti-protein L. Each ELISA was performed in triplicate. (B) Biotinylated pHLA-A3 were coated on a streptavidin plate. ScDb was incubated with pHLA at the indicated concentrations, then incubated with recombinant CD3 ϵ / δ containing a human Fc tag and detected with an HRP-conjugated anti-human Fc antibody. (C-E) Biotinylated RAS Q61 pHLA-A1 (Q61WT, Q61H, Q61L, or Q61R) were coated on streptavidin plates at the indicated concentrations. Recombinant H1-U (C), L2-U (D), or R6-U (E) scDb was added to the wells at 10 nM and detected with protein L and HRP-conjugated anti-protein L.

Figures 52A-52N. Additional peptide-pulsing data. (A) T2A3 cells were pulsed with the RAS G12V or G12WT peptides at the indicated concentrations. 5×10^4 T2A3 peptide-pulsed cells were co-cultured with 5×10^4 human T cells (E:T = 1:1) in the presence of either V2-U or V2-U2 scDb at 1 nM. Cells were incubated for 24 hours and TNF α secretion measured. (B) Quantification of G12V peptide on the surface of T2A3 cells. T2A3 cells were pulsed with the G12V peptide at the specified concentrations. G12V pHLA complexes on the cell surface were quantified using the V2 scFv and Quantibrite- and Quifikit-based methods. (C,D) G12 peptide-pulsed dendritic cells. HLA-A3+ immature dendritic cells (iDCs) were pulsed with either the G12V or G12WT peptide at the specified concentrations. 1×10^4 peptide-pulsed iDCs cells were combined with 5×10^4 human T cells (E:T = 5:1) and either V2-U or V2-U2 scDb at 1 nM. Plates were incubated for 24 hours and assayed for secreted IFN γ (C) and TNF α (D). (E-H) Q61 peptide-pulsed SigM5 cells. SigM5 cells were pulsed with the RAS Q61H, Q61R, or Q61WT peptide at the indicated concentrations. 2.5×10^4 peptide-pulsed SigM5 cells were combined with 5×10^4 human T cells (E:T = 2:1) and either H1-U (E,F) or R6-U (G,H) scDb at 1 nM. Cells were incubated for 24 hours and

assayed for secreted IFN γ (E,G). Target cell cytotoxicity was assayed using CellTiter-Glo (F,H). (I-N) Q61 peptide-pulsed dendritic cells. HLA-A1+ iDCs were pulsed with either the Q61H, Q61L, Q61R, or Q61WT peptide at the indicated concentrations. 1×10^4 peptide-pulsed iDCs cells were combined with 5×10^4 human T cells (E:T = 5:1) and H1-U (I,L), L2-U (J,M), or R6-U (K,N) scDb at 1 nM. Cells were incubated for 24 hours and assayed for secreted IFN γ (I-K) or TNF α (L-N). All experiments were performed in triplicate.

Figures 53A-53D. Western blots and co-cultures with transfected COS-7 cells. COS-7 cells were transfected with a 1:1 ratio of plasmids encoding HLA-A3 ("A3") and KRAS variants (or other negative controls). 24 hours later, cells were harvested for western blots (A) or co-cultures (B-D). (A) Western blots for KRAS and HLA-A3 in transfected COS-7. Cells were pelleted and snap frozen and analyzed via western for KRAS, HLA-A3, and β -actin protein expression. (B-D) 1×10^4 COS-7 cells were combined with 5×10^4 human T cells (E:T = 5:1) and V2-U (B) or V2-U2 scDb (C,D) at the indicated concentrations. Cells were incubated for 24 hours and supernatant assayed for secreted IFN γ (C) or TNF α (B,D). All ELISAs were performed in triplicate.

Figure 54. HLA-A3 expression in target cell lines. 5×10^5 target cells were stained with PE-conjugated anti-HLA-A3 clone GAP.A3 (shaded red) or PE-conjugated mouse isotype IgG2a control (shaded gray). Histograms show PE intensities of viable cells.

Figure 55. Sanger sequencing of *RAS* alleles in CRISPR-modified cell lines. Sanger sequencing of genomic DNA from the *KRAS* locus of NCI-H441 parental and G13D-KI clones (SEQ ID NO:695), from the *KRAS* locus of NCI-H358 parental and G12V-KI clones (SEQ ID NO:696), and from the *NRAS* locus of HL-60 parental, Q61H-KI, and Q61R-KI clones (SEQ ID NO:697). Note that *KRAS* codons are shown in the antisense orientation.

Figures 56A-56B. Effects of V2-U2 scDb on co-culture of T cells with NCI-H441 isogenic cell lines. 2×10^4 parental NCI-H441 cells or its isogenic clones were combined with 6×10^4 human T cells (E:T = 3:1) and V2-U2 scDb at the indicated concentrations. Cells were incubated for 24 hours and assayed for IFN γ release (A) or target cell cytotoxicity with CellTiter-Glo (B). All experiments were performed in triplicate.

Figure 57. Poly-functional immune response elicited by V2-U scDb. V2-U scDb at the indicated concentrations was incubated with 2.5×10^4 NCI-441 cells and 5×10^4 human T cells (E:T = 2:1). Cells were incubated for 24 hours and assayed for secreted IFN γ , TNF α ,

IL-2, granzyme B, and perforin using Luminex beads. All experiments were performed in triplicate.

Figures 58A-58B. Effects of V2-U2 scDb on co-culture of T cells with NCI-H358 isogenic cell lines. 2×10^4 parental NCI-H358 cells or its isogenic clones were combined with 6×10^4 human T cells (E:T = 3:1) and V2-U2 scDb at the indicated concentrations. Cells were incubated for 24 hours and assayed for IFN γ release (A) or target cell cytotoxicity with CellTiter-Glo (B). All experiments were performed in triplicate.

Figures 59A-59B. Effects of V2 scDbs on IFN γ secretion from T cells in co-cultures with HLA-A3+ cell lines. 2×10^4 target cells were combined with 6×10^4 human T cells (E:T = 3:1) and V2-U (A) or V2-U2 (B) scDb at the indicated concentrations. The cells were incubated for 24 hours and assayed for IFN γ release. All experiments were performed in triplicate. *** denotes $P < 0.001$ and NS denotes no statistical significance between CFPAC-1 and each of the other cell lines without the *R4S* G12V mutation, as analyzed by two-way ANOVA collapsed across different scDb concentrations for a given cell line, with Tukey's correction for multiple comparisons.

Figure 60. HLA-A1 expression in target cell lines. 5×10^5 target cells were incubated with anti-HLA-A1/A11/A26 clone 8.L.101 (shaded red) or mouse IgM isotype control (shaded gray), then stained with a PE-conjugated anti-mouse antibody. Histograms show PE intensities of viable cells.

Figure 61. Poly-functional immune response elicited by L2-U scDb. L2-U scDb at the indicated concentrations was incubated with 2.5×10^4 HL-60 cells and 5×10^4 human T cells (E:T = 2:1). Cells were incubated for 24 hour and assayed for secreted IFN γ , TNF α , IL-2, granzyme B, and perforin using Luminex beads. All experiments were performed in triplicate.

Figures 62A-62F. Testing potential cross-reactive peptides. (A) T2A3 cells were pulsed with 50 μ M of the indicated peptides. Pulsed cells were incubated with V2 phage, followed by staining with rabbit anti-M13 phage and PE-conjugated anti-rabbit antibodies or incubated with the PE-conjugated anti-HLA-A3 monoclonal antibody GAP.A3. PE MFI are plotted. (B) Western blot showing endogenous Rab-7b expression in PBMCs, monocytes (Mono), immature DCs (iDC), mature DCs (mDC), and Hs 695T cells, and overexpression of Rab-7b in HLA-A3 and RAB7B co-transfected COS-7 and HCT 116 versus HLA-A3 and GFP co-transfected controls. (C) 1×10^4 NCI-H441 cells or 5×10^4 PBMCs, Mono, iDC, or

mDC, with or without pulsing with the G12V peptide, were combined with 5×10^4 human T cells (E:T = 5:1 or 1:1) and the V2-U scDb at 1 nM. Cells were incubated for 24 hours and assayed for secreted IFN γ . Normal human cells were derived from an HLA-A3+ donor. (D) Hs 695T were transfected with HLA-A3 or HLA-A2 (negative control) encoding plasmids to assess for endogenous presentation and V2-U scDb recognition of the Rab-7b peptide. As a positive control, *HLA-A3*-transfected cells were pulsed with the G12V peptide or dimethylformamide (DMF, solvent) only. Parental NCI-H441 cells were included as a positive control and NCI-H441 *HLA-A3*-KO (A3-KO) and NCI-H441 (*KRAS* G13D/WT) clone 1 (G13D-KI) were included as negative controls. In each well, 2×10^4 target cells were combined with 5×10^4 human T cells (E:T = 5:2) and V2-U scDb at the indicated concentrations. Plates were incubated for 24 hours and assayed for secreted IFN γ . (E,F) COS-7 (E) and HCT 116 (F) cells were transfected with either vector only (GFP) or a 1:1 ratio of plasmids encoding HLA-A3 and vector only (GFP), full length *KRAS* WT, *KRAS* G12V, or Rab-7b. In each well, 2×10^4 target cells were combined with 5×10^4 human T cells (E:T = 5:2) and V2-U scDb at the indicated concentrations. Cells were incubated for 24 hour and assayed for secreted IFN γ .

Figures 63A-63C. Positional scanning of target peptides that could potentially react with V2 or L2 scDb. (A) Each amino acid of the G12V peptide was systematically changed to the other 19 amino acids. T2A3 cells were pulsed with 10 μ M of the G12V peptide library. 2.5×10^4 peptide-pulsed T2A3 cells were combined with 5×10^4 human T cells (E:T = 2:1) and V2-U scDb at 1 nM. Cells were incubated for 24 hour and assayed for secreted IFN γ . (B) Each amino acid of the Q61L peptide was systematically changed to the other 19 amino acids. SigM5 cells were pulsed with 10 μ M of the Q61L peptide library. 2.5×10^4 peptide-pulsed SigM5 cells were combined with 5×10^4 human T cells (E:T = 2:1) and L2-U scDb at 1 nM. Cells were incubated for 24 hours and assayed for secreted IFN γ . (C) SigM5 cells were pulsed with the Q61L, Q61WT, or CHD4 peptide. 2.5×10^4 peptide-pulsed SigM5 cells were combined with 5×10^4 human T cells (E:T = 2:1) and L2-U scDb at 1 nM. Cells were incubated for 24 hours and assayed for secreted IFN γ .

Figures 64A-64B. L2-U effects on tumor growth in mouse model systems. (A, B) Mice were engrafted with 1×10^7 human T cells and 5×10^5 luciferase-expressing parental HL-60 (A) or CRISPR-edited HL-60 (B) on day 0. One day later, after tumor engraftment was established by bioluminescent imaging, mice were randomized with respect to tumor

burden and intravenously injected with 1×10^7 human T cells. They were then immediately implanted with osmotic pumps delivering L2-U or isotype scDb (V2-U scDb) at 0.42g/kg/day. Tumor growth was monitored by bioluminescent imaging. N = 7 mice per group. Plotted data represent mean $\pm$ SD. * denotes $P < 0.05$ and NS denotes no statistical significance compared to isotype control according to multiple t-tests with Bonferroni-Dunn correction.

Figures 65A-65B. Body weights of NSG mice treated with scDbs. (A,B) NSG mice were implanted with tumors and treated as described in Figure 64. Body weights of the mice were serially monitored. Data shown represent mean $\pm$ SD.

Figure 66. Diagram of scFv phage library phagemid. Oligonucleotides encoding the scFv and synthesized using TRIM technology were incorporated into a pADL-10b phagemid. This phagemid contains an F1 origin, a transcriptional repressor to limit un-induced expression, a lac operator, and a lac repressor. The scFv was synthesized with a pelB periplasmic secretion signal and was subcloned downstream of the lac operator. A linker (GGGSGGGGSGGGAS; SEQ ID NO:698) connects the variable light and heavy chains of the scFv. A FLAG (DYKDDDDK; SEQ ID NO:190) epitope tag was placed immediately downstream of the variable heavy chain, which was followed in frame by the full-length M13 pIII coat protein sequence.

Figures 67A-67C. Biological and biophysical characteristics of scFv clone H2. (A) H2-scDb binding to immobilized p53^{R175H}/HLA-A*02:01 (red) or p53^{WT}/HLA-A*02:01 (gray) pHLA was assessed by ELISA. Data shown represent mean $\pm$ SD of three technical replicates. (B) H2-scDb binding to p53^{R175H}/HLA-A*02:01 was measured by single-cycle kinetics using SPR. H2-scDb was loaded at increasing concentrations, from 3, 12, 50, 200 to 800 nM. The blank- and reference-subtracted binding is shown for p53^{R175H}/HLA-A*02:01 (red) and p53^{WT}/HLA-A*02:01 (gray). H2-scDb binds to the p53^{R175H}/HLA-A*02:01 pHLA with a one-to-one binding kinetics at a K_D of 86 nM (fitted line in black). There was negligible p53^{WT}/HLA-A*02:01 binding. (C) T2 cells pulsed with p53^{R175H} or p53^{WT} peptide were co-incubated with H2-scDb and T cells at an effector:target (E:T) ratio of 2:1. IFN- γ release was measured by ELISA (left) and cell lysis was evaluated by the CellTiter-Glo assay (right). Data indicate mean $\pm$ SD of three technical replicates and are representative of three independent experiments.

Figures 68A-68D. H2-scDb activates T cells in the presence of tumor cells presenting p53^{R175H}. (A) Illustration depicting the mechanism of action of H2-scDb. (B) HLA-A*02:01 positive tumor cell lines with different HLA expression levels and p53^{R175H} status were co-incubated with H2-scDb and T cells at an E:T ratio of 2:1. IFN- γ release was measured by ELISA. Data indicate mean $\pm$ SD of six technical replicates and are representative of two independent experiments. The HLA-A*02 median fluorescence intensity (MFI) ratio is defined as MFI (anti-HLA-A*02)/MFI (isotype control). (C) Polyfunctional T-cell activation mediated by H2-scDb in response to KMS26 at an E:T ratio of 2:1 was assessed by luminescent cytotoxicity and antibody-based assays (see Supplementary Materials). EC₅₀ (M) for each assay is shown in the corresponding graphs. Data indicate mean $\pm$ SD of three technical replicates and are representative of two independent experiments. (D) Real-time live-cell imaging of T cells with GFP-labelled TYK-nu at an E:T ratio of 5:1 with or without H2-scDb. Representative phase contrast and green fluorescence images taken at 24 hours (top) and 96 hours (bottom) after mixing cells are shown.

Figures 69A-69E. Determination of H2-scDb specificity using isogenic target cell lines. (A) Methods of the generating of isogenic cell line pairs in cells with different HLA and p53 backgrounds. (B) HEK293FT and Saos-2 cell lines that were transfected with full-length p53^{WT}, full-length p53^{R175H}, or were not transfected were co-incubated with T cells at an E:T ratio of 2:1 in the presence of increasing amounts of H2-scDb. IFN- γ release was measured by ELISA. Data indicate mean $\pm$ SD of two technical replicates. (C) Cell lines expressing p53^{R175H} and transduced or not transduced with HLA-A*02:01 were co-incubated with T-cells and H2-scDb. IFN- γ release was measured by ELISA. Experiments were performed at an E:T ratio of 2:1 in three technical replicates. (D) IFN- γ release mediated by H2-scDb in response to parental tumor cell lines and their *TP53* KO counterparts at an E:T ratio of 2:1 (KMS26, TYK-nu) or 5:1 (KLE) was measured by ELISA. Data indicate mean $\pm$ SD of two (TYK-nu) or three (KMS26, KLE) technical replicates and are representative of two independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by two-tailed t-test. (E) Parental (left) or *TP53* KO (right) TYK-nu cells labeled with nuclear GFP were co-incubated with H2-scDb and T cells at an E:T ratio of 2:1 was measured by real-time live-cell imaging. Data indicate mean $\pm$ SEM of twelve technical replicates. One-way ANOVA with Tukey's multiple comparison was used to evaluate statistical significance, **** denotes $P < 0.0001$.

Figures 70A-70H. H2-Fab binds to the HLA-A*02:01 and the C-terminus of the p53^{R175H} neoantigen. (A) Overall structure of p53^{R175H}/HLA-A*02:01 bound to the H2-Fab fragment (PDB ID 6W51). HLA-A*02:01 and β 2 microglobulin (β 2M) are colored in gray and gold, respectively. The H2-Fab is colored according to the heavy (blue) and light (cyan) chains of the Fab fragment. The p53^{R175H} nine amino acid peptide is shown in light green between helices α 1 and α 2 of the HLA. (B) Structure of H2-Fab-p53^{R175H}/HLA-A*02:01 at 90° to that shown in (A). (C) Electron density map (2mFo-DFc) of the p53^{R175H} neoantigen contour at 1 σ . (D) Electron density map (2mFo-DFc) of a selected area of the H2-Fab at CDR-L3 from residues 95 to 99 contoured at 1 σ . (E) Zoom in of the interaction of H2-Fab to p53^{R175H}/HLA-A*02:01 with CDRs colored as in (A). The CDRs are labeled and colored in order from left to right: H2 (purple), H1 (magenta), L3 (yellow), H3 (orange), L1 (red), L2 (dark green). (F) Bird's-eye view of surface representation of the HLA-A*02:01 shown in grey, p53^{R175H} peptide shown in light green, and the contacting residues colored according to CDRs of the H2-Fab. (G) Schematic representation of (F). (H) Diagram of the orientation angle of the H2-Fab to p53^{R175H}/HLA-A*02:01. The docking angle of the orientation was calculated from the web server TCR3d which was based on the C^{alpha} of Cys88 of the disulfide bond of the V_L domain and the C^{alpha} of Cys96 of the disulfide bond of the V_H domain of the H2-Fab (red). The arrowed line indicates the direction of orientation with the angle between them.

Figures 71A-71F. Structural basis of H2 specificity and identification of putative cross-reactive peptides. (A) Detailed interactions of the p53^{R175H} neoantigen with HLA-A*02:01. The peptide (green) and the side chains (grey) of interacting residues of HLA-A*02:01 are represented as sticks. Hydrogen bonds are shown as dashed lines. (B) Perpendicular view of the p53^{R175H} peptide binding cleft. (C) C-terminus of the peptide (aa Val173-Cys176) with Arg174 and His175 surrounded by the interacting residues of CDR-H1 (magenta), -H2 (purple), -H3 (orange) and -L3 (yellow) shown as sticks. Hydrogen bonds are shown as dashed lines. (D) T2 cells were loaded with 10 μ M of HMTEVVRHC (SEQ ID NO:1) peptide variants from the positional scanning library and co-incubated with 1 nM H2-scDb and T cells at an E:T ratio of 2:1. IFN- γ release was measured by cytometric bead array (see Supplementary Materials) and the mean of triplicate wells was used to plot the heatmap. Black boxes represent the parental p53^{R175H} peptide. (E) Illustration of the binding pattern of H2-scDb as Seq2Logo graph, calculated by dividing the IFN- γ value by 10⁴ and using the

PSSM-Logo algorithm. (F) T2 cells were loaded with 10 μ M of p53^{R175H}, p53^{WT}, STAT2, VPS13A, or ZFP3 peptide and co-incubated with 1 nM H2-scDb and T cells at an E:T ratio of 2:1. IFN- γ secretion was measured by ELISA. Data indicate mean $\pm$ SD of three technical replicates.

5 Figures 72A-72B. *In vivo* antitumor efficacy of H2-scDb. In the early treatment model, NSG mice were engrafted with 1×10^7 human T cells and either 1×10^6 parental KMS26 (A) or 1×10^6 TP53 KO KMS26 (B) on day 0. On day 1, intraperitoneal infusion pumps were placed to administer H2-scDb or isotype control scDb. (C) In the established tumor model, mice were engrafted with 1×10^7 human T cells and 3.5×10^5 parental KMS26
10 on day 0, followed by administration of H2-scDb or isotype scDb at the specified doses on day 6. Tumor growth was monitored by bioluminescence imaging. N = 4 or 5 mice per group. Color bars denote the radiance (p/sec/cm²/sr) scale at each time point. Plotted data indicate mean $\pm$ SD. ***P* < 0.01 and NS denotes no statistical significance compared to isotype control by multiple t-test with Holm-Šidák correction.

15 Figures 73A-73C. Detection and quantification of p53^{R175H} neoantigen peptide in cells. (A) COS-7 cells transfected with constructs expressing HLA-A*02:01 and p53^{WT} or p53^{R175H} were analyzed for the presentation of the p53^{WT} HMTEVVRRC (SEQ ID NO:135) or the p53^{R175H} HMTEVVRHC (SEQ ID NO:1) peptide. Isotope labeled peptides were spiked into the assay and served as standards for absolute copy number quantification.
20 Multiple ions (indicated by different colors) fragmented from the target peptide in each sample were measured through mass spectrometer as different SRM transitions and their m/Z values were listed in the figure legend. (B) Expression of p53 protein in COS-7 cells transfected with either the full-length p53^{WT} or p53^{R175H} was assessed by Western blotting with anti-p53 antibody (clone DO-1). (C) Cells lines with endogenous HLA-A*02:01 and
25 p53^{R175H} expression were analyzed for the presentation of p53^{R175H} neoantigen peptide as described in (A).

 Figures 74A-74D. Selection of p53^{R175H}/HLA-A*02:01 reactive antibodies and their conversion into T cell-retargeting scDb. (A) Flow cytometric screening of phage clones enriched by panning. After 5 rounds of panning, phage clones from the enriched phage pool
30 were isolated by limiting dilution and grown in deep 96-well plates. Supernatants containing individual phage clones were used to assess binding to T2 cells loaded with β 2 microglobulin (β 2M) only, β 2M plus p53^{WT} peptide (HMTEVVRRC; SEQ ID NO:135), or β 2M plus

p53^{R175H} peptide (HMTEVVRHC; SEQ ID NO:1) via flow cytometry. The median fluorescence intensity (MFI) ratio was defined as MFI (p53^{R175} peptide)/MFI (p53^{WT} peptide). NC, no phage control. (B) Schematic representation of the structure of the T cell-engaging bispecific single-chain diabody (scDb) used in our experiments. V_L, variable light domain; V_H, variable heavy domain; pHLA, peptide-HLA complex; SL, short linker; LL, long linker. The graph was created with BioRender.com. (C) Screening of scDb clones via IFN- γ stimulation by p53-expressing cells. scDbs generated by linking each anti-p53^{R175H}/HLA-A*02:01 pHLA scFv clone with an anti-CD3 scFv (UCHT1) were co-incubated with T cells and COS-7 cells transfected with GFP, HLA-A*02:01 + GFP, HLA-A*02:01 + p53^{WT}, or HLA-A*02:01 + p53^{R175H} plasmids at an effector:target (E:T) ratio of 1:1. After a 20-hr incubation, the supernatant was harvested for IFN- γ detection by ELISA. Arrows indicate clones H2 and H20. A2, HLA-A*02:01. (D) Characterization of H20-scDb. H20-scDb was incubated with biotinylated p53^{R175H}/HLA-A*02:01 (red) and p53^{WT}/HLA-A*02:01 (gray) pHLA monomers coated on streptavidin microplates at the specified concentrations, then binding detected with protein L and anti-protein L HRP. Data indicate mean $\pm$ SD of three technical replicates.

Figures 75A-75E. Characteristics of scDbs generated by linking H2-scFv with anti-CD3 ϵ scFvs. (A) Expression of scDbs composed of linking H2-scFv with different anti-CD3 ϵ scFvs was assessed by anti-6x-His tag Western blotting. (B) Binding of the scDbs to the CD3 ϵ/δ heterodimer and CD ϵ was compared using ELISA. (C) The scDbs were co-incubated with T cells and T2 cells pulsed with titrated concentrations of p53^{R175H} or p53^{WT} peptide at an E:T ratio of 2:1. IFN- γ release was measured by ELISA. Data indicate mean $\pm$ SD of three technical replicates. (D) Analytical chromatogram of the purified H2-UCHT1-scDb (H2-scDb) showing absorbance at 280 nm. The retention time of the H2-scDb was marked above the peak. (E) DSF analysis of the negative derivative (RFU vs. temperature) of the H2-scDb. The melting temperature T_m at 69 °C corresponds to the peak/maximum of the first derivative of the curve and the notion of one transition state.

Figures 76A-76F. Reactivity of H2-scDb against p53^{R175H}/HLA-A*02:01-expressing tumor cells. (A) TYK-nu and its cisplatin resistant subline TYK-nu.CP-r (75) were cultured with H2-scDb and T cells at an E:T ratio of 2:1. IFN- γ release was measured by ELISA. Data indicate mean $\pm$ SD of six technical replicates and are representative of two independent experiments. (B, C) KMS26 cells were cultured with H2-scDb or an isotype scDb (scFv

against an irrelevant pHLA linked with UCHT1 scFv) in the absence or presence of T cells at an E:T ratio of 2:1. IFN- γ release was measured by ELISA (B), and cytotoxicity was assessed by luciferase assay (C). Data indicate mean $\pm$ SD of three technical replicates. ** $P < 0.01$, *** $P < 0.001$ by two tailed t-test. (D-F) H2-scDb-induced polyfunctional T-cell response. T-cell cytotoxicity and cytokine release mediated by H2-scDb in response to (D) KMS26 (cytotoxicity and other effector proteins shown in main text), (E) KLE, and (F) TYK-nu cell line at an E:T ratio of 2:1 was assessed by antibody-based assays (see Methods). EC₅₀ (M) for each analyte is shown in the corresponding graphs. Data indicate mean $\pm$ SD of three technical replicates.

Figures 77A-77B. Flow cytometric evaluation of HLA-A*02 expression. (A) Expression of HLA-A*02 on tumor cell lines was evaluated by flow cytometry. The red histogram represents staining with anti-HLA-A*02 (clone BB7.2), and the gray histogram represents staining with an isotype control. The median fluorescence intensity (MFI) ratio is defined as MFI (anti-HLA-A*02)/MFI (isotype control). (B) HLA-A*02:01-encoding retrovirus was transduced into cell lines that weakly (AU565, SK-BR-3) or do not detectably (HuCCT1, CCRF-CEM) express HLA-A*02:01. Expression of HLA-A*02:01 in the parental and transduced and sorted cell lines was evaluated by flow cytometry. The red histogram represents staining with anti-HLA-A*02 (clone BB7.2) and the gray histogram represents staining with an isotype control.

Figures 78A-78B. Determination of H2-scDb specificity using CRISPR-edited isogenic cell lines. (A) Expression of p53 protein in the parental and *TP53* KO clones of KMS26, KLE, and TYK-nu was assessed by Western blot with anti-p53 antibody (clone DO-1). (B) Cytotoxicity mediated by H2-scDb in response to parental tumor cell lines and their *TP53* KO counterparts at an E:T ratio of 2:1 (KMS26, TYK-nu) or 5:1 (KLE) was measured by the Bio-Glo (KMS-26) or CellTiter-Glo (TYK-nu, KLE) assay. Data indicate mean $\pm$ SD of two (TYK-nu) or three (KMS26, KLE) technical replicates and are representative of two independent experiments.

Figures 79A-79D. The H2-Fab-p53^{R175H}/HLA-A*02:01 complex. (A) H2-scFv was converted into full-length IgG (H2-IgG) and incubated with biotinylated p53^{R175H}/HLA-A*02:01 (red) and p53^{WT}/HLA-A*02:01 (gray) pHLA monomers coated on streptavidin microplates at the specified concentrations followed by detection with anti-human IgG HRP. Data indicate mean $\pm$ SD of three technical replicates. (B) Illustration depicting the

generation of H2-Fab from H2-IgG. (C) Size-exclusion chromatogram of the pHLA-A*02:01 in complex with the H2-Fab. Protein was monitored by A280 nm with a major peak (~100 kDa). (D) Coomassie-stained gradient SDS-PAGE gel of the eluted fractions at 11-17 mL from (C).

5 Figures 80A-80D. The neoantigen p53^{R175H} binds to HLA-A*02:01 in a canonical fashion. (A) Bird's-eye view of the p53^{R175H} neoantigen interactions with HLA-A*02:01. The peptide (green) and the side chains (grey) of interacting residues of HLA-A*02:01 are represented as sticks. Hydrogen bonds are shown as dashed lines. The N-terminal His168 is anchored by three tyrosine residues of HLA-A*02:01, one at the base of the cleft (Tyr7, not shown) and two on α 2 (Tyr159, 171), while its side chain is within hydrogen bonding distance of Lys66 (α 1) and Thr163 (α 2, not shown). Glu63 of HLA-A*02:01 α 1 forms a hydrogen bond with the backbone amino of Met169, an anchor residue of p53^{R175H} that is situated within the hydrophobic B pocket of the HLA. The main chain of Thr170 is stabilized by a hydrogen bond to Tyr99 (not shown), located at the base of the cleft while the side chain of Glu4 forms a salt-bridge with the side chain of Arg65. Positions 5-8 (Val172, Val173, Arg174, His175) of the p53^{R175H} neoantigen are stabilized by multiple hydrophobic and aliphatic residues with no direct hydrogen bonding contacts to the HLA-A*02:01. Towards the C-terminus of the neoantigen, the carboxyl group of Cys176, another anchor residue that lies within the F pocket, is secured by Tyr84 (α 1) and Lys146 (α 2), while the side chain sulfhydryl is near Thr143 on α 2. (B) Surface representation of the HLA-A*02:01 (grey) with the p53^{R175H} neoantigen shown in green as sticks. Anchor pockets B and F are circled in orange. (C) Structural alignment of the following HLA-A*02:01-bound peptides in the binding pocket: green (this work, PDB ID 6W51), cyan (p1049, PDB ID 2JCC), magenta (NY-ESO-1, PDB ID 3HAE), and light purple (WT1, PDB ID 4WUU). (D) Zoomed in view of (B) with helix α 1 transparent and residues at positions 7 (P7) and 8 (P8) shown as sticks.

Figure 81. Hydrogen bonding pattern of the H2-Fab cage-like configuration. The imidazole ring of His175 was at the center of the cage-like structure. The guanidinium group of Arg174 was at hydrogen bonding distance to the backbone carbonyl of Ala31 (CDR-H1). Another neoantigen-antibody direct contact involves the backbone carbonyl of Val173 hydrogen bonding with the side chain of Arg93 (CDR-L3).

Figures 82A-82P. Comparison of the binding orientations among TCRm-pHLA and TCR-pHLA complexes. (A) Close depiction of binding of the H2-Fab to p53^{R175H}/HLA-

A*02:01 with CDRs colored as in Fig. 4. (B) Binding of a TCR to melanoma-associated antigen 3 (MAGE-A3) and HLA-A*01:01 (PDB ID 5BRZ). Same orientation as (A). The MAGE-A3 TCR displays the canonical, diagonal binding motif to that of most known TCR topologies. (C) Recognition of the 3M4E4 Fab for the NY-ESO-1₁₅₇₋₁₆₅/HLA-A*02:01 complex (PDB ID 3HAE). Same orientation as (A). (D) Binding of the ESK1 Fab to Wilms tumor 1 peptide and HLA-A*02:01 (PDB ID 4WUU). Same orientation as (A). (E, F, G and H) Bird's-eye view of surface representation of the HLA-A*02:01/*01:01 colored in grey with the contacting residues of H2-Fab, MAGE-A3 TCR, 3M4E4 Fab, and ESK1 Fab, respectively, colored according to CDRs. (I, J, K and L) Schematic representation of E, F, G, and H, respectively. H2-Fab-p53^{R175H}/HLA-A*02:01 shows a different mode of antibody recognition compared with other Fab/TCR-pHLA complexes. Schematic representation of Fab/TCR docking angle. The docking angle was calculated from the web server TCR3d which was based on the C^{alpha} of Cys88 (or equivalent) of the disulfide bond of the V_L/α domain and the C^{alpha} of Cys96 (or equivalent) of the disulfide bond of the V_H/β domain of each antibody and TCR. The arrowed line indicates the direction of orientation with the angle between them.

Figures 83A-83D. Assessment of H2-scDb cross-reactivity. (A) A peptide library was generated by systemically substituting the amino acid at each position of the target peptide (HMTEVVRHC; SEQ ID NO:1) with each of the remaining 19 common amino acids. T2 cells were loaded with each of the variant peptides at 100 μM in the presence of 10 μg/ml β2M and anti-HLA-A*02 antibody (clone BB7.2). HLA-A*02:01 stabilized by peptide binding and was evaluated by flow cytometry (77). Black boxes represent the parental peptide. MFI, median fluorescence intensity. (B) Recognition of the p53^{R175H} positional scanning library peptides by H2-scDb. T2 cells loaded with variant peptides in the positional scanning library were incubated with 1nM H2-scDb and T cells at an E:T ratio of 2:1. IFN-γ release was measured by ELISA. Dotted lines represent 20% of parental peptide IFN-γ value. The binding motif established by the 20% reactivity cutoff, expressed in PROSITE pattern, was x-[AILMVNQTC]-[ST]-[DE]-[IV]-[IMVST]-R-H-[AILVGHSTYC] (SEQ ID NO:197). Data indicate mean ± SD of three technical replicates. (C-D) H2-scDb was co-incubated with T cells and COS-7 cells transfected with HLA-A*02:01 and full-length p53^{R175H}, STAT2, or ZFP3 at an E:T ratio of 5:1. (C) Expression of target proteins by COS-7 cells was assessed by Western blot staining. (D) IFN-γ secretion was measured by ELISA. The signals for all of the

transfectants except for p53^{R175H} were indistinguishable and are clustered near the x-axis. Data indicate mean $\pm$ SD of three technical replicates and are representative of two independent experiments.

Figures 84A-84D. Assessing *in vivo* efficacy of scDb in NSG mice. (A) Three days
 5 after the injection of KMS26 cells and human T cells, peripheral blood of mice was obtained to assess human T cell engraftment by flow cytometry. Plots shown were gated on live cells. (B) Plasma of mice was collected 3 days before and 3 and 10 days after the implantation of intraperitoneal pumps. Plasma concentration of H2-scDb was measured by ELISA. N = 9 mice. Data shown represent mean $\pm$ SEM (C) Serial monitoring of body weight of the mice
 10 in the established KMS26 model presented in Fig. 6B. N = 5 mice per group. Data shown represent mean $\pm$ SD. (D) To verify the action of H2-scDb is T cell-dependent, NSG mice were engrafted with 5×10^5 parental KMS26 cells on day 0 with and without 1×10^7 human T cells, followed by administration of the specified scDb or vehicle via intraperitoneal pumps on day 6. Tumor growth was monitored by bioluminescence imaging. N = 4 or 5 mice per
 15 group. Data shown represent mean $\pm$ SD.

DETAILED DESCRIPTION

This document provides methods and materials for assessing a mammal having cancer or suspected of having cancer and/or treating a mammal having cancer. For example, one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can
 20 target (*e.g.*, bind to) one or more modified peptides (*e.g.*, peptides present in a peptide-HLA complex such as a peptide-HLA- β 2M complex) can be used to assess a mammal having cancer or suspected of having cancer and/or to treat a mammal having a cancer (*e.g.*, a cancer expressing one or more modified peptides). In some cases, one or more molecules includes one or more antigen-binding domains that can bind to a modified peptide can be used to
 25 detect the presence or absence of one or more modified peptides in a sample obtained from a mammal having cancer or suspected of having cancer. In some cases, one or more molecules including one or more antigen-binding domains that can bind to a modified peptide can be administered to a mammal having a cancer (*e.g.*, a cancer expressing the modified peptide) to treat the mammal.

30 As used herein, a modified peptide is a peptide derived from a modified polypeptide. A modified polypeptide can be any appropriate modified polypeptide (*e.g.*, a polypeptide

having a disease-causing mutation such as a mutation in an oncogenic or a mutation in a tumor suppressor gene). A modified peptide can have one or more amino acid modifications (*e.g.*, substitutions) relative to a WT peptide (*e.g.*, a peptide derived from a WT polypeptide from which the modified polypeptide is derived). A modified peptide also can be referred to

5 as a mutant peptide. In some cases, a modified peptide can be a tumor antigen. Examples of tumor antigens include, without limitation, MANAs, tumor-associated antigens, and tumor-specific antigens. A modified peptide can be any appropriate length. In some cases, a modified peptide can be from about 7 amino acids to about 25 amino acids (*e.g.*, from about 8 amino acids to about 25 amino acids, from about 9 amino acids to about 25 amino acids,

10 from about 10 amino acids to about 25 amino acids, from about 11 amino acids to about 25 amino acids, from about 12 amino acids to about 25 amino acids, from about 13 amino acids to about 25 amino acids, from about 15 amino acids to about 25 amino acids, from about 18 amino acids to about 25 amino acids, from about 20 amino acids to about 25 amino acids, from about 7 amino acids to about 22 amino acids, from about 7 amino acids to about 20

15 amino acids, from about 7 amino acids to about 18 amino acids, from about 7 amino acids to about 15 amino acids, from about 7 amino acids to about 12 amino acids, from about 7 amino acids to about 10 amino acids, from about 7 amino acids to about 9 amino acids, from about 8 amino acids to about 22 amino acids, from about 10 amino acids to about 18 amino acids, from about 12 amino acids to about 15 amino acids, from about 8 amino acids to about 12

20 amino acids, from about 12 amino acids to about 18 amino acids, from about 18 amino acids to about 22 amino acids, or from about 9 amino acids to about 10 amino acids) in length. For example, a modified peptide can be about 9 amino acids in length. For example, a modified peptide can be about 10 amino acids in length. A modified peptide can be derived from any modified polypeptide. Examples of modified polypeptides from which modified peptides

25 described herein can be derived include, without limitation, p53 and RAS (*e.g.*, KRAS, HRAS, and NRAS). A modified peptide can include any appropriate modification. In some cases, modified peptides described herein can include one or more modifications (*e.g.*, mutations) shown in Table 1.

Table 1. Modified peptides.

Protein of origin	Mutation	Mutant Peptide	SEQ ID NO:	WT peptide	SEQ ID NO:	Peptide Codons	HLA Allele
p53	R175H	HMTEVVRHC	1	HMTEVVRRC	135	168-176	A*02:01
H/K/N RAS	Q61H	ILDTAGHEEY	2	ILDTAGQEEY	136	55-64	A*01:01
H/K/N RAS	Q61L	ILDTAGLEEY	3	ILDTAGQEEY	136	55-64	A*01:01
H/K/N RAS	Q61R	ILDTAGREEY	4	ILDTAGQEEY	136	55-64	A*01:01

A modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4) can be in a complex with any appropriate HLA. An HLA can be any appropriate HLA allele. In some cases, an HLA can be a class I HLA (*e.g.*, HLA-A, HLA-B, and HLA-C) allele. In some cases, an HLA can be a class II HLA (*e.g.*, HLA-DP, HLA-DM, HLA-DOA, HLA-DOB, HLA-DQ, and HLA-DR) allele. Examples of HLA alleles that a modified peptide described herein can complex with include, without limitation, HLA-A1 and HLA-A2. Exemplary HLA alleles for particular modified peptides are shown in Table 1. For example, a modified peptide derived from a modified p53 polypeptide (*e.g.*, HMTEVVRHC (SEQ ID NO:1)) can be in a complex with HLA-A2 and β 2M. For example a modified peptide derived from a modified H/K/N RAS polypeptide (*e.g.*, ILDTAGHEEY (SEQ ID NO:2), ILDTAGLEEY (SEQ ID NO:3), and ILDTAGREEY (SEQ ID NO:4)) can be in a complex with HLA-A1 (*e.g.*, can be in a complex with HLA-A1 and β 2M).

This document provides molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4). In some cases, a molecule including one or more antigen-binding domains that can bind to a modified peptide described herein does not target (*e.g.*, does not bind to) an uncomplexed modified peptide described herein (*e.g.*, a modified peptide described herein that is not present in a complex (*e.g.*, a peptide-HLA- β 2M complex)). In some cases, a molecule including one or more antigen-binding domains that can bind to a modified peptide described herein does not target (*e.g.*, does not bind to) a WT peptide (*e.g.*, a peptide derived from a WT polypeptide from which the modified polypeptide is derived).

A molecule including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein can be any appropriate type of molecule. In some cases, a molecule can be a monovalent molecule (*e.g.*, containing a single antigen-binding domain). In some cases, a molecule can be a multivalent molecule (*e.g.*, containing two or more antigen-binding domains and simultaneously targeting two or more antigens). For example, a bispecific molecule can include two antigen-binding domains, a trispecific molecule can include three antigen-binding domains, a quadruspecific molecule can include four antigen-binding domains, etc. Examples of molecules that contain antigen-binding domains include, without limitation, antibodies, antibody fragments, scFvs, chimeric antigen receptors (CARs), T cell receptors (TCRs), TCR mimics, tandem scFvs, bispecific T cell engagers, diabodies, scDb, scFv-Fcs, bispecific antibodies, bispecific single-chain Fcs, dual-affinity re-targeting antibodies (DARTs), and any other molecule that includes at least one variable heavy chain (VH) and at least one variable light chain (VL). Any of these molecules can be used in accordance with materials and methods described herein. In some cases, an antigen-binding domain can be a scFv. For example, a molecule including one or more antigen-binding domains (*e.g.*, one or more scFvs) that can bind to a modified peptide described herein can be a CAR. For example, a molecule including two scFvs that can bind to a modified peptide described herein can be a single-chain diabody (scDb).

In some cases, when a molecule including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4) is a multivalent molecule (*e.g.*, a bispecific molecule), a first antigen-binding domain can bind to a modified peptide described herein and a second antigen-binding domain can bind to an effector cell (*e.g.*, an antigen present on an effector cell). Examples of effector cells include, without limitation, T cells, natural killer (NK) cells, natural killer T (NKT) cells, B cells, plasma cells, macrophages, monocytes, microglia, dendritic cells, neutrophils, fibroblasts, and mast cells. Examples of antigens present on effector cells include, without limitation, CD3, CD4, CD8, CD28, NKG2D, PD-1, CTLA-4, 4-1BB, OX40, ICOS, CD27, Fc receptors (*e.g.*, CD16a), and any other effector cell surface receptors. In some cases, a molecule described herein can include a first antigen-binding domain that can bind to a modified peptide described herein and a second antigen-binding domain that can bind to an antigen present on a T cell (*e.g.*, CD3). In some cases, sequences

(*e.g.*, scFv sequences) that can bind to CD3 can be as shown in Table 4. In some cases, sequences (*e.g.*, scFv sequences) that can bind to CD3 can be as described elsewhere (see, *e.g.*, Rodrigues *et al.*, 1992 *Int J Cancer Suppl.* 7:45-50; Shalaby *et al.*, 1992 *J Exp Med.* 175:217-25; Brischwein *et al.*, 2006 *Mol Immunol.* 43:1129-43; Li *et al.*, 2005 *Immunology.* 116:487-98; WO2012162067; US20070065437; US20070065437; US20070065437; US20070065437; US20070065437; and US20070065437). In some cases, a molecule described herein can include a first antigen-binding domain that can bind to a modified peptide described herein and a second antigen-binding domain that can bind to an antigen present on a NK cell (*e.g.*, CD16a or NKG2D). In some cases, sequences (*e.g.*, scFv sequences) that can bind to CD16a can be as shown in Table 5. By binding both the modified peptide and the effector cell, the multivalent molecule can bring the cell expressing the modified peptide (*e.g.*, as part of the HLA complex) into proximity with the effector cell, permitting the effector cell to act on the cell expressing the modified peptide.

In some cases, when a molecule including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4) is a multivalent molecule (*e.g.*, a bispecific molecule), a molecule can be in any appropriate format which includes at least one VH and at least one VL. For example, a VH and a VL can be in any appropriate orientation. In some cases, a VH can be N-terminal to the VL. In some cases, a VH can be C-terminal to the VL. In some cases, a linker amino acid sequence can be positioned between the VH and VL.

In some cases, when a bispecific molecule includes tandem scFvs, the tandem scFvs can be in any appropriate orientation. Examples of tandem scFv orientations including scFv-A and scFv-B include, without limitation, VLA-LL-VHA-SL-VLB-LL-VHB, VLA-LL-VHA-SL-VHB-LL-VLB, VHA-LL-VLA-SL-VLB-LL-VHB, VHA-LL-VLA-SL-VHB-LL-VLB, VLB-LL-VHB-SL-VLA-LL-VHA, VLB-LL-VHB-SL-VHA-LL-VLA, VHB-LL-VLB-SL-VLA-LL-VHA, and VHB-LL-VLB-SL-VHA-LL-VLA, where SL is a short linker and LL is a long linker. A short linker can be from about 3 amino acids to about 10 amino acids in length. A short linker can include any appropriate amino acids (*e.g.*, glycines and serines) in any appropriate combination. A long linker can be from about 10 amino acids to about 25 amino acids in length. A long linker can include any appropriate amino acids (*e.g.*, glycines and serines) in any appropriate combination.

In some cases, when a bispecific molecule is a diabody, the diabody can be in any appropriate orientation. Examples of diabody orientations including scFv-A and scFv-B include, without limitation, VLA-SL-VHB and VLB-SL-VHA, VLA-SL-VLB and VHB-SL-VHA, VHA-SL-VLB and VHB-SL-VLA, VLB-SL-VHA and VLA-SL-VHB, VLB-SL-VLA and VHA-SL-VHB, and VHB-SL-VLA and VHA-SL-VLB, where SL is a short linker. A short linker can be from about 3 amino acids to about 10 amino acids in length. A short linker can include any appropriate amino acids (*e.g.*, glycines and serines) in any appropriate combination.

In some cases, when a bispecific molecule is a scDb, the scDb can be in any appropriate orientation. Examples of scDb orientations including scFv-A and scFv-B include, without limitation, VLA-SL-VHB-LL-VLB-SL-VHA, VHA-SL-VLB-LL-VHB-SL-VLA, VLA-SL-VLB-LL-VHB-SL-VHA, VHA-SL-VHB-LL-VLB-SL-VLA, VLB-SL-VHA-LL-VLA-SL-VHB, VHB-SL-VLA-LL-VHA-SL-VLB, VLB-SL-VLA-LL-VHA-SL-VHB, and VHB-SL-VHA-LL-VLA-SL-VLB, where SL is a short linker and LL is a long linker. A short linker can be from about 3 amino acids to about 10 amino acids in length. A short linker can include any appropriate amino acids (*e.g.*, glycines and serines) in any appropriate combination. A long linker can be from about 10 amino acids to about 25 amino acids in length. A long linker can include any appropriate amino acids (*e.g.*, glycines and serines) in any appropriate combination.

In some cases, when a bispecific molecule is a scFv-Fc, the scFv-Fc can be in any appropriate orientation. Examples of scFv-Fc orientations including scFv-Fc-A, scFv-Fc-B, and an Fc domain include, without limitation, VLA-LL-VHA-hinge-Fc and VLB-LL-VHB-hinge-Fc, VHA-LL-VLA-hinge-Fc and VHB-LL-VLB-hinge-Fc, VLA-LL-VHA-hinge-Fc and VHB-LL-VLB-hinge-Fc, VHA-LL-VLA-hinge-Fc and VLB-LL-VHB-hinge-Fc, where LL is a long linker. A long linker can be from about 10 amino acids to about 25 amino acids in length. A long linker can include any appropriate amino acids (*e.g.*, glycines and serines) in any appropriate combination. In some cases, an Fc domain in a scFv-Fc can include one or more modifications to increase heterodimerization and/or to decrease homodimerization of the scFv-Fc. In some cases, an Fc domain in a scFv-Fc can exclude a hinge domain. In some cases, an Fc domain in a scFv-Fc can be at the N-terminus of the scFv.

In some cases, when a bispecific molecule is a bispecific single-chain Fc, the bispecific single-chain Fc can be in any appropriate orientation. Examples of bispecific

single-chain Fc orientations include, without limitation, VLA-LL-VHA-SL-VHB-LL-VLB-SL-hinge-CH2-CH3-LL-hinge-CH2-CH3, VLA-LL-VHA-SL-VLB-LL-VHB-SL-hinge-CH2-CH3-LL-hinge-CH2-CH3, VHA-LL-VLA-SL-VLB-LL-VHB-SL-hinge-CH2-CH3-LL-hinge-CH2-CH3, VHA-LL-VLA-SL-VHB-LL-VLB-SL-hinge-CH2-CH3-LL-hinge-CH2-CH3, and VLA-SL-VHB-LL-VLB-VHA-SL-hinge-CH2-CH3-LL-hinge-CH2-CH3, where SL is a short linker and LL is a long linker. A short linker can be from about 3 amino acids to about 8 amino acids in length. A short linker can include any appropriate amino acids (*e.g.*, glycines and serines) in any appropriate combination. A long linker can be from about 10 amino acids to about 25 amino acids in length. A long linker can include any appropriate amino acids (*e.g.*, glycines and serines) in any appropriate combination. Any appropriate Fc domain can be used in a bispecific single-chain Fc. In some cases, an Fc domain can include an amino acid sequence derived from an IgG (*e.g.*, a natural IgG). In some cases, an Fc domain can include an amino acid sequence that includes one or more modifications (*e.g.*, one or more modifications to increase stability of the molecule and/or to increase or decrease binding to one or more Fc receptors). In some cases, an Fc domain that can be used in a bispecific single-chain Fc can exclude a hinge domain. In some cases, an Fc domain that can be used in a bispecific single-chain Fc can be at the N-terminus of the scFvs. In some cases, an Fc domain that can be used in a bispecific single-chain Fc can be as described elsewhere (see, *e.g.*, International Patent Application Publication No. WO 2017/134134 A1 at, for example, SEQ ID NOs:25-32; and International Patent Application Publication No. WO 2017/134158 A1 at, for example, Table 38; and SEQ ID NOs:25-32).

A molecule including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4) can include any appropriate complementarity determining regions (CDRs). For example, a molecule including one or more antigen-binding domains that can bind to a modified peptide described herein can include a variable heavy chain (VH) having three VH complementarity determining regions (CDR-VHs) and a variable light chain (VL) having three VL CDRs (CDR-VLs). For example, a molecule that can bind to a modified peptide derived from a modified p53 polypeptide (*e.g.*, HMTEVVRHC (SEQ ID NO:1)) can include one of each of the CDRs set forth below:

CDR-VL1: QDVNTA (SEQ ID NO:5);

CDR-VL2: SAS and SAY;

CDR-VL3: QQYSRYSPVTF (SEQ ID NO: 6), QQQSSTPVTF (SEQ ID NO: 7),
 QQSSYYPNTF (SEQ ID NO: 8), QQQWSSPDTF (SEQ ID NO: 9), QQSNAYPITF (SEQ
 5 ID NO: 10);

CDR-VH1: GFNVYASGM (SEQ ID NO: 11), GFNVYQSDM (SEQ ID NO: 12),
 GFNLYQRDM (SEQ ID NO: 13), GFNLSYYDM (SEQ ID NO: 14), GFNLNSYYM (SEQ
 ID NO: 15);

CDR-VH2: KIYPDSYTY (SEQ ID NO: 16), TIWPYSGYTY (SEQ ID NO: 17),
 10 GLLYGSDHTE (SEQ ID NO: 18), LIYYGSGYTY (SEQ ID NO: 19), MIIPGYGYTN (SEQ
 ID NO: 20); and

CDR-VH3: SRDSSFYYVYAMDY (SEQ ID NO: 21), SRDGMYAIFY (SEQ ID
 NO: 22), SRATYEEAIFY (SEQ ID NO: 23), SRGSYVSGMDY (SEQ ID NO: 24),
 SRSYYMYMDY (SEQ ID NO: 25).

15

For example, a molecule that can bind to a modified peptide derived from a modified H/K/N
 RAS polypeptide Q61H (*e.g.*, ILDTAGHEEY (SEQ ID NO:2)) can include one of each of
 the CDRs set forth below:

CDR-VL1: QDVNTA (SEQ ID NO: 5);

20

CDR-VL2: SAS;

CDR-VL3: QQVIYYPFTF (SEQ ID NO: 26), QQYDYYPFTF (SEQ ID NO: 27),
 QQSIYYPFTF (SEQ ID NO: 28), QQSSYSPWTF (SEQ ID NO: 184), QQSFSPTITF (SEQ
 ID NO: 29), QQGEYSPLTF (SEQ ID NO: 30), QQTYYPVTF, (SEQ ID NO: 31);

CDR-VH1: GFNLYSYAI (SEQ ID NO: 32), GFNISYEAM (SEQ ID NO: 33),
 25 GFNLYTSQM (SEQ ID NO: 34), GFNVFGYAI (SEQ ID NO: 35), GFNISPWDM (SEQ ID
 NO: 36), GFNISEYLM (SEQ ID NO: 37), GFNVFESAM (SEQ ID NO: 38), GFNISHYVM
 (SEQ ID NO: 39);

CDR-VH2: LLYPDYGVTS (SEQ ID NO: 40), LIYPNHGITS (SEQ ID NO: 41),
 LVYPGYYVTS (SEQ ID NO: 42), EVYPGYDVTS (SEQ ID NO: 43), QLYPSSGYTN
 30 (SEQ ID NO: 44), LLPPGLSYTN (SEQ ID NO: 45), WVGYSYDYTY (SEQ ID NO: 46),
 DFYPHSDSTY (SEQ ID NO: 47); and

CDR-VH3: SRYRSYEYSVSSYSYSAMDY (SEQ ID NO: 48), SRYSSSAMDY (SEQ ID NO: 49), SRGAYYYSSAMDY (SEQ ID NO: 50), SRYSWAGAFDY (SEQ ID NO: 51), SRSVYWSLDY (SEQ ID NO: 52), SRYGYAFDY (SEQ ID NO: 53), SRSFAYFQAMDY (SEQ ID NO: 54), SRYQSYSFDY (SEQ ID NO: 55).

5

For example, a molecule that can bind to a modified peptide derived from a modified H/K/N RAS polypeptide Q61L (*e.g.*, ILDTAGLEEY (SEQ ID NO:3) can include one of each of the CDRs set forth below:

CDR-VL1: QDVNTA (SEQ ID NO: 5);

10

CDR-VL2: SAS;

CDR-VL3: QQASRQPYTF (SEQ ID NO: 56), QQAVSYPWTF (SEQ ID NO: 57), QQTSSYPITF (SEQ ID NO: 58), QQSWYSPSTF (SEQ ID NO: 59), QQSYYPITF (SEQ ID NO: 60), QQSYSPWTF (SEQ ID NO: 61), QQAYYPPWTF (SEQ ID NO: 62), QQSYSSGPVTF (SEQ ID NO: 63), QQTYYPFTF (SEQ ID NO: 64), QQSYYPYYPWTF (SEQ ID NO: 65), QQYDRPITF (SEQ ID NO: 66);

15

CDR-VH1: GFNFSESGM (SEQ ID NO: 67), GFNISSSGI (SEQ ID NO: 68), GFNIYWYGM (SEQ ID NO: 69), GFNISASGM (SEQ ID NO: 70), GFNFSYYGM (SEQ ID NO: 71), GFNISYSNI (SEQ ID NO: 72), GFNVSRWAM (SEQ ID NO: 73), GFNFSYGGI (SEQ ID NO: 74), GFNLYAWGM (SEQ ID NO: 75), GFNVSHSAM (SEQ ID NO: 76), GFNIYYEAM (SEQ ID NO: 77)

20

CDR-VH2: HFSGDSGYTY (SEQ ID NO: 78), MVYGGSGYTN (SEQ ID NO: 79), QVYPWSGFTY (SEQ ID NO: 80), WIWGGSSYTY (SEQ ID NO: 81), WIYPFSGYTN, (SEQ ID NO: 82), MIYGTRGGTY (SEQ ID NO: 83), RVYPSGYLTY (SEQ ID NO: 84), MIYPLTGYTN (SEQ ID NO: 85), LUYGGWGSTS (SEQ ID NO: 86), TVHPDWGNTY (SEQ ID NO: 87), QIYPWNDYTY (SEQ ID NO: 88); and

25

CDR-VH3: SRYMYSGYFDY (SEQ ID NO: 89), SRWAHYSAYMDY (SEQ ID NO: 90), SRDYYSYSLDY (SEQ ID NO: 91), SRGQYLSYMDY (SEQ ID NO: 92), SREYYSRAFDY (SEQ ID NO: 93), SRYYSYAMDY (SEQ ID NO: 94), SRNMQSYMDY (SEQ ID NO: 95), SRDYYSYSDV (SEQ ID NO: 96), SRAGSSKMSAGAFDY (SEQ ID NO: 97), SRWQQYYYSFDY (SEQ ID NO: 98), SRNYAATMDY (SEQ ID NO: 99)

30

For example, a molecule that can bind to a modified peptide derived from a modified H/K/N RAS polypeptide Q61R (*e.g.*, ILDTAGREEY (SEQ ID NO:4) can include one of each of the CDRs set forth below:

CDR-VL1: QDVNTA (SEQ ID NO: 5);

5 **CDR-VL2:** SAS;

CDR-VL3: QQSYTSPLTF (SEQ ID NO: 100), QQYWYYYPITF (SEQ ID NO: 101), QQSYYAPITF (SEQ ID NO: 60), QQYYLYQPITF (SEQ ID NO: 102), QQYSNYPLTF (SEQ ID NO: 103), QQYASDPITF (SEQ ID NO: 104), QQYSYDPITF (SEQ ID NO: 105), QQYIYDPVTF (SEQ ID NO: 106), QQLMYDPITF (SEQ ID NO: 107);

10 **CDR-VH1:** GFNIYYGVM (SEQ ID NO: 108), GFNIYSYDM (SEQ ID NO: 109), GFNVQWSHM (SEQ ID NO: 110), GFNIGMYTM (SEQ ID NO: 111), GFNVFYGSM (SEQ ID NO: 112), GFNLDYGWM (SEQ ID NO: 113), GFNFSYSAM (SEQ ID NO: 114), GFNVDWAWM (SEQ ID NO: 115), GFNFGTYWM, (SEQ ID NO: 116);

CDR-VH2: MIYPDSSWTY (SEQ ID NO: 117), ISPGGSYTY (SEQ ID NO: 118),
15 RLSPPSGYTN (SEQ ID NO: 119), LVYPDSGYTN (SEQ ID NO: 120), FIGPDSTYTY (SEQ ID NO: 121), WVVPGSDYTD (SEQ ID NO: 122), DVVPDGDWTY (SEQ ID NO: 123), WVVGGSDYTY (SEQ ID NO: 124), WFLPDYDYTL (SEQ ID NO: 125); and

CDR-VH3: SRDQDFHYMNYLSYALDY (SEQ ID NO: 126), SRSAFTGYFDV (SEQ ID NO: 127), SRLILSKGGYGWAMDY (SEQ ID NO: 128), SRYTWQSMDY (SEQ ID NO: 129), SRDLGSAYAMDY (SEQ ID NO: 130), SRFHYTAFDV (SEQ ID NO: 131), SRGWYALDY (SEQ ID NO: 132), SRSYYYAFDY (SEQ ID NO: 133), SRHGEYAFDY (SEQ ID NO: 134).

Table 2. MANAbody complementarity-determining region (CDR) sequences of light (L) chains and heavy (H) chains.

Target Peptide(s)	scFv Clone	CDR L1	CDR L2	CDR L3	CDR H1	CDR H2	CDR H3
1) p53 R175H(168-176)-A2							
HMTEVVRHC (SEQ ID NO:1)	p53_R175H_A2_c12 (H2)	QDVNTA (SEQ ID NO: 5)	SAY	QQYSRYSPVTF (SEQ ID NO: 6)	GFNVYASGM (SEQ ID NO: 11)	KIYPDSDYTY (SEQ ID NO: 16)	SRDSSFYVYAMDY (SEQ ID NO: 21)
HMTEVVRHC (SEQ ID NO:1)	p53_R175H_A2_c16	QDVNTA (SEQ ID NO: 5)	SAS	QQQSSTPVTF (SEQ ID NO: 7)	GFNVYQSDM (SEQ ID NO: 12)	TIWPYSGYTY (SEQ ID NO: 17)	SRDGMYAFDY (SEQ ID NO: 22)
HMTEVVRHC (SEQ ID NO:1)	p53_R175H_A2_c115	QDVNTA (SEQ ID NO: 5)	SAS	QQSSYYPNTF (SEQ ID NO: 8)	GFNL YQRDM (SEQ ID NO: 13)	GLLYGSDHTE (SEQ ID NO: 18)	SRATYEEAFDY (SEQ ID NO: 23)
HMTEVVRHC (SEQ ID NO:1)	p53_R175H_A2_c116	QDVNTA (SEQ ID NO: 5)	SAS	QQQWSSPDTF (SEQ ID NO: 9)	GFNLSYYDM (SEQ ID NO: 14)	LIYYGSGYTY (SEQ ID NO: 19)	SRGSYVSGMDY (SEQ ID NO: 24)
HMTEVVRHC (SEQ ID NO:1)	p53_R175H_A2_c120 (H20)	QDVNTA (SEQ ID NO: 5)	SAS	QQSNAYPITF (SEQ ID NO: 10)	GFNLNSYYM (SEQ ID NO: 15)	MIIPGYGYTN (SEQ ID NO: 20)	SRSYMYMDY (SEQ ID NO: 25)
2) H/K/N RAS Q61H(55-64)-A1							
ILDTAGHEEY (SEQ ID NO:2)	H/K/N RAS Q61H_A1_c11	QDVNTA (SEQ ID NO: 5)	SAS	QQVIYYPFTF (SEQ ID NO: 26)	GFNL YSYAI (SEQ ID NO: 32)	LLYPDYGVTS (SEQ ID NO: 40)	SRYRSYEYSVSSY AMDY (SEQ ID NO: 48)
ILDTAGHEEY (SEQ ID NO:2)	H/K/N RAS Q61H_A1_c12	QDVNTA (SEQ ID NO: 5)	SAS	QQYDYYPFTF (SEQ ID NO: 27)	GFNISYEAM (SEQ ID NO: 33)	LIYPNHGITS (SEQ ID NO: 41)	SRYSSSAMDY (SEQ ID NO: 49)

ILDTAGHEEY (SEQ ID NO:2)	H/K/N RAS Q61H_A1_c13	QDVNTA (SEQ ID NO: 5)	SAS	QQSIYYPFTF (SEQ ID NO: 28)	GFNL YTSQM (SEQ ID NO: 34)	LVYPGYVTS (SEQ ID NO: 42)	SRGAYYSSAMDY (SEQ ID NO: 50)
ILDTAGHEEY (SEQ ID NO:2)	H/K/N RAS Q61H_A1_c14	QDVNTA (SEQ ID NO: 5)	SAS	QQYDYYPFTF (SEQ ID NO: 27)	GFNVFGYAI (SEQ ID NO: 35)	EVYPGYDVTS (SEQ ID NO: 43)	SRYSWAGAFDY (SEQ ID NO: 51)
ILDTAGHEEY (SEQ ID NO:2)	H/K/N RAS Q61H_A1_c15	QDVNTA (SEQ ID NO: 5)	SAS	QQSSYSPWTF (SEQ ID NO: 184)	GFNISPWDM (SEQ ID NO: 36)	QLYPSSGYTN (SEQ ID NO: 44)	SRSVYWSLDY (SEQ ID NO: 52)
ILDTAGHEEY (SEQ ID NO:2)	H/K/N RAS Q61H_A1_c18	QDVNTA (SEQ ID NO: 5)	SAS	QQSFSTPITF (SEQ ID NO: 29)	GFNISEYLM (SEQ ID NO: 37)	LLPPGLSYTN (SEQ ID NO: 45)	SRYGYAFDY (SEQ ID NO: 53)
ILDTAGHEEY (SEQ ID NO:2)	H/K/N RAS Q61H_A1_c19	QDVNTA (SEQ ID NO: 5)	SAS	QQGEYSPLTF (SEQ ID NO: 30)	GFNVFESAM (SEQ ID NO: 38)	WVYGSYDYTY (SEQ ID NO: 46)	SRSFAYFQAMDY (SEQ ID NO: 54)
ILDTAGHEEY (SEQ ID NO:2)	H/K/N RAS Q61H_A1_c110	QDVNTA (SEQ ID NO: 5)	SAS	QQTYTTPVTF (SEQ ID NO: 31)	GFNISHYVM (SEQ ID NO: 39)	DFYPHSDSTY (SEQ ID NO: 47)	SRYQSYSFDY (SEQ ID NO: 55)
3) H/K/N RAS Q61L(55-64)-A1							
ILDTAGLEEY (SEQ ID NO:3)	H/K/N RAS Q61L_A1_c11	QDVNTA (SEQ ID NO: 5)	SAS	QQASRQPYTF (SEQ ID NO: 56)	GFNFSESGM (SEQ ID NO: 67)	HFSGDSGYTY (SEQ ID NO: 78)	SRYMYSGYFDY (SEQ ID NO: 89)
ILDTAGLEEY (SEQ ID NO:3)	H/K/N RAS Q61L_A1_c12	QDVNTA (SEQ ID NO: 5)	SAS	QQAVSYPWTF (SEQ ID NO: 57)	GFNISSGI (SEQ ID NO: 68)	MVYGGSGYTN (SEQ ID NO: 79)	SRWAHYSAYMDY (SEQ ID NO: 90)
ILDTAGLEEY (SEQ ID NO:3)	H/K/N RAS Q61L_A1_c13	QDVNTA (SEQ ID NO: 5)	SAS	QQTSSYPITF (SEQ ID NO: 58)	GFNIYWYGM (SEQ ID NO: 69)	QVYPWVGFTY (SEQ ID NO: 80)	SRDYYSYSLDY (SEQ ID NO: 91)

ILDTAGLECY (SEQ ID NO:3)	H/K/N RAS Q61L_A1_cl4	QDVNTA (SEQ ID NO: 5)	SAS	QQSWYSPSTF (SEQ ID NO: 59)	GFNISASGM (SEQ ID NO: 70)	WIWGGSSYTY (SEQ ID NO: 81)	SRGQYLSYMDY (SEQ ID NO: 92)
ILDTAGLECY (SEQ ID NO:3)	H/K/N RAS Q61L_A1_cl5	QDVNTA (SEQ ID NO: 5)	SAS	QQSYYPITF (SEQ ID NO: 60)	GFNFSYGM (SEQ ID NO: 71)	WIYFSGYTN (SEQ ID NO: 82)	SREYYSRAFDY (SEQ ID NO: 93)
ILDTAGLECY (SEQ ID NO:3)	H/K/N RAS Q61L_A1_cl6	QDVNTA (SEQ ID NO: 5)	SAS	QQSYYSPWTF (SEQ ID NO: 61)	GFNISYNI (SEQ ID NO: 72)	MIYGTRGGTY (SEQ ID NO: 83)	SRYYSYAMDY (SEQ ID NO: 94)
ILDTAGLECY (SEQ ID NO:3)	H/K/N RAS Q61L_A1_cl7	QDVNTA (SEQ ID NO: 5)	SAS	QQAYYPPWTF (SEQ ID NO: 62)	GFNVSRWAM (SEQ ID NO: 73)	RVYPSGYLTY (SEQ ID NO: 84)	SRNMQSYMDY (SEQ ID NO: 95)
ILDTAGLECY (SEQ ID NO:3)	H/K/N RAS Q61L_A1_cl8	QDVNTA (SEQ ID NO: 5)	SAS	QQSYSSGPVTF (SEQ ID NO: 63)	GFNFSYGGI (SEQ ID NO: 74)	MIYPLTGYTN (SEQ ID NO: 85)	SRDYYSVDV (SEQ ID NO: 96)
ILDTAGLECY (SEQ ID NO:3)	H/K/N RAS Q61L_A1_cl9	QDVNTA (SEQ ID NO: 5)	SAS	QQTYYYPVTF (SEQ ID NO: 64)	GFNLYAWGM (SEQ ID NO: 75)	LVYGGWGSTS (SEQ ID NO: 86)	SRAGSSKMSAGA (SEQ ID NO: 97)
ILDTAGLECY (SEQ ID NO:3)	H/K/N RAS Q61L_A1_cl10	QDVNTA (SEQ ID NO: 5)	SAS	QQSYYPYPWTF (SEQ ID NO: 65)	GFNVSHSAM (SEQ ID NO: 76)	TVHPDWGNTY (SEQ ID NO: 87)	SRWQQYYYSFDY (SEQ ID NO: 98)
ILDTAGLECY (SEQ ID NO:3)	H/K/N RAS Q61L_A1_cl11	QDVNTA (SEQ ID NO: 5)	SAS	QQYDRPITF (SEQ ID NO: 66)	GFNIYYEAM (SEQ ID NO: 77)	QIYPWNDYTY (SEQ ID NO: 88)	SRNYYAATMDY (SEQ ID NO: 99)
4) H/K/N RAS Q61R(55-64)-A1							
ILDTAGRECY (SEQ ID NO:4)	H/R/N RAS Q61R_A1_cl1	QDVNTA (SEQ ID NO: 5)	SAS	QQSYTSPLTF (SEQ ID NO: 100)	GFNIYYGVM (SEQ ID NO: 108)	MIYPDSSWY (SEQ ID NO: 117)	SRDQDFHYMNY ALDY (SEQ ID NO: 126)

ILDTAGREEY (SEQ ID NO:4)	H/R/N RAS Q61R_A1_c12	QDVNTA (SEQ ID NO: 5)	SAS	QQYWYYYPITF (SEQ ID NO: 101)	GFNIYSYDM (SEQ ID NO: 109)	ISPGGSYTY (SEQ ID NO: 118)	SRSAFTGYFDV (SEQ ID NO: 127)
ILDTAGREEY (SEQ ID NO:4)	H/R/N RAS Q61R_A1_c13	QDVNTA (SEQ ID NO: 5)	SAS	QQSYYPITF (SEQ ID NO: 60)	GFNVQWSHM (SEQ ID NO: 110)	RLSPPSGYTN (SEQ ID NO: 119)	SRLILSKGGYGWAMD Y (SEQ ID NO: 128)
ILDTAGREEY (SEQ ID NO:4)	H/R/N RAS Q61R_A1_c15	QDVNTA (SEQ ID NO: 5)	SAS	QQYYLYQPITF (SEQ ID NO: 102)	GFNIGMYTM (SEQ ID NO: 111)	LVYPDSGYTN (SEQ ID NO: 120)	SRYTWQSM DY (SEQ ID NO: 129)
ILDTAGREEY (SEQ ID NO:4)	H/R/N RAS Q61R_A1_c16	QDVNTA (SEQ ID NO: 5)	SAS	QQYSNYPLTF (SEQ ID NO: 103)	GFNVFYGSM (SEQ ID NO: 112)	FIGPDSTYTY (SEQ ID NO: 121)	SRDLGSAYAMDY (SEQ ID NO: 130)
ILDTAGREEY (SEQ ID NO:4)	H/R/N RAS Q61R_A1_c17	QDVNTA (SEQ ID NO: 5)	SAS	QQYASDPITF (SEQ ID NO: 104)	GFNLDYGWM (SEQ ID NO: 113)	WVVPGSDYTD (SEQ ID NO: 122)	SRFHYTAFDV (SEQ ID NO: 131)
ILDTAGREEY (SEQ ID NO:4)	H/R/N RAS Q61R_A1_c113	QDVNTA (SEQ ID NO: 5)	SAS	QQYSYDPITF (SEQ ID NO: 105)	GFNFSYSAM (SEQ ID NO: 114)	DVVPDGDW TY (SEQ ID NO: 123)	SRGWYALDY (SEQ ID NO: 132)
ILDTAGREEY (SEQ ID NO:4)	H/R/N RAS Q61R_A1_c118	QDVNTA (SEQ ID NO: 5)	SAS	QQYIYDPVTF (SEQ ID NO: 106)	GFNVDWAWM (SEQ ID NO: 115)	WVVGGS DYTY (SEQ ID NO: 124)	SRSYYYAFDY (SEQ ID NO: 133)
ILDTAGREEY (SEQ ID NO:4)	H/R/N RAS Q61R_A1_c119	QDVNTA (SEQ ID NO: 5)	SAS	QQLMYDPITF (SEQ ID NO: 107)	GFNFGTYWM (SEQ ID NO: 116)	WFLPDYDYTL (SEQ ID NO: 125)	SRHGEYAFDY (SEQ ID NO: 134)

In some cases, a molecule including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4) can include any appropriate set of CDR sequences (*e.g.*, any of the CDR sequence sets described herein).

A molecule including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4) can include any appropriate sequence. For example, a molecule that can bind to a modified peptide derived from a modified p53 polypeptide (*e.g.*, HMTEVVRHC (SEQ ID NO:1)) can include, without limitation, the scFv sequence set forth in any one of SEQ ID NO:137, SEQ ID NO:138, SEQ ID NO:139, SEQ ID NO:140, or SEQ ID NO:141. For example, a molecule that can bind to a modified peptide derived from a modified H/K/N RAS polypeptide (*e.g.*, ILDTAGHEEY (SEQ ID NO:2), ILDTAGLEEY (SEQ ID NO:3), or ILDTAGREEY (SEQ ID NO:4)) can include, without limitation, the scFv sequence set forth in any one of SEQ ID NO:142, SEQ ID NO:143, SEQ ID NO:144, SEQ ID NO:145, SEQ ID NO:146, SEQ ID NO:147, SEQ ID NO:148, SEQ ID NO:149, SEQ ID NO:150, SEQ ID NO:151, SEQ ID NO:152, SEQ ID NO:153, SEQ ID NO:154, SEQ ID NO:155, SEQ ID NO:156, SEQ ID NO:157, SEQ ID NO:158, SEQ ID NO:159, SEQ ID NO:160, SEQ ID NO:161, SEQ ID NO:162, SEQ ID NO:163, SEQ ID NO:164, SEQ ID NO:165, SEQ ID NO:166, SEQ ID NO:167, SEQ ID NO:168, or SEQ ID NO:169. Examples of sequences (*e.g.*, scFv sequences) that can bind to particular modified peptides are shown in Table 3 and Table 12. In some cases, a molecule including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4) can have a sequence that deviates from a sequence shown in Table 3 and Table 12, sometimes referred to as a variant sequence. For example, a molecule including one or more antigen-binding domains that can bind to a modified peptide described herein can have at least 75% sequence identity (*e.g.*, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, at least 96% sequence identity, at least 97% sequence identity, at least 98% sequence identity, at least

99% sequence identity, or more) to any of the sequences shown in Table 3 and Table 12, provided the variant sequence maintains the ability to bind to a modified peptide described herein. For example, a molecule including one or more antigen-binding domains that can bind to a modified peptide described herein can have one or more (e.g., one, two, three, four, 5 five, six, seven, eight, nine, ten, or more) modifications (e.g., one or more amino acid substitutions) as compared to the sequences shown in Table 3 and Table 12, provided the variant sequence maintains the ability to bind to a modified peptide described herein. In some cases, a molecule including one or more antigen-binding domains that can bind to a modified peptide described herein can include any appropriate set of CDR sequences 10 described herein, and any sequence deviations from a sequence shown in Table 3 and Table 12 can be in the scaffold sequence(s).

A molecule including one or more antigen-binding domains (e.g., scFvs) that can bind to a modified peptide described herein (e.g., a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID 15 NO:4) can be attached (e.g., covalently or non-covalently attached) to a label (e.g., a detectable label). A detectable label can be any appropriate label. In some cases, a label can be used to assist in detecting the presence or absence of one or more modified peptides described herein. For example, a molecule described herein that is labelled can be used *in vitro* to detect cancer cells (e.g., cancer cells expressing a modified peptide described herein) 20 in a sample obtained from a mammal. In some cases, a label (e.g., a detectable label) can be used to assist in determining the location of one or more modified peptides described herein. For example, molecule described herein that is labelled can be used *in vivo* to monitor anti-tumor therapy and/or to detect cancer cells (e.g., cancer cells expressing a modified peptide described herein) in a mammal. Examples of labels that can be attached to a molecule 25 described herein include, without limitation, radionuclides, contrast agents used in magnetic resonance imaging (MRI), computed tomography (CT), ultrasound (US), and other imaging modalities, chromophores, enzymes, and fluorescent molecules (e.g., green fluorescent protein and near-IR fluorescence).

A molecule including one or more antigen-binding domains (e.g., scFvs) that can bind 30 to a modified peptide described herein (e.g., a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID

NO:4) can be attached (*e.g.*, covalently or non-covalently attached) to a therapeutic agent. A therapeutic agent can be any therapeutic agent. In some cases, a therapeutic agent can be an anti-cancer agent. Examples of therapeutic agents that can be attached to a molecule described herein include, without limitation, anti-cancer agents such as monomethyl
5 auristatin E (MMAE), monomethyl auristatin F (MMAF), maytansine, mertansine/emtansine (DM1), ravtansine/soravtansine (DM4), SN-38, calicheamicin, D6.5, dimeric pyrrolobenzodiazepines (PBDs), α -amantin (AAMT), PNU-159682, ricin, pseudomonas exotoxin A, diphtheria toxin, and gelonin.

This document also provides methods for using one or more molecules including one
10 or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4). For example, one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can target (*e.g.*, bind to) one or more modified peptides can be used to assess a mammal having cancer or
15 suspected of having cancer and/or to treat a mammal having a cancer (*e.g.*, a cancer expressing one or more modified peptides such as a p53 R175H MANA, a RAS Q61H/L/R MANA, and/or a RAS G12V MANA). In some cases, one or more molecules includes one or more antigen-binding domains that can bind to a modified peptide can be used to detect the presence or absence of one or more modified peptides in a sample obtained from a
20 mammal having cancer or suspected of having cancer. In some cases, one or more molecules including one or more antigen-binding domains that can bind to a modified peptide can be administered to a mammal having a cancer (*e.g.*, a cancer expressing the modified peptide) to treat the mammal. Administration of one or more molecules including one or more antigen-binding domains that can bind to a modified peptide described herein to a mammal (*e.g.*,
25 human) having a cancer can be effective to treat the mammal.

Any type of mammal can be assessed and/or treated as described herein. Examples of mammals that can be assessed and/or treated as described herein include, without limitation, primates (*e.g.*, humans and non-human primates such as chimpanzees, baboons, or monkeys), dogs, cats, pigs, sheep, rabbits, mice, and rats. In some cases, a mammal can be a human.

30 A mammal can be assessed and/or treated for any appropriate cancer. In some cases, a cancer can express one or more modified peptides (*e.g.*, one or more MANAs) described

herein. A cancer can be a primary cancer. A cancer can be a metastatic cancer. A cancer can include one or more solid tumors. A cancer can include one or more non-solid tumors.

Examples of cancers that can be assessed as described herein (*e.g.*, based at least in part on the presence of one or more modified peptides described herein) and/or treated as described

5 herein (*e.g.*, by administering one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein) include, without limitation, blood cancers (*e.g.*, Hodgkin's lymphoma, non-Hodgkin's lymphoma, acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), multiple myeloma, MDS, and myeloproliferative diseases), lung cancers, pancreatic cancers, gastric cancers, colon
10 cancers (*e.g.*, colorectal cancers), ovarian cancers, endometrial cancers, biliary tract cancers, liver cancers, bone and soft tissue cancers (*e.g.*, sarcomas), breast cancers, prostate cancers, esophageal cancers, stomach cancers, kidney cancers, head and neck cancers, brain cancers (*e.g.*, glioblastoma multiforme and astrocytomas), thyroid cancers, germ cell tumors, and melanomas.

15 When assessing a mammal having cancer or suspected of having cancer, one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4) can be used to assess for the presence or absence of one or more modified peptides described herein.

20 For example, the presence, absence, or level of one or more modified peptides described herein in a sample obtained from a human can be used to determine whether or not the human has a cancer. In some cases, the presence of one or more modified peptides described herein in a sample obtained from a mammal can be used to identify the mammal as having a cancer. For example, a mammal can be identified as having a cancer when a sample obtained
25 from the mammal has one or more modified peptides described herein.

Any appropriate sample obtained from a mammal can be assessed for the presence, absence, or level of one or more modified peptides described herein. For example, biological samples such as tissue samples (*e.g.*, breast tissue, and cervical tissue such as from a Papanicolaou (Pap) test), fluid samples (*e.g.*, blood, serum, plasma, urine, saliva, sputum, and
30 cerebrospinal fluid), and solid samples (*e.g.* stool) can be obtained from a mammal and assessed for the presence, absence, or level of one or more modified peptides described

herein. Any appropriate method can be used to detect the presence, absence, or level of one or more modified peptides described herein. For example, sequencing techniques including, but not limited to, Sanger sequencing, chemical sequencing, nanopore sequencing, sequencing by ligation (SOLiD sequencing), sequencing with mass spectrometry, whole
5 exome sequencing, whole genome sequencing, and/or next-generation sequencing can be used to determine the presence, absence, or level of one or more modified peptides described herein in a sample obtained from a mammal.

When treating a mammal having cancer, one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described
10 herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4) can be administered to a mammal having cancer to treat the mammal. In some cases, a mammal can have a cancer expressing one or more modified peptides described herein. For example, one or more molecules including one or more antigen-binding domains that can bind to a modified
15 peptide described herein can be administered to a mammal having a cancer expressing that modified peptide to treat the mammal. For example, one or more molecules including one or more scFvs that can bind to a modified peptide described herein (*e.g.*, one or more scDBs) can be administered to a mammal having a cancer expressing that modified peptide to treat the mammal.

20 In some cases, one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4) can be administered to a mammal (*e.g.*, a mammal having a cancer) once or multiple times over a period of time ranging from days to weeks.

25 In some cases, one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4) can be formulated into a composition (*e.g.*, a pharmaceutically acceptable composition) for administration to a mammal (*e.g.*, a mammal
30 having a cancer). For example, one or more antigen-binding domains that can bind to a modified peptide described herein can be formulated together with one or more

pharmaceutically acceptable carriers (additives), excipients, and/or diluents. In some cases, a pharmaceutically acceptable carrier, excipient, or diluent can be a naturally occurring pharmaceutically acceptable carrier, excipient, or diluent. In some cases, a pharmaceutically acceptable carrier, excipient, or diluent can be a non-naturally occurring (*e.g.*, an artificial or synthetic) pharmaceutically acceptable carrier, excipient, or diluent. Examples of pharmaceutically acceptable carriers, excipients, and diluents that can be used in a composition described herein include, without limitation, sucrose, lactose, starch (*e.g.*, starch glycolate), cellulose, cellulose derivatives (*e.g.*, modified celluloses such as microcrystalline cellulose and cellulose ethers like hydroxypropyl cellulose (HPC) and cellulose ether hydroxypropyl methylcellulose (HPMC)), xylitol, sorbitol, mannitol, gelatin, polymers (*e.g.*, polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), crosslinked polyvinylpyrrolidone (crospovidone), carboxymethyl cellulose, polyethylene-polyoxypropylene-block polymers, and crosslinked sodium carboxymethyl cellulose (croscarmellose sodium)), titanium oxide, azo dyes, silica gel, fumed silica, talc, magnesium carbonate, vegetable stearin, magnesium stearate, aluminum stearate, stearic acid, antioxidants (*e.g.*, vitamin A, vitamin E, vitamin C, retinyl palmitate, and selenium), citric acid, sodium citrate, benzyl alcohol, lysine hydrochloride, trehalose dihydrate, sodium hydroxide, parabens (*e.g.*, methyl paraben and propyl paraben), petrolatum, dimethyl sulfoxide, mineral oil, serum proteins (*e.g.*, human serum albumin), glycine, sorbic acid, potassium sorbate, water, salts or electrolytes (*e.g.*, saline, protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, and zinc salts), colloidal silica, magnesium trisilicate, polyacrylates, waxes, wool fat, lecithin, and corn oil. In some cases, a pharmaceutically acceptable carrier, excipient, or diluent can be an antiadherent, a binder, a colorant, a disintegrant, a flavor (*e.g.*, a natural flavor such as a fruit extract or an artificial flavor), a glidant, a lubricant, a preservative, a sorbent, and/or a sweetener.

A composition (*e.g.*, a pharmaceutical composition) containing one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4) can be formulated into any appropriate dosage form. Examples of dosage forms include solid or liquid forms including, without limitation, gums, capsules, tablets (*e.g.*, chewable tablets, and

enteric coated tablets), suppositories, liquids, enemas, suspensions, solutions (*e.g.*, sterile solutions), sustained-release formulations, delayed-release formulations, pills, powders, and granules.

A composition containing one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4) can be designed for oral, parenteral (including subcutaneous, intramuscular, intravenous, and intradermal), or intratumoral administration. Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions that can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient. The formulations can be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets.

A composition containing one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4) can be administered using any appropriate technique and to any appropriate location. A composition including one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein can be administered locally (*e.g.*, intratumorally) or systemically. For example, a composition provided herein can be administered locally by intratumoral administration (*e.g.*, injection into tumors) or by administration into biological spaces infiltrated by tumors (*e.g.* intraspinal administration, intracerebellar administration, intraperitoneal administration and/or pleural administration). For example, a composition provided herein can be administered systemically by oral administration or by intravenous administration (*e.g.*, injection or infusion) to a mammal (*e.g.*, a human).

Effective doses can vary depending on the risk and/or the severity of the cancer, the route of administration, the age and general health condition of the subject, excipient usage,

the possibility of co-usage with other therapeutic treatments such as use of other agents, and the judgment of the treating physician. An effective amount of a composition containing one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4) can be any amount that treats a cancer present within the subject without producing significant toxicity to the subject. If a particular subject fails to respond to a particular amount, then the amount of one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein can be increased (*e.g.*, by two-fold, three-fold, four-fold, or more). After receiving this higher amount, the mammal can be monitored for both responsiveness to the treatment and toxicity symptoms, and adjustments made accordingly. The effective amount can remain constant or can be adjusted as a sliding scale or variable dose depending on the subject's response to treatment. Various factors can influence the actual effective amount used for a particular application. For example, the frequency of administration, duration of treatment, use of multiple treatment agents, route of administration, and severity of the condition (*e.g.*, cancer) may require an increase or decrease in the actual effective amount administered.

The frequency of administration of one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4) can be any frequency that effectively treats a mammal having a cancer without producing significant toxicity to the mammal. For example, the frequency of administration of one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein can be from about two to about three times a week to about two to about three times a year. In some cases, a subject having cancer can receive a single administration of one or more antibodies described herein. The frequency of administration of one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein can remain constant or can be variable during the duration of treatment. A course of treatment with a composition containing one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified

peptide described herein can include rest periods. For example, a composition containing one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein can be administered every other month over a two-year period followed by a six-month rest period, and such a regimen can be repeated multiple times. As with the effective amount, various factors can influence the actual frequency of administration used for a particular application. For example, the effective amount, duration of treatment, use of multiple treatment agents, route of administration, and severity of the condition (*e.g.*, cancer) may require an increase or decrease in administration frequency.

An effective duration for administering a composition containing one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4) can be any duration that effectively treats a cancer present within the mammal without producing significant toxicity to the mammal. In some cases, the effective duration can vary from several months to several years. In general, the effective duration for treating a mammal having a cancer can range in duration from about one or two months to five or more years. Multiple factors can influence the actual effective duration used for a particular treatment. For example, an effective duration can vary with the frequency of administration, effective amount, use of multiple treatment agents, route of administration, and severity of the condition being treated.

In certain instances, a cancer within a mammal can be monitored to evaluate the effectiveness of the cancer treatment. Any appropriate method can be used to determine whether or not a mammal having cancer is treated. For example, imaging techniques or laboratory assays can be used to assess the number of cancer cells and/or the size of a tumor present within a mammal. For example, imaging techniques or laboratory assays can be used to assess the location of cancer cells and/or a tumor present within a mammal.

In some cases, one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4) can be administered to a mammal having a cancer as

a combination therapy with one or more additional cancer treatments (*e.g.*, anti-cancer agents). A cancer treatment can include any appropriate cancer treatments. In some cases, a cancer treatment can include surgery. In some cases, a cancer treatment can include radiation therapy. In some cases, a cancer treatment can include administration of one or more
5 therapeutic agents (*e.g.*, one or more anti-cancer agents). Examples of anti-cancer agents include, without limitation, platinum compounds (*e.g.*, a cisplatin or carboplatin), taxanes (*e.g.*, paclitaxel, docetaxel, or an albumin bound paclitaxel such as nab-paclitaxel), altretamine, capecitabine, cyclophosphamide, etoposide (vp-16), gemcitabine, ifosfamide, irinotecan (cpt-11), liposomal doxorubicin, melphalan, pemetrexed, topotecan, vinorelbine,
10 luteinizing-hormone-releasing hormone (LHRH) agonists (*e.g.*, goserelin and leuprolide), anti-estrogens (*e.g.*, tamoxifen), aromatase inhibitors (*e.g.*, letrozole, anastrozole, and exemestane), angiogenesis inhibitors (*e.g.*, bevacizumab), poly(ADP)-ribose polymerase (PARP) inhibitors (*e.g.*, olaparib, rucaparib, and niraparib), radioactive phosphorus, anti-CTLA-4 antibodies, anti-PD-1 antibodies, anti-PD-L1 antibodies, IL-2 and other cytokines,
15 other bispecific antibodies, and any combinations thereof. In cases where one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein are used in combination with one or more additional cancer treatments, the one or more additional cancer treatments can be administered at the same time or independently. For example, a composition including one or more molecules
20 including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein can be administered first, and the one or more additional cancer treatments administered second, or vice versa.

Also provided herein are kits that include one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described
25 herein (*e.g.*, a modified peptide including an amino acid sequence set forth in any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4). For example, a kit can include a composition (*e.g.*, a pharmaceutically acceptable composition) containing one or more molecules including one or more antigen-binding domains (*e.g.*, scFvs) that can bind to a modified peptide described herein. In some cases, a kit can include instructions for
30 performing any of the methods described herein. In some cases, a kit can include at least one dose of any of the compositions (*e.g.*, pharmaceutical compositions) described herein. In

some cases, a kit can provide a means (*e.g.*, a syringe) for administering any of the compositions (*e.g.*, pharmaceutical compositions) described herein.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

5

EXAMPLES

Example 1: Identification of MANAbody Clones and Conversion of MANAbody Clones into T cell-based Therapeutic Formats

In this study, a phage display library was designed and built, which displayed a single chain variable fragment (scFv) on the phage surface. The scFvs present in the library were
10 based on the humanized 4D5 (trastuzumab) framework with amino acid variability introduced at key positions of the scFv's complementarity determining regions (CDRs).

Phage display library was used to identify scFvs that specifically recognized mutation-containing peptides folded into a complex with a recombinant HLA allele alpha chain and beta-2 microglobulin (β 2M). These complexes, also referred to herein as
15 monomers, mimic the natural peptide/HLA complexes on a cancer cell surface.

Peptide-HLA targets can include mutant peptides (*e.g.*, MANAs) shown in Table 1. scFvs that can specifically bind to peptide-HLA targets in Table 1 are shown in Table 3. These scFvs can also be referred to as MANAbodies for their ability to bind to MANAs.

20

Table 3. MANAbody scFv sequences.

Target Peptide(s)	Target HLA Allele	scFv clone name	scFv sequence	SEQ ID NO:
1) p53 R175H(168-176)-A2				
HMTEVVRHC (SEQ ID NO:1)	HLA-A2	p53_R175H_A2_cl2 (H2)	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS AYFLYSGVPSRFSRSGTDFTLTISSLQPEDFATYYCQQYSRYSPVTFGQG TKVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFN VYASGMHWVRQAPGKGLEWVAKIYPDSDYTYYADSVKGRFTISADTSKN TAYLQMNSLRAEDTAVYYCSRDSSEFYVYAMDYWGQGLVTVSS	137
HMTEVVRHC (SEQ ID NO:1)	HLA-A2	p53_R175H_A2_cl6	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSRSGTDFTLTISSLQPEDFATYYCQQQSSTPVTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNV YQSDMHWVRQAPGKGLEWVA TIWPYSGYTYYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRDDGMVAFDYWGQGLVTVSS	138
HMTEVVRHC (SEQ ID NO:1)	HLA-A2	p53_R175H_A2_cl15	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSRSGTDFTLTISSLQPEDFATYYCQQSSYYPNTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNL YQRDMHWVRQAPGKGLEWVAGLLYGSDHTEYADSVKGRFTISADTSKN TAYLQMNSLRAEDTAVYYCSRATYEEAFDYWGQGLVTVSS	139
HMTEVVRHC (SEQ ID NO:1)	HLA-A2	p53_R175H_A2_cl16	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSRSGTDFTLTITSLQPEDFATYYCQQQWSSPDTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNL SYYDMHWVRQAPGKGLEWVALIYYGSGYTYYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRGSYVSGMDYWGQGLVTVSS	140
HMTEVVRHC (SEQ ID NO:1)	HLA-A2	p53_R175H_A2_cl20 (H20)	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSRSGTDFTLTISSLQPEDFATYYCQQSNAYPITFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNL NSYYMHWVRQAPGKGLEWVAMIIPGYGTYNYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRSYMYMDYWGQGLVTVSS	141

2) H/K/N RAS Q61H(55-64)-A1				
ILDTAGHEEY (SEQ ID NO:2)	HLA-A1	H/K/N RAS Q61H_A1_cl1 (H1)	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQVITYYPFTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNLT YSYAIHWVRQAPGKGLEWVALLYPDYGVTSYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRYSRYSEYSVSSYSAMDYWGQGTLVTVSS	142
ILDTAGHEEY (SEQ ID NO:2)	HLA-A1	H/K/N RAS Q61H_A1_cl2	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQYDYYPFTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNI SYEAMHWVRQAPGKGLEWVALIYPNHGITSYADSVKGRFTISADTSKNTA YLMNSLRAEDTAVYYCSRYSAMDYWGQGTLVTVSS	143
ILDTAGHEEY (SEQ ID NO:2)	HLA-A1	H/K/N RAS Q61H_A1_cl3	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQSIYYPFTFGQGTK VEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNLY TSQMHWVRQAPGKGLEWVALVYPGYVTSYADSVKGRFTISADTSKNTA YLMNSLRAEDTAVYYCSRGAYYSAMDYWGQGTLVTVSS	144
ILDTAGHEEY (SEQ ID NO:2)	HLA-A1	H/K/N RAS Q61H_A1_cl4	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQYDYYPFTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNV FGYAIHWVRQAPGKGLEWVAEVYPGYDVTSYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRYSWAGAFDYWGQGTLVTVSS	145
ILDTAGHEEY (SEQ ID NO:2)	HLA-A1	H/K/N RAS Q61H_A1_cl5	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQSSYSPWTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNI SPWDMHWVRQAPGKGLEWVAQLYPSSGYTNYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRVSYYWSLDYWGQGTLVTVSS	146

ILD TAGHEY (SEQ ID NO:2)	HLA-A1	H/K/N RAS Q61H_A1_cl8	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSFSTPTITFGQGTK VEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNISE YLMHWVRQAPGKGLEWVALLPPGLSYTNYADSVKGRFTISADTSKNTAY LQMNSLRAEDTAVYYCSRYGYYAFDYWGQGTLVTVSS	147
ILD TAGHEY (SEQ ID NO:2)	HLA-A1	H/K/N RAS Q61H_A1_cl9	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQGEYSPLTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNV FESAMHWVRQAPGKGLEWVAVVYGSYDYTYADSVKGRFTISADTSKN TAYLQMNSLRAEDTAVYYCSRFAFYQAMDYWGQGTLVTVSS	148
ILD TAGHEY (SEQ ID NO:2)	HLA-A1	H/K/N RAS Q61H_A1_cl10	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQTYYTPVTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNI SHYVMHWVRQAPGKGLEWVADFYPHSDSTYYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRYQSYSFDYWGQGTLVTVSS	149
3) H/K/N RAS Q61L(55-64)-A1				
ILD TAGLEY (SEQ ID NO:3)	HLA-A1	H/K/N RAS Q61L_A1_cl1	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQASRQPYTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNF SESGMHWVRQAPGKGLEWVAHFSGDSGYTYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRYMYYSGYFDYWGQGTLVTVSS	150
ILD TAGLEY (SEQ ID NO:3)	HLA-A1	H/K/N RAS Q61L_A1_cl2 (L2)	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQAVSYPWTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNI SSSGIHWVRQAPGKGLEWVAMVYGGSGYTNYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRWAHYSAYMDYWGQGTLVTVSS	151

ILDTAGLECY (SEQ ID NO:3)	HLA-A1	H/K/N RAS Q61L_A1_c13	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQTSSYPITFGQGTK VEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNIY WYGMHWVRQAPGKGLEWVAQVYPWSGFTYYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRDIYYSYSLDYWGQGLVTVSS	152
ILDTAGLECY (SEQ ID NO:3)	HLA-A1	H/K/N RAS Q61L_A1_c14	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSWYSPSTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNI SASGMHWVRQAPGKGLEWVAWIWGGSSYTYADSVKGRFTISADTSKN TAYLQMNSLRAEDTAVYYCSRQQYLSYMDYWGQGLVTVSS	153
ILDTAGLECY (SEQ ID NO:3)	HLA-A1	H/K/N RAS Q61L_A1_c15	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYAPITFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNF SYYGMHWVRQAPGKGLEWVAWIYPFSGYTNYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSREYYSRAFDYWGQGLVTVSS	154
ILDTAGLECY (SEQ ID NO:3)	HLA-A1	H/K/N RAS Q61L_A1_c16	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYSPWTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNI SYSNIHWVRQAPGKGLEWVAMIYGRGGTYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRYYSYAMDYWGQGLVTVSS	155
ILDTAGLECY (SEQ ID NO:3)	HLA-A1	H/K/N RAS Q61L_A1_c17	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQAYYPPWTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNV SRWAMHWVRQAPGKGLEWVARVYPSGYLTYYADSVKGRFTISADTSKN TAYLQMNSLRAEDTAVYYCSRNMQSYM DYWGQGLVTVSS	156
ILDTAGLECY (SEQ ID NO:3)	HLA-A1	H/K/N RAS Q61L_A1_c18	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYSSGPVTFGQG TKVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFN FSYGGIHWVRQAPGKGLEWVAMIYPLTGYTNYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRDIYYSVDVWGQGLVTVSS	157

ILDTAGLEEEY (SEQ ID NO:3)	HLA-A1	H/K/N RAS Q61L_A1_c19	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQTYYPFTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNL YAWGMHWVRQAPGKGLEWVALVYGGWGSTSYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRAGSSKMSAGAFDYWGQGLVTVSS	158
ILDTAGLEEEY (SEQ ID NO:3)	HLA-A1	H/K/N RAS Q61L_A1_c110	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYPYPWTFGQ GTKVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGF NVSHSAMHWVRQAPGKGLEWVATVHPDWGNTYYADSVKGRFTISADTS KNTAYLQMNSLRAEDTAVYYCSRWQQYYYSFDYWGQGLVTVSS	159
ILDTAGLEEEY (SEQ ID NO:3)	HLA-A1	H/K/N RAS Q61L_A1_c111	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQYDRPITFGQGTK VEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNIY YEAMHWVRQAPGKGLEWVAQIYPWN DYTYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRNYAAATMDYWGQGLVTVSS	160
4) H/K/N RAS Q61R(55-64)-A1				
ILDTAGREEY (SEQ ID NO:4)	HLA-A1	H/R/N RAS Q61R_A1_c11	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYTSPLTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNI YYGVMHWVRQAPGKGLEWVAMIYPDSSW TYADSVKGRFTISADTSKN TAYLQMNSLRAEDTAVYYCSR DQDFHYMNYLSYALDYWGQGLVTVS S	161
ILDTAGREEY (SEQ ID NO:4)	HLA-A1	H/R/N RAS Q61R_A1_c12	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQYWYYYPITFGQG TKVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFN IYSYDMHWVRQAPGKGLEWVAISPGGSYTYADSVKGRFTISADTSKNTA YLQMNSLRAEDTAVYYCSRSAFTGYFDVWGQGLVTVSS	162

ILDTAGREEY (SEQ ID NO:4)	HLA-A1	H/R/N RAS Q61R_A1_cl3	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYPITFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNV QWSHMHWVRQAPGKGLEWVARLSPPSGYTNYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRLLILSKGGYGWAMDYWGQGTLVTVSS	163
ILDTAGREEY (SEQ ID NO:4)	HLA-A1	H/R/N RAS Q61R_A1_cl5	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQYYLYQPITFGQG TKVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFN IGMYTMHWVRQAPGKGLEWVALVYPDSGYTNYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRYTWQSMDYWGQGTLVTVSS	164
ILDTAGREEY (SEQ ID NO:4)	HLA-A1	H/R/N RAS Q61R_A1_cl6 (R6)	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQYSNYPLTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNV FYGSMHWVRQAPGKGLEWVAFIGPDSITYYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCSRDLGSAYAMDYWGQGTLVTVSS	165
ILDTAGREEY (SEQ ID NO:4)	HLA-A1	H/R/N RAS Q61R_A1_cl7	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQYASDPITFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNL DYGWMHWVRQAPGKGLEWVAWVVPGSDYTDYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRPHYTAFDVWGQGTLVTVSS	166
ILDTAGREEY (SEQ ID NO:4)	HLA-A1	H/R/N RAS Q61R_A1_cl13	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQYSYDPITFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNF SYSAMHWVRQAPGKGLEWVADVVPDGDWITYYADSVKGRFTISADTSKN TAYLQMNSLRAEDTAVYYCSRGWYALDYWGQGTLVTVSS	167
ILDTAGREEY (SEQ ID NO:4)	HLA-A1	H/R/N RAS Q61R_A1_cl18	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQYIYDPVTFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFNV DWAWMHWVRQAPGKGLEWVAWVVGSDYITYYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRSYYYAFDYWGQGTLVTVSS	168

ILDTAGREEY (SEQ ID NO:4)	HLA-A1	H/R/N RAS Q61R_A1_c119	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQLMYDPITFGQGT KVEIKRTGGSGGGGGGASEVQLVESGGGLVQPGGSLRLSCAASGFINF GTYYWMHWVRQAPGKGLEWVAWFLPDYDYTLYADSVKGRFTISADTSKN TAYLQMNSLRAEDTAVYYCSRHGEYAFDYWGQGTLVTVSS	169

Flow cytometry data for scFvs that specifically recognized a p53 peptide containing the R175H mutation in complex with HLA-A2 (HMTEVVRHC; SEQ ID NO: 1) are shown in Figure 1. The scFvs specifically stained the HLA allele-matched cell lines when these cells are pulsed with the mutant peptide, but not the WT peptide or not pulsed with peptide at all. Flow cytometry data for scFvs specific for H/K/N RAS Q61H, Q61L, and Q61R peptides are shown in Figures 2-4.

To demonstrate that MANAbody clones can be utilized as a therapeutic modality, selected MANAbody clones were engineered into bispecific antibodies having one antibody-fragment binding to a mutant peptide presented in the context of HLA and having one antibody-fragment binding to a CD3 protein on the T cell surface (Table 4). The bispecific antibodies are referred to as single-chain diabody (scDb) hereafter. Specifically, bispecific antibodies targeting a mutant p53 R175H peptide presented in the context of HLA-A2 and CD3 were engineered, and bispecific antibodies targeting mutant H/K/N RAS Q61H, Q61L, and Q61R peptides presented in the context of HLA-A1 and CD3 were engineered.

Representative scDb co-culture results are shown in Figure 5 for five p53 R175H HLA-A2 MANAbody scFv clones combined with anti-CD3 scFv. T cells were co-cultured with COS-7 cells co-transfected with plasmids encoding HLA-A2, full-length p53 variants, and/or GFP in the presence of the specified concentration of scDb. As a read out of T cell activation by cognate antigen on target cells, the release of IFN γ in the co-culture media supernatant was measured by ELISA. Only when COS-7 cells were co-transfected with HLA-A2 and mutant p53 R175H plasmids was there significant T cell release of IFN γ over background, with the level of IFN γ dependent on the concentration of scDb included in the well. T cells co-cultured with COS-7 cells co-transfected with HLA-A2 and WT p53 released only background levels of IFN γ . Representative scDb co-culture results are shown in Figures 6-8 for H/K/N RAS Q61H, Q61L, and Q61R HLA-A1 MANAbody scFv clones combined with an anti-CD3 clone into a scDb. In these co-cultures, only when COS-7 cells were co-transfected with HLA-A1 and mutant full-length KRAS Q61H, Q61L, or Q61R plasmids was there significant T cell release of IFN γ over background. T cells co-cultured with COS-7 cells co-transfected with HLA-A1 and WT KRAS released only background levels of IFN γ , similar to the levels of IFN γ seen in no scDb wells.

To evaluate whether the H/K/N RAS Q61H, Q61L, and Q61R scDBs can react against all 3 RAS isoforms with the cognate Q61 mutations, COS-7 cells were transfected with HLA-A1 and plasmids encoding for full-length HRAS, KRAS, or NRAS that are WT, or harbored Q61H, Q61K, Q61L, or Q61R mutation. The transfected COS-7 cells were then
5 co-cultured with T cells and representative H/K/N RAS Q61H, Q61L, and Q61R scDBs. Figure 9a shows that the H/K/N RAS Q61H scDb only induced IFN γ in the presence of COS-7 cells co-transfected with HLA-A1 and the Q61H mutant HRAS, KRAS, or NRAS. Likewise, H/K/N RAS Q61L scDb only induced IFN γ in the presence of COS-7 cells co-transfected with HLA-A1 and the Q61L mutant HRAS, KRAS, or NRAS (Figure 9b) and
10 H/K/N RAS Q61R scDb only induced IFN γ in the presence of COS-7 cells co-transfected with HLA-A1 and the Q61R mutant HRAS, KRAS, or NRAS (Figure 9c).

To evaluate the ability of MANAbody clones to recognize tumor cells, tumor cell lines with endogenous cognate HLA and mutations were co-cultured with T cells and scDBs. An endogenous p53 R175H HLA-A2 positive cell line TYKnu, along with its isogenic p53
15 knockout control, was cultured with T cells and p53 R175H HLA-A2 scDb. IFN γ release was only induced against the parental TYKnu cell line but not the p53 knockout TYKnu (Figure 10). An endogenous NRAS Q61L HLA-A1 positive cell line HL-60, along with its isogenic HLA-A1 knockout control, was cultured with T cells and H/K/N RAS Q61L HLA-A1 scDb. IFN γ release was only seen against the parental HL-60 cell line but not the HLA-A1
20 A1 knockout HL-60 (Figure 11). Together, these findings suggest that bispecific antibodies containing MANAbody clones can target tumor cells expressing MANAs presented in the context of HLA molecules.

To evaluate the efficacy of using MANAbody clones as a therapeutic modality, target cell viability of p53 R175H HLA-A2 and H/K/N RAS Q61L HLA-A1 scDb co-cultures was
25 assayed using Promega's CellTiter-Glo reagent. CellTiter-Glo measures ATP concentration in a well, which is proportional to the number of viable cells. Percent target cell viability was measured by subtracting the CellTiter-Glo value from T cell only wells and normalizing to target cell only wells. Only when parental TYKnu cells were incubated with T cells in the presence of the p53 R175H HLA-A2 scDb, was there significant target cell death (Figure
30 12). No target cell death was observed among the TYKnu p53 knockout wells. Similarly, only when parental HL-60 cells were incubated with T cells in the presence of the H/K/N

RAS Q61L HLA-A1 scDb, was there significant target cell death (Figure 13). No target cell death was observed among the HL-60 HLA-A1 knockout wells.

Together, these findings demonstrate that MANAbodies can be used to redirect and activate T cells to kill tumor cells expressing particular mutant protein and HLA allele pairs
5 (e.g., p53 R175H with HLA-A2 and H/K/N RAS Q61L with HLA-A1).

Table 4. Anti-human CD3 scFv sequences

Clone Name	Clone scFv Sequence	SEQ ID NO:
humanized UCHT1 (hUCHT1v9)	DIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGVPSRFS	170
	GSGGTDYTLTISSLQPEDFATYYCQQGNTLPWTFGQGTKVEIKGGSGGGSGGGSEV QLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQAPGKGLEWVALINPYKGVSTYNQ KFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCARSGYYGSDSWYFDVWGQGTLLTVSS	
murine UCHT1 (mUCHT1)	DIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKPDGTVKLLIYYTSRLHSGVPSKFS	171
	GSGGTDYSLTISNLEQEDIATYFCQQGNTLPWTFAGGTKLEIKGGSGGGSGGGSEVQ LQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQSHGKNLEWMGLINPYKGVSTYNQK FKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCARSGYYGSDSWYFDVWGAGTTTVTVSS	
diL2K	DIVLTQSPATLSLSPGERATLSCRASQSVSYMNWYQQKPGKAPKRWIYDTSKVASGVPARFS	172
	GSGGTDYSLTINSLEAEDAATYYCQQWSSNPLTFGGGTKVEIKGGSGGGSGGGSDV QLVQSGAEVKKPGASVKASGYTFTRYTMHWVRQAPGQGLEWIGYINPSRGYTNYAD SVKGRFTITTDKSTSTAYMELSSLRSEDATYYCARYYDDHYCLDYWGQGTITTVTVSS	
hXR32	QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNKRAPWTP	173
	ARFSGSLLGKAAALTITGAQAEDEADYYCALWYSNLWVFGGGTKLTVLGGGSGGGSGGG GGSEVQLVESGGGLVQPGGSLRLSCAASGFTFTNTYAMNWVRQAPGKGLEWVARIRSKYNN YATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGNFGNSYVSWFAYWG QGTLVTVSS	
L2K-07/TR66	DIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFS	174
	GSGGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELKGGSGGGSGGGGSDI KLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPQQGLEWIGYINPSRGYTNYNQ KFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTLLTVSS	
OKT3	QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLASGVPAHFR	175
	GSGGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINGGGSGGGSGGGSQV QLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPQQGLEWIGYINPSRGYTNYNQ KFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTLLTVSS	

PSMA-CD3	QTVVTQEPSLTVSPGGTVTLTCGSSTGAVTSGNYPNWVQQKPGQAPRGLIGTKFLAPGTPA RFGSLLGGKAALTLSGVQPEDEAEYYCVLWYSNRWVFGGGTKLTVLGGGSGGGSGGG GSEVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAMNWVRQAPGKGLEWVAIRISKYNNY ATYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISYWAYWGQ GTLVTVSS	176
28F11	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRAATGIPARFS GSGGTDFTLTISLLEPEDFAVYYCQQRSNWPPLTFGGGTKVEIKGGGSGGGSGGGSQV QLVESGGGVVQGRSLRLSCAASGFKFSGYGMHWVRQAPGKGLEWVAVIWYDGSKKYYV DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARQMGYWHFDLWGRGTLVTVSS	177
27H5-VL1	EIVLTQSPRTLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYGASSRAATGIPDRFS GSGGTDFTLTISRLDPEDFAVYYCQQYGSSPITFGQGTRLEIKGGGSGGGSGGGSQVQ LVESGGGVVQGRSLRLSCAASGFTFRSYGMHWVRQAPGKGLEWVAIIWYDGSKKNYADS VKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGTGYNWFDPWGQGTLVTVSS	178
27H5-VL2	DILMTQSPSSLSASVGDRVTITCRASQGISSALAWYQQKPGKAPKLLIYYASSLQSGVPSRFS GSGGTDYTLTISSLQPEDFATYYCQQYYSTLTFGGGTKVEIKGGGSGGGSGGGSQVQL VESGGGVVQGRSLRLSCAASGFTFRSYGMHWVRQAPGKGLEWVAIIWYDGSKKNYADSV KGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGTGYNWFDPWGQGTLVTVSS	179
23F10	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRAATGIPARFS GSGGTDFTLTISLLEPEDFAVYYCQQRSNWPPLTFGGGTKVEIKGGGSGGGSGGGSQV QLVQSGGGVVQSGRSLRLSCAASGFKFSGYGMHWVRQAPGKGLEWVAVIWYDGSKKYYV DSVKGRFTISRDN SKNTLYLQMNSLRGEDTAVYYCARQMGYWHFDLWGRGTLVTVSS	180
15C3-VL1	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRAATGIPARFS GSGGTDFTLTISLLEPEDFAVYYCQQRSNWPWTFGQGTKVEIKGGGSGGGSGGGSQV QLVQSGGGVVQGRSLRLSCV ASGFTFSSYGMHWVRQAPGKGLEWVAAIWYNGRKQDYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCTRGTGYNWFDPWGQGTLVTVSS	181
15C3-VL2	AIQLTQSPSSLSASVGDRVTITCRASQGISSALAWYQQKPGKAPKLLIYDASSLESVPSRFSG SGSGTDFTLTISLQPEDFATYYCQQFNSTPITFGQGTRLEIKGGGSGGGSGGGSQVQLV QSGGGVVQGRSLRLSCV ASGFTFSSYGMHWVRQAPGKGLEWVAAIWYNGRKQDYADSV KGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCTRGTGYNWFDPWGQGTLVTVSS	182

hu12F6	QIVLSQSPAILSASPGEKVTMTCRASSSVSYMHWYQQKPGSSPKPWYIATSNLASGVPARFS GSGSGTSYSLTISRVEAEDAATYYCQQWSSNPPTFGGGTKLETKRGGGGSGGGSGGGSQ VQLQQSGAELARPGASVKMSCKASGYTFTSYTMHWVKQRPGQGLEWIGYINPSSGYTKYN QKFKDKATLTADKSSSTAYMQLSSLTSEDSAVYYCARWQDYDYVFDYWGQGTTTLTVSS	183

Table 5. Anti-human CD16a scFv sequences

Clone Name	Clone scFv Sequence	SEQ ID NO:
3G8	DIVLTQSPASLAVSLGQRATISCKASQSVDFDGDSEFMNWWYQQKPGQPPKLLIYTTSNLESGIP ARFSASGSGTDFTLNIHPVEEEDTATYYCQQSNEDPYTFGGGTKLELKGGGGSGGGSGGG GSQVTLKESGPGLQPSQTLSLTCSFSGFSLRTSGMGVGVWIRQPSGKGLEWLAHIWWDDDKR YNPALKSRLTISKDTSSNQVFLKIASVDTADTATYYCAQINPAWFAYWGQGTLLVTVSA	185
Humanized 3G8 clone Mu 3	DIVLTQSPDSLAVSLGERATINCKASQSVDFDGDSEFMNWWYQQKPGQPPKLLIYTTSNLESGV PDRFSGSGSGTDFTLTIRPLQAEDVAVYYCQQSNEDPYTFGQGTKLEIKGGGGSGGGSGGG GSQVTLKESGPALVKPTQTLTLCTFSGFSLSTSGMGVGVWIRQPPGKALEWLAHIWWDDDK RYNPALKSRLTISKDTSKNQVVLMTNMDPVDATYYCAQINPAWFAYWGQGTLLVTVSS	186
muA9	DIELTQESALTTSPGETVTLTCRSNTGTVTTSNYANWVQEKPDHLFTGLIGHTNNRAPGVPA RFSGSLIGDKAALTITGAQTEDEAIYFCALWYNNHWVFGGGTKLEIKGGGGSGGGSGGGG SEVQLQESGAELVRPGTSVKISCKASGYTFTNHWLVGWVKQRPGHGLEWIGDIYPGGGYTNY NEKFKGKATVTADTSSRTAYVQVRSLTSEDSAVYFCARSASWYFDVWQGQTTTVTVSS	187
huA9	DIELTQSPSSLASVGDRVTITCRSNTGTVTTSNYASWYQQKPGKAPGGLIGHTNNRAPGVPS RFSGSLSGADATLTISLKPEDLATYYCALWYNNHWVFGQGTKLEIKGGGGSGGGSGGGGS EVQLQESGGGLVQPGDSLRLSCAASGFTFSNYWLGWIRQAPGKGPEWVGDIYPGGGYTNY NEKFKGRFTISADTSKNIAYLQVNSLRAEDTAVYYCARSAWYFDVWQGQGTLLVTVSS	188

LSIV21	SYVLTQPSSVSVAPGQTATISCGGHNIGSKNVHWYQQRPQQSPVLVIYQDNKRPSGIPERFSG SNSGNTATLTISGTQAMDEADYYCQVWDNYSVLFGGGTKLTVLGGGGSGGGSGGSQV QLVQSGAEVKKPGESLKVSCKASGYTFTSYMHWVRQAPGQGLEWMGIINPSGGSTSYAQ KFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARGSAYYYDFADYWQGQTLVTVSS	189

Materials and Methods

Cells and Cell Lines.

RPMI-6666 cells (ATCC, Manassas, VA) were cultured in RPMI-1640 (ATCC) with 20% FBS (GE Hyclone, Logan, Utah, USA), and 1% penicillin streptomycin (Life Technologies). T2 cells (ATCC) and TYKnu (JCRB, Japan) were cultured in RPMI-1640 (ATCC) with 10% FBS (GE Hyclone), and 1% penicillin streptomycin (Thermo Fisher). SigM5 cells (DSMZ, Brunswick, Germany) and HL-60 cells (ATCC) were cultured in Iscove's MDM (ATCC) with 20% FBS (GE Hyclone), and 1% penicillin streptomycin (Thermo Fisher). COS-7 cells (ATCC) was cultured in McCoy's 5A (Modified) Medium (Thermo Fisher) with 10% FBS (GE Hyclone), and 1% penicillin streptomycin (Thermo Fisher). COS-7 cells (ATCC, CRL-1651™) were cultured in DMEM (high glucose, pyruvate; Thermo Fisher) with 10% FBS (GE Hyclone), and 1% Penicillin-Streptomycin (Thermo Fisher). 293FT cells (Thermo Fisher) were cultured in high-glucose D-MEM (Thermo Fisher), with 10% FBS (GE Hyclone), 0.1 mM MEM Non-Essential Amino Acids (NEAA, Thermo Fisher), 6 mM L-glutamine (Thermo Fisher), 1 mM MEM Sodium Pyruvate (Thermo Fisher), 500 µg/ml geneticin (Thermo Fisher), and 1% Penicillin-Streptomycin (Thermo Fisher). All cell lines were maintained at 37°C under 5% CO₂.

PBMCs were obtained by Ficoll-Paque PLUS (GE Healthcare) gradient centrifugation of whole blood from healthy volunteer donors. To activate and expand T cells, PBMCs were cultured with 15 ng/ml OKT3 (BioLegend, San Diego, CA), 100 IU/mL recombinant human interleukin-2 (Aldesleukin, Prometheus Therapeutics and Diagnostics, San Diego, CA), and 5 ng/ml recombinant human interleukin-7 (BioLegend, San Diego, CA) in RPMI-1640 (ATCC) with 10% FBS (GE Hyclone), 1% Penicillin-Streptomycin (Life Technologies) at 37°C under 5% CO₂ for 3 days. After 3 days, the expanded T cells were kept in the same cytokine-containing media without OKT3.

Phage Display Library Construction.

Oligonucleotides were synthesized by GeneArt (Thermo Fisher Scientific) using trinucleotide mutagenesis (TRIM) technology. The oligonucleotides were incorporated into the pADL-10b phagemid (Antibody Design Labs, San Diego, CA). This phagemid contains

an F1 origin, a transcriptional repressor to limit uninduced expression, a lac operator, and a lac repressor. The scFv was synthesized with a pelB periplasmic secretion signal and was subcloned downstream of the lac operator. A FLAG (DYKDDDDK; SEQ ID NO:190) epitope tag was placed immediately downstream of the variable heavy chain, which was followed in frame by the full-length M13 pIII coat protein sequence.

Ten ng of the ligation product was mixed on ice with 10 μ L of electrocompetent SS320 cells (Lucigen, Middleton, WI) and 14 μ L of double-distilled water. This mixture was electroporated (200 ohms, 25 microFarads, 1.8kV) using a Gene Pulser electroporation system (Bio-Rad, Hercules, CA) and allowed to recover in Recovery Media (Lucigen) for 45 minutes at 37°C. Cells transformed with 60 ng of ligation product were pooled and plated on a 24-cm x 24-cm plate containing 2xYT medium supplemented with carbenicillin (100 μ g/mL) and 2% glucose. Cells were grown at 37°C for 6 hours and placed at 4°C overnight. To determine the transformation efficiency for each series of electroporations, aliquots were taken and titered by serial dilution. Cells grown on plates were scraped into 850 mL of 2xYT medium with carbenicillin (100 μ g/mL) plus 2% glucose for a final OD₆₀₀ of 5-15. Two mL of the 850 mL culture were taken and diluted ~1:200 to reach a final OD₆₀₀ of 0.05-0.07. To the remaining culture, 150 mL of sterile glycerol were added before snap freezing to produce glycerol stocks. The diluted bacteria were grown to an OD₆₀₀ of 0.3-0.5, infected with M13K07 Helper phage at an MOI of 4 (Antibody Design Labs) and shaken at 37°C for 1 hour. The culture was centrifuged and the cells were resuspended in 2xYT medium with carbenicillin (100 μ g/mL), kanamycin (50 μ g/mL), and IPTG (50 mM, Thermo Fisher) and grown overnight at 30°C for phage production. The following morning, the bacterial culture was aliquoted into 50 mL Falcon tubes and pelleted twice at high speed to obtain clarified supernatant. The phage-laden supernatant was precipitated on ice for 40 minutes with a 20% PEG-8000/2.5M NaCl solution at a 4:1 ratio of PEG/NaCl:supernatant. After precipitation, phage was centrifuged at 12,000 g for 40 minutes and resuspended in 1x TBS, 2 mM EDTA, and 1x Complete Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). Phages from multiple tubes were pooled and re-precipitated. The total number of transformants obtained was determined to be 3.6×10^{10} . The library was aliquoted and stored in 15% glycerol at -80°C.

Next-generation sequencing of the complete phage library.

DNA from the libraries was amplified using primers that flank the CDR-H3 region. The sequences at the 5'-ends of these primers incorporated molecular barcodes to facilitate unambiguous enumeration of distinct phage sequences. The protocols for PCR-amplification and sequencing are described by Kinde *et al.* (2011 *PNAS*. 108:9530-35). Sequences processed and translated using a custom SQL database and both the nucleotide sequences and amino acid translations were analyzed using Microsoft Excel.

Peptides and HLA-Monomers.

Mutant and WT peptides (listed in Table 1) were predicted to bind to HLA alleles using NetMHC version 4.0. All peptides were synthesized at a purity of >90% by Peptide 2.0 (Chantilly, VA). Peptides were resuspended in DMSO or DMF at 10 mg/mL and stored at -20°C. HLA monomers were synthesized by refolding recombinant HLA with peptide and beta-2 microglobulin, purified by gel-filtration, and biotinylated (Fred Hutchinson Immune Monitoring Lab, Seattle, WA). Monomers were confirmed to be folded prior to selection by performing an ELISA using W6/32 antibody (BioLegend, San Diego, CA).

Selection for phage binding to mutant peptide-HLA monomers.

scFv-bearing phage clones specific to the mutant pHLA complexes were identified similar to methods described elsewhere (see, *e.g.*, Skora *et al.*, 2015 *PNAS*.112:9967-72). The panning schema involved an enrichment phase, a competition phase, and a final selection phase.

The phage display library stored at -20°C in 15% glycerol, were regrown within a week of starting the panning process. A colony of phage-competent SS320 cells (Lucigen, Middleton, WI) was inoculated in a 37°C overnight culture of 2xYT medium (Sigma-Aldrich, St. Louis, MO) supplemented with tetracycline (20 µg/mL), and the next day grown to 2 L of mid-log phase (OD₆₀₀ of 0.3-0.5) bacteria. Bacteria were infected with the phage library at an MOI of 0.5 and M13K07 Helper phage (Antibody Design Labs, San Diego, CA) at an MOI of 4 along with the addition of 2% (W/V) glucose (Sigma-Aldrich, St. Louis, MO) and allowed to shake for 1 hour at 37°C. The culture was centrifuged and the cells were resuspended in 2xYT medium with carbenicillin (100 µg/mL), kanamycin (50 µg/mL), and 50 µM IPTG and subsequently shaken and grown overnight at 30°C for phage production.

The following morning, the bacterial culture was aliquoted into 50 mL Falcon tubes and pelleted twice at high speed to obtain clarified supernatant. The phage-laden supernatant was precipitated on ice for 40 minutes with a 20% PEG-8000/2.5M NaCl solution at a 1:4 ratio of PEG/NaCl:supernatant. After precipitation, phage was centrifuged at 12,000 x g for 40
5 minutes and resuspended in 1 mL of 1X TBS with 2 mM EDTA, 0.1% sodium azide, and 1x Complete Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO).

Biotinylated pHLA monomer complexes were conjugated to M-280 streptavidin magnetic Dynabeads (Life Technologies, Carlsbad, CA). The biotinylated pHLA were incubated with either 25 μ L of Dynabeads beads per 1 μ g of pHLA in blocking buffer (PBS,
10 0.5% BSA, 2mM EDTA, and 0.1% sodium azide) for 1 hour at room temperature (RT). After the initial incubation, the complexes were washed and resuspended in 100 μ L of blocking buffer.

During the enrichment phase (Round 1), approximately 2×10^{13} phage, representing ~500-fold coverage of the library, were negatively selected for 1 hour at RT with a mixture
15 of 1 mL unconjugated washed Dynabeads, 1 mg free streptavidin protein (RayBiotech, Norcross, GA) to remove any phage recognizing either the unconjugated Dynabead and streptavidin. After negative selection, beads were isolated with a DynaMag-2 magnet (Life Technologies, Carlsbad, CA) and the supernatant containing unbound phage was transferred for positive selection for 1 hour at RT against the 1 μ g of mutant pHLA conjugated to
20 Dynabeads. Prior to elution, beads were washed 10 times with 1 mL of TBST (1x TBS with 0.5% Tween-20). Phage were eluted by resuspending the beads in 1 mL of 0.2 M glycine, pH 2.2. After a 10 minutes incubation, the solution was neutralized by the addition of 150 μ L of 1 M Tris, pH 9.0. Neutralized phages were used to infect 10 mL cultures of mid-log-phage SS320s, with the addition of M13K07 helper phage (MOI of 4) and 2% glucose. Bacteria
25 were then incubated as previously described and the phages were precipitated the next morning with PEG/NaCl.

During the selection phase (Rounds 2-5), phage from the previous round was subjected to negative selection against HLA-matched cell lines without the mutations of interest, corresponding WT pHLA monomer conjugated to Dynabeads, unrelated pHLA
30 monomerconjugated to Danybeads, and free streptavidin. After negative selection, beads were isolated with a DynaMag-2 magnet and unbound phage was transferred for positive

selection. This was performed by incubating phage with 1 μ g (Round 2), 0.5 μ g (Rounds 3, 4), or 0.25 μ g (Round 5) mutant pHLA conjugated to the magnetic Dynabeads. Prior to elution, beads were washed 10 times in 1 mL TBST. Phage were eluted from magnetic Dynabeads and used to infect mid-log phase SS320 cells as described above.

5 *Flow Cytometry.*

Monoclonal phage flow cytometry staining was performed by selecting individual colonies of SS320 cells transformed with a limiting dilution of phage obtained from the final selection round. Individual colonies were inoculated into 200 μ L of 2xYT medium containing 100 μ g/mL carbenicillin and 2% glucose and grown for three hours at 37°C. The cells were then infected with 1.6×10^7 M13K07 helper phage and incubated for 1 hour at 37°C with shaking. The cells were pelleted, resuspended in 300 μ L of 2xYT medium containing carbenicillin (100 μ g/mL), kanamycin (50 μ g/mL), and 50 μ M IPTG, and grown overnight at 30°C for phage production. Cells were pelleted and the phage-laden supernatant was used for staining.

For peptide pulsing, HLA-matched cells were washed once with PBS and once with serum-free RPMI-1640 before incubation at 10^6 cells per mL in serum-free RPMI-1640 containing 50 μ g/mL peptide and 10 μ g/mL human beta-2 microglobulin (ProSpec, East Brunswick, NJ) overnight at 37°C. The pulsed cells were pelleted, washed once in cold staining buffer (PBS containing 0.5% BSA, 2 mM EDTA, and 0.1% sodium azide), and resuspended in 50 μ L of stain buffer. Phage staining was performed on ice with 50 μ L monoclonal phage supernatant for 1 hour, followed by one 800 μ L wash in cold staining buffer. Cells were then stained with 1 μ g of rabbit anti-M13 antibody (Novus Biologics, Centennial, CO) in 100 μ L total volume on ice for 1 hour and washed once with 800 μ L of cold staining buffer. Cells were then stained with 5 μ L anti-rabbit-PE (Biolegend) on ice for 1 hour in 100 μ L total volume, followed by incubation with 200 μ L LIVE/DEAD Fixable Near-IR Dead Cell Stain (Thermo Fisher) for 10 minutes at room temperature per manufacturer's instructions. Cells were washed once in 800 μ L of staining buffer. Stained cells were analyzed using an iQue Screener (IntelliCyt, Albuquerque, NM).

Bispecific Antibody Production.

gBLOCKs encoding bispecific antibodies were ordered from IDT (Skokie, Illinois). gBLOCKs were cloned into the pcDNA3.4 plasmid (Thermo Fisher) by NEBuilder HiFi DNA Assembly (New England Biolabs, Ipswich, MA) following the manufacturer's protocol. 293FT cells (Thermo Fisher) were transfected with the bispecific antibody pcDNA3.4 plasmids using Lipofectamine 3000 (Life Technologies) per the manufacturer's instructions in a T75 flask. Following a 5-8 day incubation, media was harvested and centrifuged at 500g for 10 minutes at 4°C. Bispecific antibody protein was purified using a Clontech Capture™ His-Tagged Purification Mixiprep Kit (Takara Bio, Mountain View, CA) per manufacturer's instructions. Bispecific antibody protein was desalted into PBS using Zeba spin 7k MWCO desalting columns per the manufacturer's instructions. Bispecific antibody concentration was quantified using Mini-PROTEAN® TGX Stain-Free™ Precast Gels (Biorad, Hercules, California) using a standard curve of protein of known concentration. Stain-free gels were imaged using the ChemiDoc XRS+ Imager (Biorad).

Bispecific Antibody Co-Culture Assay.

COS-7 cells were transfected with various combinations of pcDNA3.1 or pcDNA3.4 (Life Technologies) plasmids encoding HLA-A2, HLA-A1, p53(WT, R175H), HRAS(WT, Q61H, Q61K, Q61L, Q61R), KRAS(WT, Q61H, Q61K, Q61L, Q61R), NRAS(WT, Q61H, Q61K, Q61L, Q61R) with Lipofectamine 3000 (Life Technologies) per manufacturer's instructions in a T75 flask. A total of 50,000 T cells were combined with transfected 50,000 COS-7 cells, 25,000 TYKnu cells, or 25,000 HL-60 cells and the specified concentration of bispecific antibody in a 96-well plate, and the co-culture was allowed to incubate for 20 hours at 37°C under 5% CO₂. Following co-culture, conditioned media was collected and assayed for secreted IFN γ by Quantikine® ELISA (R&D Systems, Minneapolis, MN). Alternatively, following coculture, target cell viability was measured using CellTiter-Glo (Promega, Madison, WI) per the manufacturer's instructions.

CRISPR Cell Line Engineering.

The Alt-R CRISPR system (Integrated DNA Technologies, IDT) was used to modify the p53 of the TYKnu cell line and the HLA allele of HL-60 cell line. Alt-R® CRISPR Cas9

crRNAs (IDT) targeting *TP53* exon 3 (CCCCGGACGATATTGAACAA; SEQ ID NO:191), and *HLA-A* exon 2 (CAGACTGACCGAGCGAACCT; SEQ ID NO:192) as well as Alt-R® CRISPR-Cas9 tracrRNA (IDT) were resuspended at 100 μM with Nuclease-Free Duplex Buffer (IDT). The crRNAs and tracrRNA were duplexed at a 1:1 molar ratio for 5 minutes at 5 95°C according to the manufacturer's instructions. The duplexed RNA was allowed to cool to room temperature prior to mixing with Cas9 Nuclease (IDT) at a 1.2:1 molar ratio for 15 minutes. To knock out p53 of TYKnu cells, 40 pmols of the Cas9 RNP complexed with p53 gRNA were mixed with 200,000 TYKnu cells in 20 μL of OptiMEM. This mixture was loaded into a 0.1 cm cuvette (Biorad) and electroporated at 120V and 16ms using an ECM 10 2001 (BTX). To knock out the HLA-A alleles in HL-60 cells, 40 pmols of the Cas9 RNP complexed with HLA-1 gRNA were mixed with 200,000 HL-60 cells in 20 μL of OptiMEM. This mixture was loaded into a 0.1 cm cuvette (Biorad) and electroporated at 150V and 16 ms using an ECM 2001 (BTX). After electroporation, cells were immediately transferred to complete growth medium and cultured for 10 days, changing media and 15 passaging as needed.

p53 and HLA-A modified polyclonal pools were plated at a density of 0.5 cells per well in 96 well plates and cultured for 2 weeks. Single colonies were harvested and plated into 2 replicate 96-well plates. Genomic DNA was harvested from one of the plates using the Quick-DNA™ 96 Kit (Zymo Research, Irvine, CA), PCR amplified using Q5® Hot Start 20 High-Fidelity 2X Master Mix (New England Biolabs), and Sanger sequenced (Genewiz, South Plainfield, NJ) to select for clones with the desired modifications.

Example 2: Targeting a Neoantigen Derived From a common TP53 Mutation

TP53 is the most commonly mutated cancer driver gene, but despite extensive efforts, no drug targeting mutant *TP53* has been approved for treatment of the large number of 25 patients whose tumor contain p53 mutations. This Example describes the identification of an antibody highly specific to the most common *TP53* mutation (R175H) in complex with a common HLA-A allele on the cell surface. For example, this Example describes the identification of a TCRm antibody specific to the HLA-A*02:01-restricted p53^{R175H} neoantigen, the structural basis of its specificity, and its conversion to a bispecific antibody 30 that can lyse cancer cells in a fashion dependent on the presence of the neoantigen. Such an

immunotherapeutic agent that targets a common *TP53* mutation can be used to target cancers containing other tumor suppressor gene mutations.

Results

The p53^{R175H} neoantigen is presented on the surface of cancer cells

5 The p53^{R175H} (aa 168-176, HMTEVVRHC; SEQ ID NO:1) and p53^{WT} (HMTEVVRRC; SEQ ID NO:135) peptides were predicted on the NetMHCpan 4.0 server to bind HLA-A*02:01 at 5177.6 nM (rank 9.6%) and 7121.5 nM (11.6%), respectively. To provide experimental evidence of and to quantify such presentation, peptides eluted from HLA molecules were analyzed in four different cell culture systems using a mass
10 spectrometry (MS)-based method. First, the human HLA-A*02:01 and either p53^{R175H} or p53^{WT} were co-expressed in monkey COS-7 cells. MS analysis of the peptides immunopurified with an anti-HLA antibody detected the p53^{R175H} peptide at approximately 1500 copies per cell (Fig. 20, Table 6). Though relatively abundant p53^{R175H} peptide was detected, the p53^{WT} peptide was not observed. Second, MS analysis was performed on three
15 human cancer cell lines, KMS26, TYK-nu, and KLE, all of which harbor p53^{R175H} mutations and carry an HLA-A*02:01 allele. The p53^{R175H} peptide was detected on all three cell lines, and, as expected, at much lower levels than in the COS-7 cells in which the mutant *TP53* and HLA genes were exogenously introduced (Fig. 20, Table 6). Based on comparisons with heavy isotope labeled controls, it was estimated that there were 2.4, 1.3, and 1.5 copies of
20 cell-surface p53^{R175H}/HLA-A*02:01 complexes on the cell surfaces of KMS26, TYK-nu, and KLE cell lines, respectively (Table 6).

Table 6. Quantitative assessment of the p53^{R175H} neoantigen peptide. The amount of p53^{R175H} neoantigen peptide (HMTEVVRHC; SEQ ID NO:1) present in COS-7 cells transfected with HLA-A*02:01 and p53^{R175H} or p53^{WT}, as well as cells lines that endogenously express HLA-A*02:01 and p53^{R175H}, were quantified using mass spectrometry.

5

Cell line	HLA and p53 status	No. cells (millions) used for analysis	Abundance (femtomole)	Copy no. / cell
COS-7	Exogenous HLA-A*02:01/p53 ^{R175H}	224.0	156	1587.3
COS-7	Exogenous HLA-A*02:01/p53 ^{WT}	224.0	Not detectable	Not detectable
KMS26	Endogenous HLA-A*02:01/p53 ^{R175H}	157.0	0.48	2.4
KLE	Endogenous HLA-A*02:01/p53 ^{R175H}	92.4	0.18	1.5
TYK-nu	Endogenous HLA-A*02:01/p53 ^{R175H}	168.3	0.28	1.3

*Identification of scFv-expressing phage clones specific for the HLA-A*02:01-restricted p53^{R175H} peptide and conversion to scDb format*

To identify TCR-mimic single-chain variable fragments (scFvs) selectively targeting mutant pHLA complexes, an scFv-displaying phage library was screened with an estimated complexity >1x10¹⁰. Positive selection against HLA-A*02:01 pHLA monomers containing the p53^{R175H} peptide were combined with negative selection against pHLA monomers containing the p53^{WT} and irrelevant peptides. Selected phage clones were amplified and assessed for binding to T2 cells presenting the mutant or wild-type (WT) peptide via flow cytometry (Fig. 21).

Twenty-three phage clones with median fluorescence intensity (MFI) ratios of p53^{R175H} to p53^{WT} >4 were then converted to T cell-retargeting bispecific antibodies (Fig. 21). This was achieved through linking each individual scFv to an anti-CD3 scFv (UCHT1) in a single-chain diabody (scDb) format (Fig. 22). The scDb format was chosen after evaluating several previously described bispecific antibody formats, such as bispecific T-cell engager (BiTE), dual-affinity re-targeting antibody (DART), and diabody in pilot

experiments. The ability of scDb to activate T cells was assessed by interferon- γ (IFN- γ) release after co-incubation with COS-7 cells overexpressing HLA-A*02:01 and either full-length p53^{R175H} or p53^{WT} proteins. Two scDb clones, named H2-scDb and H20-scDb and derived from phage clones H2 and H20, respectively, showed the most potent and specific T-cell activation in the presence of p53^{R175H}/HLA-A*02:01 (Fig. 23). The specificity of these scDbs was further evaluated by titration enzyme-linked immunosorbent assay (ELISA). Both H2 and H20 bound to p53^{R175H}/HLA-A*02:01 at low concentrations, as expected. At high concentrations, H20-scDb also bound to p53^{WT}/HLA-A*02:01, while H2-scDb did not bind to the wild type pHLA complex even at very high concentrations of the scDb (Fig. 1A, Fig. 24). H2-scDb was therefore chosen for further analysis. As assessed by surface plasmon resonance (SPR), the H2-scDb bound to p53^{R175H}/HLA-A*02:01 with a K_D =86 nM, a k_{on} of $1.76 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, and a k_{off} of $1.48 \times 10^{-2} \text{ s}^{-1}$ (Fig. 14B). The k_{on} of $1.76 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ suggested a lack of conformational change of the p53^{R175H}/HLA-A*02:01 upon binding. No detectable binding of the H2-scDb to p53^{WT}/HLA-A*02:01 was observed in the SPR experiments (Fig. 14B).

Next, it was examined whether anti-CD3 arms of the scDb other than the original UCHT1, could influence the ability of H2 to induce T-cell activation. The H2 scFv was linked to a panel of commonly used anti-CD3 ϵ scFvs, including UCHT1, hUCHT1v9, OKT3, TR66, and hXR32. It was found that, among the anti-CD3 scFvs tested, UCHT1 activated T cells at the lowest p53^{R175H} peptide concentration when linked to the H2 scFv (Fig. 25), and used this particular bispecific antibody for further experiments.

H2-scDb specifically recognizes cancer cells expressing the p53^{R175H} neoantigen

It was next evaluated the ability of H2-scDb to recognize cancer cell lines harboring the p53^{R175H} mutations and expressing various levels of HLA-A*02:01. H2-scDb elicited T-cell responses in a dose-dependent manner when T cells were co-cultured with three lines that expressed moderate to high levels of HLA-A*02:01 (Fig. 15A). This activation was noted even at very low (sub-nanomolar) concentrations of the bispecific antibody. The T-cell responses were polyfunctional, as indicated by the release of cytotoxic granule proteins granzyme B and perforin, cytotoxicity, and the production of cytokines IFN- γ , tumor necrosis factor α (TNF- α), interleukin-2 (IL-2), and others (Fig. 15B, Fig. 26). Clustering of

T cells around tumor cells, leading to their lysis in the presence of H2-scDb, was also visualized by real-time live-cell imaging (Fig. 15C). The specificity of the bispecific antibody for both the p53^{R175H} peptide and HLA-A*02:01 was evident from the observation that much lower levels of IFN- γ were induced by cells harboring a p53^{R175H} mutation but low
 5 levels of expression of HLA-A*02:01 (AU565 or SK-BR3) or by cells without p53^{R175H} but relatively high levels of HLA-A*02:01 expression (Fig. 15A, Fig. 27).

It was further validated the specificity of H2-scDb using nine pairs of isogenic cell lines that differed with respect to HLA-A*02:01 expression or p53^{R175H} mutation. First, human HEK293FT (*TP53*^{WT}/HLA-A*02:01) or Saos-2 (*TP53*^{null}/HLA-A*02:01) cells were
 10 transfected with plasmids expressing either full-length p53^{R175H} or p53^{WT}. H2-scDb induced robust T-cell activation when co-cultured with both cell lines overexpressing p53^{R175H} but not with p53^{WT}-overexpressing or parental cells (Fig. 16A). Second, HLA-A*02:01-encoding retrovirus were transduced into four cell lines (AU565, SK-BR-3, HuCCT1, CCRF-CEM) that harbored the p53^{R175H} mutation but had low levels of HLA-A*02:01 expression (Fig.
 15 28). Exogenous expression of HLA-A*02:01 in all four lines conferred T-cell activation by H2-scDb (Fig. 16B). Third, *TP53* was genetically disrupted in KMS26, TYK-nu, and KLE cancer cell lines that carry endogenous p53^{R175H} using a CRISPR-based technology (Fig. 29A). T-cell activation, as assessed by IFN- γ secretion, was reduced to control levels when *TP53* was knocked out in all three cell lines (Fig. 16C). The cytotoxicity mediated by H2-
 20 scDb was similarly mitigated by *TP53* knock-out (KO) in these cells (Fig. 16D, Fig. 29B).

*Overall structure of the H2-Fab–p53^{R175H}/HLA-A*02:01 ternary complex*

To understand the structural basis for the high specificity of the H2 clone for p53^{R175H}/HLA-A*02:01, the H2 fragment antigen-binding (H2-Fab)–p53^{R175H}/HLA-A*02:01 complex was purified (Fig. 30) and its crystal structure was determined by molecular
 25 replacement and refined to 3.5 Å resolution (PDB ID 6W51, Table 7). There were four H2-Fab and four p53^{R175H}/HLA-A*02:01 per asymmetric unit (Fig. 17A, B). All four H2-Fab were firmly positioned on the p53^{R175H}/HLA-A*02:01, without evidence of rocking with a root-mean-square deviation (rmsd) of 0.45 to 0.51 Å. The total buried surface area of the H2-Fab–p53^{R175H}/HLA-A*02:01 interface was 1173 Å², with roughly equal contributions
 30 from the heavy and light chains (644 Å² and 529 Å², respectively, Table 8). Although the

entire structure was refined to a resolution of 3.5 Å, particularly clear electron densities were observed for the p53^{R175H} neoantigen, the CDRs of the H2-Fab, and the HLA-A*02:01 (Fig. 17C, D).

5 Table 7. X-ray Crystallography data collection and refinement statistics.

Space group	P 1 2 ₁ 1
Cell constants a, b, c (Å) α, β, γ (°)	113.29, 123.89, 137.07 90.00, 100.38, 90.00
Resolution (Å)	49.21 – 3.50 49.17 – 3.50
Number of reflections	104482
Number of unique reflections	43734
% Data completeness (in resolution range)	99.3 (49.21-3.50) 99.4 (49.17-3.50)
R_{merge}	0.25
CC _{1/2} (%)	0.938
$\langle I/\sigma(I) \rangle$	1.31 (at 3.48Å)
R, R_{free}	0.216 , 0.267 0.221 , 0.272
R_{free} test set	2349 reflections (5.01%)
Wilson B-factor (Å ²)	61.6
Anisotropy	0.581
L-test for twinning	$\langle L \rangle = 0.38, \langle L^2 \rangle = 0.21$
F _o ,F _c correlation	0.90
Total number of atoms	25900
Average B, all atoms (Å ²)	69.0

*Binding of the p53^{R175H} peptide to HLA-A*02:01*

10 The p53^{R175H} neoantigen occupies the binding cleft $\alpha 1$ - $\alpha 2$ of HLA-A*02:01, burying a solvent accessible surface area of 870 Å², slightly larger than other peptide/HLA-A*02:01 complexes (Fig. 18A, B, Fig. 31A) and with the C-terminal arginine at position 7 (P7, Arg7, Arg174 in p53) and histidine at position 8 (P8, His8, His175 in mutant p53) pointing up, out of the groove. In contrast, the N-terminus of the peptide is situated deep within the peptide binding cleft, anchored by multiple residues in the HLA-A*02:01 (Fig. 18A, B, Fig. 31A).

The anchor residues of the peptide, a methionine at position 2 (P2, Met2, Met169 in p53) and a cysteine residue at position 9 (P9) (Fig. 31B), departed from the canonical anchor residues—leucine at P2 and valine or leucine at P9. Peptides that bind to HLA-A*02:01 through either a methionine at P2 or a cysteine at P9 have been reported, but not both. Based on alignments with structures of other HLA-A*02:01 peptides in complex with TCR or TCRm, the unconventional anchoring of p53^{R175H} did not result in drastic peptide conformational change or positioning (Fig. 31C, D).

*Structural basis for the recognition of p53^{R175H}/HLA-A*02:01 by the H2-Fab*

The recognition of the HLA-A*02:01 by the H2-Fab was mediated by all six CDRs. There were a total of 79 contacts between the H2-Fab CDRs and the α 1 and α 2 of HLA-A*02:01, with the light chain contributing to 61% of those contacts. The H2-Fab buried a solvent accessible surface area of 818 Å² within the HLA, of which 427 Å² were contributed by the light chain and 391 Å² by the heavy chain (Table 8). In contrast, only four of the six H2-Fab CDRs (H1, H2, H3 and L3) interacted with the p53^{R175H} peptide. Overall, the H2-Fab made 36 contacts with the p53^{R175H} neoantigen, including five hydrogen bonds and numerous van der Waals interactions. Importantly, His175 at P8 made 47% of all direct contacts with the H2-Fab. The CDR-H1, H2, and H3 of the heavy chain and CDR-L3 of the light chain formed a cage-like configuration around the C-terminus of the p53^{R175H} peptide, trapping Arg174 at P7 and His175 at P8 into position by providing a stable interaction (Fig. 18C). The imidazole side chain of His175 at P8 was anchored by a hydrogen bonding network with Asp54 (CDR-H2) and Tyr94 (CDR-L3) (Fig. 18C, Fig. 32). Tyr52 (CDR-H2) acted as a ceiling and capped the cage-like structure around His8 by forming π - π interactions (Fig. 18C, Fig. 32).

Table 8. Structural comparison of H2-Fab–p53^{R175H}/ HLA-A*02:01 with various TCR and Fab antibody-pHLA. Total bonds were calculated using a 4 Å cutoff which includes both hydrogen bonds and van der Waals. PDB, Protein Data Bank; BSA, buried surface area; α , TCR α chain; β , TCR β chain; H, V_H domain; L, V_L domain; pep, HLA presented peptide.

	H2-Fab	MAGE-A3 TCR	NY-ESO-1 Fab (3M4E4)	ESK-1 Fab
Affinity (K _D)	86 nM	7.1 nM	95 nM	13.2 nM
PDB	6W51	5BRZ	3HAE	4WUU

Angle of rotation (°)	27°	-57°	134°	112°
Total bonds	115	114	167	183
Peptide bonds (bold ≥10)	4, 5, 6, 7, 8	1, 4, 5, 7, 8	1, 2, 4, 5 , 6, 7, 8, 9	1 , 4
Peptide bonds	36 (31%)	16 (14%)	89 (53%)	19 (10%)
Bonds from β/H	21	10	47	10
Bonds from α/L	15	6	42	9
HLA bonds	79	98	78	164
Bonds from β/H	31	20	47	130
Bonds from α/L	48	78	31	34
HLA ≥5 bonds (bold ≥10)	61, 65 , 72, 80, 146, 155	66, 154 , 155, 157, 158, 163	65 , 66, 72 , 73	58 , 62, 63 , 65, 66 , 155, 161, 162, 166, 167 , 169, 170
BSA				
BSA total	1173	1027	1366	1084
BSA β/H pep	253	158	256	72
BSA α/L pep	102	112	260	93
BSA β/H HLA	391	247	523	601
BSA α/L HLA	427	510	327	318

Viewed from the axis of the C-terminus to the N-terminus of the p53^{R175H} peptide, the CDRs were arranged in the order H2, H1, L3, H3, L1, L2 (Fig. 17E, F, G). Interestingly, the axis of the H2-Fab was nearly parallel to the axis of the peptide within the HLA groove with a binding angle of 27° (Fig. 17G, H). This orientation was quite different from that of most previously described TCRs or TCRm antibodies to pHLA complexes, in which the axes are diagonal (Fig. 33).

Assessing candidate cross-reactive peptides

One of the major challenges confronting new immunotherapeutic antibodies is off-target binding, which can result in toxicity to normal cells. Several powerful approaches to profile TCR and TCRm specificity have been developed to address this important issue. Scanning mutagenesis was employed to identify peptides in the human proteome to which H2-scDb might cross-react. A peptide library was generated by systemically substituting amino acids at each position of the target p53^{R175H} peptide (HMTEVVRHC; SEQ ID NO:1) with each of the remaining 19 common amino acids. T2 cells loaded with each of the 171 variant peptides were then used to assess T-cell activation by measuring IFN-γ release following incubation with H2-scDb (Fig. 18D). In congruence with the X-ray structural

analysis, any changes in P8, where the mutant histidine residue lies, and any change in P7, which is encased with P8 by the CDR loops, abolished recognition of the peptide.

Importantly, peptides with substitutions at these positions retained their ability to bind to HLA-A*02:01 (Fig. 34), but not to the H2-scDb. Other non-anchor residues at positions 3-6
5 also highly favored the parental amino acids present in the target peptide. This recognition pattern is illustrated as a Seq2Logo graph (Fig. 18E).

Next, a nonamer binding motif, x-[AILMVNQTC]-[ST]-[DE]-[IV]-[IMVST]-R-H-[AILVGHSTYC] (SEQ ID NO:197), was generated using 20% target peptide reactivity as a cutoff for permissive amino acids at each position (Fig. 35). A search of this motif in the
10 UnitProtPK human protein database using ScanProsite yielded 3 homologous peptides from STAT2 (PLTEIIRHY; SEQ ID NO:185), VP13A (LQSEVIRHY; SEQ ID NO:186), and ZFP3 (QNSEIIRHI; SEQ ID NO:187) (Table 9). None of these 3 peptides were predicted to be potent binders of HLA-A*02:01 by NetMHCpan 4.0 (% rank all >2.0) and had lower predicted binding affinity than the parental p53^{R175H} peptide (Table 9). However, to
15 experimentally exclude the possibility of cross-reactivity, T2 cells were pulsed with each of these peptides. H2-scDb activated T cells only in the presence of T2 pulsed with the p53^{R175H} peptide (Fig. 18F). Additionally, COS-7 cells were co-transfected with expression plasmids for HLA-A*02:01 and STAT2 or ZFP3; full-length VP13A was not tested due to its large size (3000 aa). Again, no T-cell activation was detected in the co-culture assay with
20 COS-7 cells expressing the two proteins containing the candidate cross-reactive peptides (Fig. 36).

Table 9. Putative cross-reactive peptides identified through peptide scanning. A binding motif of H2-scDb was determined by positional scanning of the p53^{R175H} HMTEVVRHC (SEQ ID NO:1) peptide. Three peptides that conformed to this motif in the UniProtPK human protein database were identified using ScanProsite. NetMHCpan4.0 was used to predict the binding affinity and % rank of these peptides to HLA-A*02:01. The parental p53^{R175H} target peptide is listed for comparison.

UniProt ID	Name	Peptide position	Sequence	SEQ ID NO	HLA-A*02:01 Affinity (nM)	%Rank
NA	p53 ^{R175H}	168-176	HMTEVVRHC	1	5177.6	9.7
P52630	STAT2	640-648	PLTEIIRHY	185	32385.4	46.8
Q96RL7	VP13A	2840-2848	LQSEVIRHY	186	21897.4	27.6
Q96NJ6	ZFP3	348-356	QNSEIIRHI	187	31174.4	43.9

Antitumor activity of the H2-scDb in vivo

To determine whether H2-scDb could control tumor growth *in vivo*, KMS26 cells were engrafted into NOD-SCID-*Il2rg*^{-/-} (NSG) mice through intravenous injection, establishing widespread, actively growing cancer cells throughout the body. Two models were used to assess the effects of the H2-scDb in combination with human T cells. In an early treatment model, mice were randomized based on luminescence quantification of tumor burden and H2-scDb was subsequently administered through intraperitoneal infusion pumps at 0.3 mg/kg/day, starting one day after tumor inoculation. An irrelevant isotype scDb was administered in parallel as control. H2-scDb markedly suppressed the growth of parental KMS26 cells (Fig. 19A). In contrast, the H2-scDb had no effect on KMS26 cells in which the *TP53* gene had been disrupted using CRISPR (Fig. 19B). In the second model, mice were randomized 6 days after tumor inoculation. The H2-scDb was administered at two doses (0.15 and 0.3 mg/kg/day). Both doses resulted in major tumor regressions and were well

tolerated as assessed by the absence of significant changes in body weight (Fig. 19C, Fig. 37).

Taken together, these results demonstrate that --- can be used to target p53, the most common mutation of the most commonly mutated tumor suppressor gene in human cancers, and, as such, can specifically target cancer cells harboring the mutation.

Materials and Methods

Cell lines and primary T cells

COS-7, T2 (174 x CEM.T2), Raji, HH, AU565, SK-BR-3, KLE, HCT116, SW480, NCI-H441, Saos-2, and CCRF-CEM cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). KMS26, TYK-nu, and HuCCT1 were purchased from Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan). SigM5 was obtained from DSMZ (Braunschweig, Germany). HEK293FT was obtained from Invitrogen (Thermo Fisher Scientific, Waltham, MA). T2, Raji, Jurkat, HH, AU565, NCI-H441, TOV-112D, CCRF-CEM, KMS26, TYK-nu, and HuCCT1 were cultured in RPMI-1640 (ATCC, 30-2001) with 10% FBS (GE Healthcare, SH30070.03) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, 15140163). COS-7, SK-BR-3, HCT116, SW480, and Saos-2 were culture in McCoy's 5A modified media (Thermo Fisher Scientific, 16600108) with 10% FBS and 1% Penicillin-Streptomycin. SigM5 was cultured in IMDM (Thermo Fisher Scientific, 12440061) with 20% FBS and 1% Penicillin-Streptomycin. HEK293FT was cultured in DMEM (high glucose, pyruvate, Thermo Fisher Scientific, 11995065) with 10% FBS, additional 2 mM GlutaMAX (Thermo Fisher Scientific, 35050061), 0.1mM MEM non-essential amino acids (Thermo Fisher Scientific, 11140050), 1% Penicillin-Streptomycin, and 500 µg/mL Geneticin (Thermo Fisher Scientific, 10131027). PBMCs were isolated from leukapheresis samples (Stem Cell Technologies, Vancouver, BC) by standard density gradient centrifugation with Ficoll Paque Plus (GE Healthcare, 17-1440-03). T cells were expanded from PBMCs with addition of the anti-human CD3 antibody (OKT3, BioLegend, San Diego, 317347) at 15 ng/mL for three days. T cells were cultured in RPMI-1640 with 10% FBS, 1% Penicillin-Streptomycin, 100 IU/mL recombinant human IL-2 (aldesleukin,

Prometheus Therapeutics and Diagnostics, San Diego, CA), and 5 ng/mL recombinant human IL-7 (BioLegend, 581908). All cells were grown at 37°C in 5% CO₂ with humidification.

Detection of neoantigen peptide

5 HLA-A*02:01-restricted p53^{R175H} peptide was directly detected and quantified in COS-7 cells transfected with HLA-A*02:01 and p53R175H and in human cancer cell lines expressing HLA-A*02:01 and p53^{R175H} through MANA-SRM. In particular, the dual-reduction approach described in MANA-SRM was critical for this detection because a cystine and a methionine coexist in the p53^{R175H} peptide. 100 femtomole heavy-isotope
10 labeled peptide HMTEVVRHC (SEQ ID NO:1; New England Peptide Inc, Gardner, MA) were spiked into each sample before the assay. The MANA-SRM assays were performed at Complete Omics (Baltimore, Maryland).

Phage display library construction

The scFv-bearing phage library was described elsewhere (see, e.g., Miller et al., *J. Biol. Chem.* 294:19322-19334 (2019); and Skora et al., *Proc. Natl. Acad. Sci. U. S. A.* 112:9967-9972 (2015)). Oligonucleotides were synthesized by GeneArt (Thermo Fisher Scientific) using trinucleotide mutagenesis (TRIM) technology to diversify complementarity-determining region (CDR)-L2, CDR-L3, CDR-H1, CDR-H2, and CDR-H3. A FLAG (DYKDDDDK; SEQ ID NO:190) epitope tag was placed immediately downstream of the
20 scFv, which was followed in frame by the full-length M13 pIII coat protein sequence. The total number of transformants obtained was determined to be 3.6×10^{10} .

Peptides and monomers

All peptides were synthesized at a purity of >90% by Peptide 2.0 (Chantilly, VA) or ELIM Biopharm (Hayward, CA), except for the positional scanning library, where crude
25 peptides were used. Peptides were resuspended in dimethylformamide at 10 mg/mL and stored at -20°C. Peptide-HLA monomers were synthesized by refolding recombinant HLA with peptide and β 2 microglobulin (β 2M), purified by gel-filtration, and biotinylated (Fred Hutchinson Immune Monitoring Lab, Seattle, WA). Monomers were confirmed to be folded

prior to selection by performing an ELISA using W6/32 antibody (BioLegend, 311402), which recognizes only folded HLA.

Selection of mutant pHLA specific phage clone

Phage clones bearing scFvs specific to p53^{R175H}/HLA-A*02:01 pHLA were identified using an approach described elsewhere (Skora et al., *Proc. Natl. Acad. Sci. U. S. A.* 112:9967-9972 (2015)). Biotinylated HLA-A*02:01 pHLA monomer complexes were conjugated to 25 μ L of M-280 streptavidin magnetic Dynabeads (Thermo Fisher Scientific, 11206D) per 1 μ g of pHLA. During the enrichment phase (Round 1), approximately 4×10^{13} phage, representing ~ 1000 -fold coverage of the library, were negatively selected with a mixture of unconjugated Dynabeads and free streptavidin protein (RayBiotech, Norcross, GA, 228-11469). After negative selection, supernatant containing unbound phage was transferred for positive selection using 1 μ g of p53^{R175H}/HLA-A*02:01 pHLA. Beads were then washed and phage was eluted to infect mid-log-phage SS320 bacteria, with the addition of M13K07 helper phage (MOI of 4). Bacteria were then grown overnight at 30°C for phage production and the phage was precipitated the next morning with PEG/NaCl.

During the selection phase (Rounds 2-5), phage from the previous round was subjected to two stages of negative selection: 1) against cell lines without p53^{R175H}/HLA-A*02:01 (RPMI-6666, Jurkat, Raji, SigM5, HH, T2, and NCI-H441) and 2) against p53^{WT}/HLA-A*02:01 pHLA, unrelated HLA-A*02:01 pHLA, and free streptavidin. For negative selection using cell lines, phage was incubated with a total number of $5 \times 10^6 - 1 \times 10^7$ of cells at 4°C overnight. After negative selection, beads were isolated and unbound phage was transferred for positive selection by incubating with 1 μ g (Round 2), 0.5 μ g (Round 3), or 0.25 μ g (Round 4, 5) of p53^{R175H}/HLA-A*02:01 pHLA. Phage was then eluted and amplified by infecting SS320 as described above.

After five rounds of selection, SS320 cells were infected with a limiting dilution of the enriched phage. A total of 190 individual colonies of SS320 were picked and phage DNA was PCR amplified by primers flanking the CDRs (Forward: GGCCATGGCAGATATTCAGA (SEQ ID NO:198), Reverse: CCGGGCCTTTATCATCATC (SEQ ID NO:199)) using Q5 Hot Start High-Fidelity 2X Master Mix (New England BioLabs, M0494L) and Sanger sequenced by GENEWIZ (South

Plainfield, NJ). Sequences flanking the CDRs were trimmed using DNA Baser Sequence Assembler v4 (Arges, Romania) and the sequences spanning the CDRs were clustered using the CD-HIT Suite. Colonies containing unique phage clones were selected and grown overnight in 400 μ L of media in deep 96-well plates (Thermo Fisher Scientific, 278743) with the addition of M13K07 helper phage. Bacteria were pelleted the next day and the phage-laden supernatants were used for downstream analysis.

Peptide pulsing

For peptide pulsing, T2 cells were washed with serum-free RPMI-1640 media before incubation at $5 \times 10^5 - 1 \times 10^6$ cells per mL in serum-free RPMI-1640 containing specified concentration of peptide for 2 hours at 37°C. For experiments assessed by flow cytometry, 10 μ g/mL human β 2M (ProSpec, East Brunswick, NJ, PRO-337) was added with the peptides and is specified in the figure legend of such experiments.

Flow cytometry

Phage staining of peptide-pulsed T2 cells was performed with 50 μ L phage supernatant on ice for 1 hour, followed by staining with 1 μ g of rabbit anti-M13 antibody (Novus Biologicals, NB100-1633), and anti-rabbit-PE (BioLegend, 406421). HLA-A*02 staining was performed by staining cells with fluorescently labeled anti-human HLA-A*02 (BB7.2, BioLegend, 343308) or mouse isotype IgG2b, κ (BioLegend, 402206). Stained cells were analyzed using an LSRII flow cytometer (Becton Dickinson, Mansfield, MA) or an iQue Screener (IntelliCyt, Albuquerque, NM).

ELISAs

Streptavidin-coated, 96-well plates (R&D Systems, Minneapolis, MN, CP004) were coated with 50 ng of biotinylated HLA-A*02:01 pHLA monomers in 50 μ L of blocking buffer (PBS with 0.5% BSA, 2 mM EDTA, and 0.1% sodium azide) at 4°C overnight. Plates were washed with 1X TBST (TBS + 0.05% Tween-20) using a BioTek 405 TS plate washer (BioTek, Winooski, VT). Serial dilutions of single chain diabodies (scDbs) were incubated on the plate for 1 hour at RT, washed then incubated with 1 μ g/mL recombinant protein L (Thermo Fisher Scientific, 77679) for 1 hour at RT, washed, then incubated with anti-protein L HRP (1:10000, Abcam, ab63506) for 1 hour at RT. Plates were washed, 50 μ L of 3,3',5,5'-

Tetramethylbenzidine (TMB) substrate (BioLegend, San Diego, CA, 4211101) was added to each well, and the reaction was quenched with 50µl 2N sulfuric acid (Thermo Fisher Scientific). Absorbance at 450 nm was measured with a Synergy H1 Multi-Mode Reader (BioTek).

5 *Bispecific antibody production*

Single chain diabodies (scDbs) were produced by cloning gBlocks (IDT, Coralville, Iowa) encoding each of the variants in the format of, from N- to C-terminus: IL-2 signal sequence, anti-pHLA variable light chain (V_L), GGGGS linker (SEQ ID NO:200), anti-CD3 variable heavy chain (V_H), (GGGGS)₃ linker (SEQ ID NO:201), anti-CD3 V_L, GGGGS linker (SEQ ID NO:200), anti-pHLA V_H, and 6 x HIS tag into linearized pcDNA3.4 vector (Thermo Fisher Scientific, A14697). The proteins were expressed by the Eukaryotic Tissue Culture Core Facility of Johns Hopkins University. Briefly, 1 mg of plasmid DNA was transfected with PEI at a ratio of 1:3 into 1 L of FreeStyle 293-F cells at a density of 2-2.5x10⁶ cells per mL and incubated at 37°C. Five days after transfection, culture media was collected and filtered through a 0.22-µm unit. The scDbs were purified using HisPur Ni-NTA Resin (Thermo Fisher Scientific, 88222) and desalted into PBS pH 7.4 or 20 mM Tris pH 9.0, 150 mM NaCl using 7k MWCO Zeba Spin desalting columns (Thermo Fisher Scientific, 89890). Proteins were quantified using a 4-15% Mini-PROTEAN TGX gel (Bio-Rad, Hercules, CA, 4568085) and/or nanodrop (Thermo Fisher Scientific). Proteins were stored at 4°C for short term storage or snap frozen with the addition of 7% glycerol and stored at -80°C for long term storage. Alternatively, the scDb protein was produced by GeneArt (Thermo Fisher Scientific) in Expi293s, purified with a HisTrap column (GE Healthcare, 17-5255-01) followed by size exclusion chromatography with a HiLoad Superdex 200 26/600 column (GE Healthcare, 28989335).

25 *Surface plasmon resonance affinity measurements of p53^{R175H}/HLA-A*02:01 to H2-scDb*

Biotinylated p53^{R175H}/HLA-A*02:01, p53^{WT}/HLA-A*02:01, and H2-scDb binding experiments were performed at 25°C using a Biacore T200 SPR instrument (GE Healthcare). Approximately 100-110 response units (RU) of biotinylated p53^{R175H}/HLA-A*02:01 and p53^{WT}/HLA-A*02:01 were captured in flow cells (Fc) 2 and 4, respectively, using a streptavidin chip. Single-cycle kinetics were performed by injecting increasing

concentrations (3, 12, 50, 200 to 800 nM) of purified clone H2-scDb flowed over Fc 1-4. Binding responses for kinetic analysis were both blank- and reference-subtracted. Both binding curves were fit with a 1:1 binding model using Biacore Insight evaluation software.

CRISPR-mediated knockout of TP53

5 The Alt-R CRISPR system (IDT) was used to knock out the *TP53* gene from KMS26, TYK-nu, and KLE cell lines. CRISPR Cas9 crRNAs targeting *TP53* exon 3 (p53-5: CCCCCGACGATATTGAACAA (SEQ ID NO:191) or p53-6: CCCCTTGCCGTCCCAAGCAA (SEQ ID NO:202)) as well as CRISPR-Cas9 tracrRNA were resuspended at 100 μ M with Nuclease-Free Duplex Buffer. The crRNAs and tracrRNA
10 were duplexed at a 1:1 molar ratio for 5 minutes at 95°C. The duplexed RNA was allowed to cool to room temperature prior to mixing with Cas9 Nuclease at a 1.2:1 molar ratio for 15 minutes. A total of 40 pmols of the Cas9 RNP complexed with *TP53* gRNA were mixed with 200,000 cells in 20 μ L of OptiMEM. This mixture was loaded into a 0.1 cm cuvette (Bio-Rad, 1652089) and electroporated at 120 V and 16 ms using an ECM 2001 (BTX,
15 Holliston, MA). Cells were immediately transferred to complete growth medium and cultured for 7 days. Single cell clones were established by limiting dilution and genomic DNA was harvested using a Quick-DNA 96 Kit (Zymo Research, Irvine, CA, D3012). A region flanking the CRISPR cut site was PCR amplified (forward primer: GCTGCCCTGGTAGGTTTTCT (SEQ ID NO:203), reverse primer:
20 GAGACCTGTGGGAAGCGAAA (SEQ ID NO:204)) and Sanger Sequenced to select for clones with the desired *TP53* status.

Immunoblotting analysis

 Cells were lysed in cold RIPA buffer (Thermo Fisher Scientific, 89901) supplemented with protease inhibitor cocktail (Thermo Fisher Scientific, 87785). Protein
25 concentration was determined using a BCA assay (Thermo Fisher Scientific, 23227). Equal amounts of total protein (20-50 μ g) were loaded in each lane of a 4-15% Mini-PROTEAN TGX gel (Bio-Rad, 4568085) and transferred to polyvinylidene difluoride membranes after electrophoresis. The membranes were incubated with appropriate primary antibodies (p53 [DO-1], 1:1000, Santa Cruz, sc-126; STAT2, 1:1000, Thermo Fisher Scientific, 44-362G;
30 ZFP3, 1:1000, Thermo Fisher Scientific, PA5-62726; β -actin [13E5], 1:1000, Cell Signaling

Technology, 5125S; β -actin [8H10D10], 1:1000, Cell Signaling Technology, 3700S) and species-specific HRP-conjugated secondary antibodies (1:5000-10000). Signal was detected by a ChemiDoc MP chemiluminescence system (Bio-Rad).

Transfection of cell lines

5 gBlocks (IDT) encoding HLA and target proteins were cloned into pcDNA3.1 or pcDNA3.4 vectors (Thermo Fisher Scientific, V79020, A14697). COS-7, Saos-2, and HEK293FT cells were transfected at 70-80% confluency using Lipofectamine 3000 (Thermo Fisher Scientific, L3000015) and incubated at 37°C overnight. A total of 15 μ g and 30 μ g plasmid (1:1 ratio of HLA plasmid/target protein plasmid in co-transfections) was used for
10 T25 and T75 flasks, respectively.

Viral transduction of cell lines

HLA-A*02:01-encoding retrovirus was produced using the MSCV retroviral expression system (Clontech, Mountain View, CA, 634401). In brief, a gBlock encoding HLA-A*02:01-T2A-GFP (IDT) was cloned into the pMSCVpuro retroviral vector by HiFi
15 DNA assembly (New England Biolabs, Ipswich, MA, E2621L). The pMSCVpuro-HLA-A*02:01-T2A-GFP plasmid was then co-transfected with a pVSV-G envelope vector into the GP2-293 packaging cell line. Viral supernatant was harvested 48 hours after transfection and concentrated 20-fold using Retro-X Concentrator (Clontech, 631456). RediFect Red-Fluc-GFP lentivirus particles (Perkin Elmer, Waltham, MA, CLS960003) was used for generating
20 luciferase-expressing cell lines.

For transduction, non-tissue culture-treated 48-well plates were coated with 200 μ L of 10 μ g/mL RetroNectin (Clontech, T100B) per well overnight at 4°C and blocked with 10% FBS for 1 hour at RT. Viral particles and 2×10^5 target cells were added to each well in a total volume of 500 μ L cell culture media and spun at 2000 x g for 1 hour then incubated at
25 37°C. Selection with 1 μ g/mL puromycin (Thermo Fisher Scientific, A1113803) began three days later. Transduced cells were sorted based on presence of GFP using FACS Aria Fusion (BD Biosciences, San Jose, CA) 10-14 days after transduction.

in vitro scDb co-incubation assays

To each well of a 96-well flat-bottom plate, the following components were combined in a final volume of 100 μ L RPMI-1640 with 10% FBS, 1% Penicillin-Streptomycin, and 100 IU/mL IL-2: bispecific antibody diluted to the specified concentration, 5×10^4 human T cells, and $1-5 \times 10^4$ target cells (COS-7, T2, or other tumor cell lines). The effector to target cell ratio is specified in the figure legend for each experiment. The co-culture plate was incubated for 20 hours at 37°C and conditioned media was assayed for cytokine and cytotoxic granule protein secretion using the Human IFN- γ Quantikine Kit (R&D Systems, Minneapolis, MN, SIF50), Human IFN- γ Flex Set Cytometric Bead Array (BD, 558269), or the MILLIPLEX Luminex assays (Millipore Sigma, HSTCMAG28SPMX13, HCD8MAG-15K) read on the Bioplex 200 platform (Bio-Rad). Cytotoxicity was assayed by CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, G7571) or Bio-Glo Luciferase Assay (Promega, G7941) per manufacturer's instructions. For CellTiter-Glo assays, percent cytotoxicity was calculated by subtracting the luminescence signal from the average of the T cell only wells and normalizing to the no scDb condition: $1 - (\text{scDb well} - \text{T cell only}) / (\text{no scDb well} - \text{T cell only}) \times 100$. For Bio-Glo assays, percent cytotoxicity was calculated by normalizing luminescence signal to the no scDb condition: $1 - (\text{scDb well}) / (\text{no scDb well}) \times 100$.

Real-time live-cell imaging

A total of 1×10^4 CellTracker Green CMFDA (Thermo Fisher Scientific, C7025)-labeled target cells were plated in each well of a 96-well flat bottom plate and allowed to attach for 8 hours before adding T cells and scDb at the indicated E:T ratio and concentrations, respectively. Each condition was plated in triplicate. Plates were imaged every 3 hours using the IncuCyte ZOOM Live-Cell analysis system (Essen Bioscience, Ann Arbor, MI) for a total of 60 hours. Four images per well at 10X zoom were collected at each time point. Cell confluence in each well was quantified using the phase contrast channel.

*Expression, purification and refolding of p53^{R175H}/HLA-A*02:01*

Plasmids for HLA-A*02:01 and β 2M were received from the NIH Tetramer Facility (Atlanta, GA) and separately transformed into BL21(DE3) cells. Each was expressed in inclusion bodies using auto-induction media as described elsewhere (Skora et al., *Proc. Natl.*

Acad. Sci. U. S. A. 112:9967-9972 (2015); Martayan et al., *The Journal of Immunology* 182:3609-3617 (2009); and Huang et al., *Bioinformatics* 26:680-682 (2010)). Purification of the HLA-A*02:01 and β 2M inclusion bodies was achieved with a series of detergent washes followed by solubilization with 8 M urea. Refolding of the HLA-A*02:01, β 2M, and mutant p53^{R175H} peptide was performed as described elsewhere (Myszka, *J. Mol. Recognit.* 12:279-284 (1999)). Briefly, HLA-A*02:01 and β 2M inclusion bodies were combined in a refolding buffer containing 100 mM Tris pH 8.3, 400 mM L-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 2 mM PMSF, and 30 mg of the mutant p53^{R175H} peptide (amino acid 168-176, HMTEVVRHC (SEQ ID NO:1)) dissolved in 1 mL of DMSO.

The resultant solution was stirred at 4°C for 2 days, with two further additions of HLA-A*02:01 inclusion bodies on day 2, concentrated to 10 mL and purified by size exclusion chromatography on a HiLoad 26/60 Superdex 75 Prep grade column (GE Healthcare, 28989334). For incubation with the H2-Fab, purified pHLA-A*02:01 was concentrated to ~1-3 mg/mL and stored at -80°C until use.

Production of the H2-Fab antibody fragment

The light chain (LC) and heavy chain (HC) variable region sequences of H2 scFv were grafted, linked with the respective constant region sequences of human IgG1 and separately cloned into a pcDNA3.4 vector (Thermo Fisher Scientific, A14697). Both chains were preceded by a mouse IgKVIII signal peptide. Before large-scale expression of full-length antibody, optimization of the LC:HC DNA ratio for transfection was performed to determine optimal recombinant protein yields. For a 1 L expression, a total of 50 μ g of purified plasmids (1:1 LC:HC ratio) were transfected with PEI at a ratio of 1:3 into Freestyle 293-F cells at a density of 2-2.5x10⁶ cells per mL and incubated at 37°C for 7 days. The media was harvested via centrifugation, filtered through a 0.22- μ m unit and the full-length antibody was purified via protein A affinity chromatography on a HiTrap MabSelectTM SuReTM column (GE Healthcare, 29-0491-04). Full-length antibody was eluted using a linear gradient of 0-100 mM sodium citrate, pH 3.5. The protein A fractions containing pure H2 antibody were pooled, quantified by SDS-PAGE gel electrophoresis and dialyzed into 20 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA.

For generation of H2-Fab fragments, ~1-3 mg of full-length antibody was mixed with 0.5 mL of a 50 % Immobilized Papain slurry (Thermo Fisher Scientific, 20341) pre-activated with digestion buffer (20 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA) containing 20 mM cysteine-HCl. The mixture was incubated at 37°C overnight with constant shaking at 200 rpm. The H2 antibody digest was separated from the immobilized resin by a gravity resin separator and washed with 10 mM Tris-HCl, pH 7.5. Newly generated H2-Fab fragments were further purified by cation-exchange chromatography using a Mono-S column (GE Healthcare, 17516801) and eluted using a linear gradient of 0-500 mM NaCl.

The H2-Fab fragments were concentrated, mixed with equimolar p53^{R175H}/HLA-A*02:01 and incubated at 4°C overnight. The H2-Fab-p53^{R175H}/HLA-A*02:01 mixture was evaluated by size exclusion chromatography on a Superdex™ 200 Increase 10/300 column (GE Healthcare, 28990944). The fractions of ~ 98% pure pHLA-A*02:01-H2-Fab complex were pooled, concentrated to 12.6 mg/mL and exchanged into a buffer containing 25 mM HEPES, pH 7.0, 200 mM NaCl.

Crystallization, data collection and structure determination

Crystals of the ternary complex H2-Fab-p53^{R175H}/HLA-A*02:01 were grown in hanging drop by vapor diffusion in drops set up with a TTP mosquito robot with a reservoir solution of 0.2 M ammonium chloride and 20% (w/v) PEG 3350 MME. Crystals were flash-frozen in mother liquor. Data were collected at National Synchrotron Light Source-II at beamlines 17-ID-1(AMX) on a DECTRIS Eiger X 9M detector and 17-ID-2 (FMX) on a DECTRIS Eiger X 16M detector. Datasets were indexed, integrated and scaled using fastdp, XDS, and aimless. Monoclinic crystals of H2-Fab-p53^{R175H}/HLA-A*02:01 diffracted to 3.5 Å. The structure for the H2-Fab-p53^{R175H}/HLA-A*02:01 complex was determined by molecular replacement with PHASER using PDB ID 6O4Y (11) and 6UJ9 as the search models. The data was refined to a final resolution of 3.5 Å using iterative rounds of refinement with REFMAC5 and manual rebuilding in Coot. Structures were validated using Coot and PDB Deposition tools. The model has 94.11% of the residues in preferred and allowed regions according to Ramachandran statistics (Table 7). Figures were rendered in PyMOL (v2.2.3, Schrödinger, LLC, New York, NY). Buried areas were calculated with PDBePISA. The angle that determines the relative orientation between the pHLA and the

Fab/TCR was calculated by the dot product of the vector defined by the position of the alpha carbon of the P1-P9 of the peptide and the vector defined by the disulfide bonds in the V_H and V_L domains.

Mouse xenograft model

5 Female NOD.*Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* (NSG) mice at 6-10 weeks were acquired from the Jackson Laboratory (Bar Harbor, Maine, 005557) and treated in compliance with the institutional Animal Care and Use Committee approved protocol. In the early treatment model, mice were inoculated intravenously with 1×10^6 luciferase-expressing KMS26 or KMS26-TP53 KO cells and 1×10^7 *in vitro* expanded human T cells via lateral tail vein
10 injection on day 0. On day 1, mice were randomized based on luminescence quantification using the IVIS imaging system and Living Image software (Perkin Elmer) to ensure similar pretreatment tumor burden. After randomization, two-week micro-osmotic pumps (ALZET, Cupertino, CA, 1002) filled with H2-scDb or isotype control scDb (scFv against an irrelevant pHLA linked with UCHT1scFv) that had been primed in 1 mL PBS overnight at 37°C were
15 placed intraperitoneally using sterile surgical technique. Tumor growth was serially monitored by bioluminescent imaging. In the established tumor model, mice were inoculated with 3.5×10^5 luciferase-expressing KMS26 cells and 1×10^7 human T cells via lateral tail vein injection on day 0. On day 6, H2-scDb or isotype control scDb was administered similarly as in the early treatment model.

20 *Statistical analysis*

Data are presented as means $\pm$ SD. Statistical analyses were carried out using specific tests indicated in the figure legend. A *P* value of < 0.05 was used to denote statistical significance. All analyses were performed using Prism version 8.0 (GraphPad, San Diego, CA).

25 *Example 3: Bispecific Antibodies Targeting Mutant RAS Neoantigens*

Mutations in the *RAS* oncogenes occur in multiple cancers and new approaches to target these mutations have been the subject of intense research for decades. Most of these efforts have been focused on conventional small molecule drugs rather than antibody-based therapies because the RAS proteins are intracellular.

This Example identifies scFvs specific for peptides derived from two recurrent RAS mutations, G12V and Q61H/L/R, which are presented on cancer cells in the context of two common human leukocyte antigen alleles, HLA-A3 and HLA-A1, respectively. The scFvs did not recognize the peptides derived from the wildtype (WT) form of RAS proteins or other related peptides. Given their extremely low antigen density on cancer cells, a very sensitive immunotherapeutic agent was developed to kill cells harboring *RAS* gene mutations. Single chain diabodies (scDbs) specific for peptides derived from G12V or Q61H/L/R were capable of inducing T cell activation and killing of target cancer cells expressing endogenous levels of the mutant RAS proteins and cognate HLA alleles.

Results

MANAs derived from clinically relevant RAS gene mutations

In silico predictions suggested that the 10-mer peptide from codons 5 to 14 (KLVVVGAVGV, “G12V[5-14]”; SEQ ID NO:209), in which the underlined valine residue (V) represents the G12V mutation, would bind to HLA-A*02:01 (henceforth referred to as HLA-A2) (see, e.g., Skora et al., *Proc Natl Acad Sci U S A* 112:9967-9972 (2015); and Andreatta, *Bioinformatics* 32:511-517 (2016)). A MANAbody (called D10) that bound to this pHLA-A2 complex and did not bind to the wildtype (WT) counterpart, with selective binding demonstrated using pHLA complexes attached to artificial surfaces as well as using cells pulsed with mutant peptides, was previously reported (Skora et al., *Proc Natl Acad Sci U S A* 112:9967-9972 (2015)). However, it could not be demonstrated that the D10 MANAbody could bind to cells that expressed endogenous levels of the mutant *KRAS* gene or even to cells that overexpressed an exogenous mutant *KRAS* gene. It was hypothesized that despite the predictions of the *in silico* algorithms, the KRAS protein was not processed to the G12V[5-14] peptide and transported to the cell surface. There is indeed precedent for such *in silico* predictions to be inaccurate (Schmidt et al., *J Biol Chem* 292:11840-11849 (2017)). To evaluate this possibility, a highly-sensitive mass spectrometry (MS)-based approach (MANA-SRM) was developed to analyze HLA-bound peptides. Human HLA-A2 and full-length human KRAS containing G12V were expressed in SV40 virus-immortalized monkey kidney COS-7 cells and HLA-bound peptides were immunopurified. MS analysis of eluted peptides showed that the G12V[5-14] peptide could not be detected, even when the

mutant *KRAS* gene was overexpressed (Table 10). It was concluded that this peptide was not processed and presented on HLA-A2+ cells harboring a *KRAS* G12V mutant gene.

- 5 Table 10. MANA-SRM quantification. Mutation-associated Neoantigen (MANA) peptides per cell were quantified using the mass-spectrometry based method MANA-SRM. COS-7 cells were co-transfected with plasmids encoding HLA alleles and full-length *KRAS* variants. To assess endogenous expression of MANAs, cell lines bearing HLA alleles and *RAS* variants of interest were analyzed.

Cell Line	Transfection Condition	Endogenous HLA alleles	Predicted binding peptide by NetMHCv4.0	SEQ ID NO:	Predicted Affinity by NetMHCv4.0 (nM)	Detected Copy Number Per Cell
COS-7	HLA-A2 + <i>KRAS</i> G12V	N/A	KLVVVGAVGV	209	300.18	N/A
COS-7	HLA-A3 + <i>KRAS</i> G12V	N/A	VVGAVGVGK	206	277.44	24
			VVVGAVGVGK	205	396.16	102
COS-7	HLA-A2 + <i>KRAS</i> G12V	N/A	N/A		N/A	N/A
COS-7	HLA-A1 + <i>KRAS</i> Q61H	N/A	ILDTAGHEEY	2	185.91	583
COS-7	HLA-A2 + <i>KRAS</i> Q61H	N/A	N/A		N/A	N/A
COS-7	HLA-A1 + <i>KRAS</i> Q61L	N/A	ILDTAGLEEY	3	66.03	512
COS-7	HLA-A2 + <i>KRAS</i> Q61L	N/A	N/A		N/A	N/A
COS-7	HLA-A1 + <i>KRAS</i> Q61R	N/A	ILDTAGREEY	4	322.23	127
NCI-H441	N/A	A*02:01, A*03:01	VVVGAVGVGK	205	396.16	9
CFPAC-1	N/A	A*02:01, A*03:01	VVVGAVGVGK	205	396.16	3
RD	N/A	A*01:01, A*01:01	ILDTAGHEEY	2	185.91	8
HL-60	N/A	A*01:01, A*01:01	ILDTAGLEEY	3	66.03	4
Hs 940.T	N/A	A*01:01, A*01:01	ILDTAGREEY	4	322.23	1

Thus, attention was to two peptides, a 9-mer (VVGAVGVGK; SEQ ID NO:206) from codons 8 to 16 (“G12V[8-16]”) and a 10-mer (VVVGAVGVGK; SEQ ID NO:205) from codons 7 to 16 (“G12V[7-16]”), containing the RAS G12V mutation that were
 5 predicted by NetMHCv4.0 to bind HLA-A*03:01 (henceforth referred to as HLA-A3) with high affinity (Andreata, *Bioinformatics* 32:511-517 (2016)). HLA-A3 is one of the most common HLA-A alleles. MANA-SRM was used to confirm the *in silico* predictions that these peptide complexes would be displayed on the cell surface before screening for MANAbodies. Human HLA-A3 and full-length human KRAS G12V were expressed in
 10 COS-7 cells, then immunopurified with an anti-human HLA antibody. MS analysis of peptides eluted from the captured peptide-HLA complexes detected the G12V[7-16] peptide at 102 copies per cell and the G12V[8-16] peptide at considerably lower levels (24 copies per cell) (Fig. 45A, Table 10). Importantly, when COS-7 cells were transfected with HLA-A2 and KRAS G12V as a negative control, neither of these peptides were detected (Table 10).
 15 MANA-SRM analysis was then performed on two human cancer cell lines, the lung adenocarcinoma line NCI-H441 and the pancreatic ductal adenocarcinoma line CFPAC-1, both expressing endogenous levels of HLA-A3 and harboring the *KRAS* G12V mutation. The CFPAC-1 and NCI-H441 lines presented on average 3 and 9 copies per cell, respectively, of the G12V[7-16] peptide (Fig. 45, B and C, Table 10), and the G12V[8-16]
 20 peptide was not detected. Given the higher abundance of the 10-mer G12V[7-16] (henceforth referred to as “G12V”) peptide, efforts were focused on targeting this MANA.

Another commonly mutated residue in *RAS* genes is the glutamine at codon 61. RAS codon 61 mutations are found in a wide variety of cancers, including melanomas, multiple myelomas, thyroid, and bladder cancers. Using NetMHCv4.0, it was predicted that a 10-mer
 25 RAS peptide (codons 55 to 64) could bind with high affinity to HLA-A*01:01 (henceforth referred to as HLA-A1), another common HLA-A allele (Majers et al., *Hum Immunol* 68:779-788 (2007)). This peptide (ILDTAGQEEY; SEQ ID NO:136) as well as the flanking amino acids are conserved across all three RAS proteins. Using MANA-SRM, cell surface expression of 10-mer peptides from codons 55 to 64 containing the Q61H, Q61L, and Q61R
 30 mutations (ILDTAGH^HEEY (SEQ ID NO:2), ILDTAGL^LEEY (SEQ ID NO:3), and ILDTAGR^REEY (SEQ ID NO:4), respectively) were previously evaluated, and in the

transfected COS-7 overexpression system, an average of 583, 512, and 127 copies of the Q61H, Q61L, and Q61R peptides per cell were found (Wang et al., *Cancer Immunol Res* 7:1748-1754 (2019)). These peptides were also identified to be presented on cell lines expressing endogenous levels of Q61 mutant RAS proteins, notably with four copies of the Q61L peptide per cell found in the acute promyelocytic leukemia cell line HL-60 (Table 10).

Together, these data show that G12V and Q61 mutant RAS proteins can be processed into peptides that are presented on the surface of cancer cell lines, albeit at antigen densities below what is considered the minimum required for recognition by conventional antibody-based immunotherapeutic agents.

10 *Identification of scFv-expressing phage clones targeting HLA-restricted RAS mutant peptides*

To develop MANAbodies targeting the above-named RAS peptides, a second phage library displaying scFvs was built. This library was designed based on principles described elsewhere (Skora et al., *Proc Natl Acad Sci U S A* 112:9967-9972 (2015)), but with important modifications. In particular, precursor library DNA was synthesized using trinucleotide mutagenesis (TRIM) technology, permitting fine tuning of the amino acid diversity at particular codons considered most critical for antigen binding. Diversity was introduced in five of the six complementarity-determining regions (CDRs), with the most amino acid diversity as well as length diversity incorporated into the third CDR of the heavy chain (CDR-H3) (Fig. 46, A and B). The completed library was estimated to contain $\sim 3.6 \times 10^{10}$ unique clones. As a quality control step, a portion of the library was subjected to massively parallel sequencing of the heavy chain CDR3 region, demonstrating that the expected and actual amino acid diversity were in excellent alignment (Fig. 46C).

For the RAS G12V HLA-A3 MANA target, HLA-A3 and beta-2-microglobulin were folded together with the chemically synthesized G12V peptide or its WT [7-16] (G12WT) counterpart to form pHLA complexes (Table 11). These pHLA-A3 were then used to screen the phage display library described above. The screening process consisted of four to six rounds of phage selection, similar to the scheme described previously, with key differences outlined in the Materials and Methods. Over the course of this selection, phage were negatively selected against soluble streptavidin, streptavidin magnetic beads, denatured HLA-A3, unrelated pHLA-A3, and G12WT pHLA-A3 and positively selected against the

G12V pHLA-A3. Following the screening process, individual phage clones were amplified and subjected to enzyme-linked immunosorbent assay (ELISA) to assess their binding specificity (Fig. 47A). While multiple clones initially appeared to be promising on ELISAs with plaste-bound pHLA complexes, one phage clone (V2) stood out upon subsequent flow cytometry assays on cells displaying these complexes (Fig. 47B, Table 12). The V2 phage had substantially greater binding to G12V pHLA-A3 compared to other pHLA-A3, including those formed with HLA-A3 folded with the G12WT peptide, the G12C or G12D variant, or an unrelated CTNNB peptide (Fig. 47C, Table 11). V2 phage also failed to detectably interact with HLA-A3 folded with the G12V[8-16] or G12WT[8-16] peptides (Fig. 47C, Table 11).

Table 11. Peptides for pHLA complexes. Peptides used for pHLA complexes were predicted to bind to the HLA allele of interest using NetMHCv4.0.

Peptide Name	Sequence	SEQ ID NO	HLA-A allele	NetMHCv4.0 predicted HLA affinity (nM)
KRAS G12WT[8-16]	VVGAGGVGK	208	HLA-A*03:01	277.44
KRAS G12WT[7-16]	VVVGAGGVGK	207	HLA-A*03:01	396.16
KRAS G12C[8-16]	VVGACGVGK	210	HLA-A*03:01	221.43
KRAS G12C[7-16]	VVVGACGVGK	211	HLA-A*03:01	375.53
KRAS G12D[8-16]	VVGADGVGK	212	HLA-A*03:01	1172.08
KRAS G12D[7-16]	VVVGADGVGK	213	HLA-A*03:01	938.8
KRAS G12V[8-16]	VVGAVGVGK	206	HLA-A*03:01	202.62
KRAS G12V[7-16]	VVVGAVGVGK	205	HLA-A*03:01	341.99
CTNNB	TTAPSLSGK	214	HLA-A*03:01	43.24
RAS Q61WT[55-64]	ILDTAGQEEY	136	HLA-A*01:01	86.69
RAS Q61H[55-64]	ILDTAGHEEY	2	HLA-A*01:01	185.91
RAS Q61K[55-64]	ILDTAGKEEY	215	HLA-A*01:01	324.9
RAS Q61L[55-64]	ILDTAGLEEY	3	HLA-A*01:01	66.03
RAS Q61R[55-64]	ILDTAGREEY	4	HLA-A*01:01	322.23
CMV	YSEHPTFTSQY	216	HLA-A*01:01	12.72

Table 12. scFv sequences. Amino Acid (AA) sequences of anti-CD3 scFvs used for bispecific antibody construction. Affinities of select anti-CD3 clones were gathered from the literature.

scFv name	antigen	Affinity (nM)	variable light chain (AA)	SEQ ID NO	variable heavy chain (AA)	SEQ ID NO
V2	RAS G12V [7-16] pHLA-A3	8.7	DIQMTQSPSSLSASVGDRVTITCRAS QDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFLT TISS LQPEDFATYYCQQSYYYFRPITFGQG TKVEIK	699	EVQLVESGGGLVQPGGSLRLSCAASGF NLSYSDIHWVRQAPGKGLEWVA VVMPT DSGHTNYADSVKGRFTISADTSKNTAY LQMNSLRAEDTAVYYCSRATNIPVYAF DYWGQGTLVTVSS	700
			DIQMTQSPSSLSASVGDRVTITCRAS QDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFLT TISS LQPEDFATYYCQQVIYYPFTFGQGT KVEIK	701	EVQLVESGGGLVQPGGSLRLSCAASGF NLYSYAIHWVRQAPGKGLEWVA LLYP DYGVTSYADSVKGRFTISADTSKNTAY LQMNSLRAEDTAVYYCSRYRSYEYSVS SYSYSAMDYWGQGTLVTVSS	702
L2	RAS Q61L [55-64] pHLA-A1	65	DIQMTQSPSSLSASVGDRVTITCRAS QDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFLT TISS LQPEDFATYYCQQAVSYPPWTFGQGT KVEIK	703	EVQLVESGGGLVQPGGSLRLSCAASGF NISSGIHWVRQAPGKGLEWVA MVG GSGYTNYADSVKGRFTISADTSKNTAY LQMNSLRAEDTAVYYCSRWAHYSAY MDYWGQGTLVTVSS	704
			DIQMTQSPSSLSASVGDRVTITCRAS QDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFLT TISS LQPEDFATYYCQQYSNYPLTFGQGT KVEIK	705	EVQLVESGGGLVQPGGSLRLSCAASGF NVFYGSMHWVRQAPGKGLEWVA FIGP DSTYTYADSVKGRFTISADTSKNTAY LQMNSLRAEDTAVYYCSRDLGSAYAM DYWGQGTLVTVSS	706
R6	RAS Q61R [55-64] pHLA-A1	not determined				

UCHT1 "U"	human CD3	1.4 - 2.2	DIQMTQTSSLSASLGDRVTISCRAS QDIRNYLNWYQQKPDGTVKLLIYYT SRLHSGVPSKFSGSGSGTDYSLTISNL EQEDIAITYFCQQQGNLTPWTFAGGTK LEIK	707	EVQLQQSGPELVKPGASMKISKASGY SFTGYTMNWVKQSHGKNLEWMGLINP YKGVSTYNQKFKDKATLTVDKSSSTAY MELLSLTSEDSAVYYCARSGYYGDS WYFDVWGAGTTVTVSS	708
UCHT1.v9 "U2"	human CD3	2.5 - 4.7	DIQMTQSPSSLSASVGDRVTITCRAS QDIRNYLNWYQQKPGKAPKLLIYYT SRLESGVPSRFSGSGSGTDYTLTISSL QPEDFATYYCQQGNLTPWTFGQGTK VEIK	709	EVQLVESGGGLVQPGGSLRLSCAASGY SFTGYTMNWVRQAPGKGLEWVALINP YKGVSTYNQKFKDRFTISVDKSKNTAY LQMNSLRAEDTAVYYCARSGYYGDS WYFDVWGQGTLLVTVSS	710
diL2K	human CD3	87	DIVLTQSPATLSLSPGERATLSCRASQ SVSYMNWYQQKPGKAPKRWIYDTS KVASGVPARFSGSGSGTDYSLTINSL EAEDAATYYCQQWSSNPLTFGGGTK VEIK	711	DVQLVQSGAEVKKPGASVKVCKASG YTFTRYTMHWVRQAPGQGLEWIGYIN PSRGYTNYADSVKGRFTITTDKSTAY MELSSLRSEDTATYYCARYYDDHYCL DYWGQGTITVTVSS	712
hXR32	human CD3	3.6 - 6.1	QAVVTQEPSLTVSPGGTVTLTCRSST GAVTTSNYANWVQQKPGQAPRGLI GGTNKRAPWTPARFSGSLGGKAAL TITGAQAEDEADYYCALWYSNLWV FGGGTKLTVL	713	EVQLVESGGGLVQPGGSLRLSCAASGF TFNTYAMNWNVRQAPGKGLEWVARIRS KYNNTATYYADSVKDRFTISRDDSKNS LYLQMNSLKTEDTAVYYCVRHGNFGN SYVSWFAYWGQGTLLVTVSS	714
L2K	human CD3	-	DIQLTQSPAIMASAPGEKVTMTCRAS SSVSYMNWYQQKSGTSPKRWIYDTS KVASGVPPYRFSGSGSGTSYSLTISSM EAEDAATYYCQQWSSNPLTFGAGTK LELK	715	DIKLQQSGAELARPGASVKMSCKTSGY TFTRYTMHWVKQRPQGQGLEWIGYINPS RGYTNYNQKFKDKATLTTDKSSSTAY MQLSSLTSEDSAVYYCARYYDDHYCL DYWGQGTTLTVSS	716

OKT3	human CD3	1.2 - 1.5	QIVLTQSPAIMSASPGEKVTMTCSASSVSYMNWYQQKSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAITYYCQQWSSNPFTFGSGTKLEIN	717	QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPQGQLEWIGYINPSRGYTNYNQKFKDKA TLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSS	718
28F11	human CD3	-	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASN RATGIPARFSGSGGTDFLTLSLLEPEDFAVYYCQQRSNWPPLTFGGGKVEIK	719	QVQLVESGGGVVQGRSLRLSCAASGFKFSGYGMHWVRQAPGKGLEWVA VIWYDGSKKYYVDSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARQMGYWHFDLWGRGTLVTVSS	720
27H5-VL1	human CD3	-	EIVLTQSPRTLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGA SS RATGIPDRFSGSGGTDFLTISRLDPEDFAVYYCQYQGSSPITFGQGTRL EIK	721	QVQLVESGGGVVQGRSLRLSCAASGFTFRSYGMHWVRQAPGKGLEWVA IHWYDGSKKNYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGTGYNWFD PWGQGTLVTVSS	722
23F10	human CD3	-	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASN RATGIPARFSGSGGTDFLTLSLLEPEDFAVYYCQQRSNWPPLTFGGGKVEIK	719	QVQLVQSGGGVVQGRSLRLSCAASGFKFSGYGMHWVRQAPGKGLEWVA VIWYDGSKKYYVDSVKGRFTISRDN SKNTLYLQMNSLRGEDTAVYYCARQMGYWHFDLWGRGTLVTVSS	723
15C3-VL1	human CD3	-	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASN RATGIPARFSGSGGTDFLTLSLLEPEDFAVYYCQQRSNWPWTFGGQGTKVEIK	724	QVQLVQSGGGVVQGRSLRLSCV ASGFTFSSYGMHWVRQAPGKGLEWVA AIWYNGRKQDYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCTRGTGYNWFD PWGQGTLVTVSS	725

15C3-VL2	human CD3	-	AIQLTQSPSSLASVGDREVITITCRASQ GISSALAWYQQKPGKAPKLLIYDASS LESGVPSRFSGSGGTDFTLTISSLQP EDFATYYCQQFNSYPITFGQGTRLEI K	726	QVQLVQSGGGVVQPGRSLRLSCVASGF TFSSYGMHWVRQAPGKGLEWVAIW YNGRKQDYADSVKGRFTISRDN SKNTL YLMNSLRAEDTAVYYCTRTGTGYNWF DPWGQGTLVTVSS	725
hu12F6	human CD3	-	QIVLSQSPAILSASPGEKVTMTCRASS SVSYMHWYQQKPGSSPKPWYATSN LASGVPARFSGSGGTSYSLTISRVE AEDAATYYCQQWSSNPPTFGGGTKL ETKR	727	QVQLQQSGAEELARPGASVKMSCKASG YTFTSYTMHWVKQRPGQGLEWIGYINP SSGYTKYNQKFKDKATLTADKSSSTAY MQLSSLTSEDSAVYYCARWQDYDVYF DYWGQGTTTLTVSS	728

The scFv component of the phage was then expressed and purified in bacteria for further characterization. The recombinant V2 scFv retained the same binding profile as its phage counterpart (Fig. 47D), with only minimal binding to the WT pHLA complex at the highest concentration tested (Fig. 38A). TAP-deficient T2 cells modified to express HLA-A3 (T2A3) were pulsed with the G12V peptide or a variety of control peptides, and the cells were assessed for binding to V2 scFv or HLA-A3-specific monoclonal antibodies. Flow cytometric analysis showed remarkably specific binding of V2 scFv to G12V peptide pulsed T2A3 cells compared to cells pulsed with the G12WT, G12C, or G12D RAS peptides (Fig. 38B, Fig. 47E). As in the ELISA assay, the V2 scFv was unable to detectably bind to cells pulsed with 9-mer [8-16] peptides, whether mutant or WT. Surface plasmon resonance (SPR) binding analysis of the V2 scFv demonstrated a K_D value of 8.7 nM for G12V pHLA-A3, while no appreciable binding to G12WT pHLA-A3 was identified (Fig. 38C).

A similar screening procedure was used for RAS Q61H, Q61L, and Q61R pHLA-A1 MANA targets (see Materials and Methods). Using phage staining and T cell-based assays, one phage clone was identified displaying high specificity for each of the three targets: clone H1 for Q61H, clone L2 for Q61L, and clone R6 for Q61R (Fig. 48, A to F and Table 12). These clones bound to their cognate mutant pHLA with no detectable binding to the RAS Q61WT pHLA-A1 (Fig. 38, D to F). To further assess binding to pHLA complexes on the cell surface, the HLA-A1+ acute myeloid leukemia line SigM5 was pulsed with cognate peptides or controls. All three clones specifically bound to the expected pHLA complexes derived from mutant *RAS* genes (Fig. 38G). Given the efficient presentation of the Q61L peptide on transfected COS-7 cells (27) and its higher level of binding to peptide-pulsed SigM5 cells, most subsequent experiments focused on Q61L clone L2. SPR analysis of the L2 clone revealed a K_D of 65 nM for Q61L pHLA-A1 and no appreciable binding to Q61WT pHLA-A1 (Fig. 38H).

T cell-engaging bispecific antibodies can recognize mutant RAS-derived pHLA complexes

A variety of T cell-engaging bispecific antibody formats have been developed for targeting T cells to specific ligands. However, there are little data available on whether any of these formats can recognize targets when they are presented on the cell surface at low densities. To inform this point, six bispecific formats were evaluated: diabodies, single chain

diabodies (scDBs), bispecific T cell engagers (BiTEs), dual affinity retargeting molecules (DARTs), bivalent scFv-Fcs, and trivalent scFv-Fcs (Fig. 49A). The scFv-Fcs were heterodimerized via the Knob-Into-Hole method. The V2 scFv was used as the pHLA-targeting moiety and different anti-CD3 antibodies were used for engaging T cells (Tables 12 and 13). In several of the formats, different configurations of the heavy (“H”) and light (“L”) chains of the V2 and anti-CD3 scFvs were tested (Fig. 49B).

Table 13. V2 bispecific antibody sequences. Amino acid sequences of the G12V pHLA-A3-targeting V2 bispecific antibodies tested. The anti-CD3 clone UCHT1 is referred to as "U" and anti-CD3 clone UCHT1.v9 is referred to as "U2". The bivalent scFv-Fc was the product of co-expression of V2-FcHole and an anti-CD3-FcKnob. The trivalent scFv-Fc was the product of co-expression of V2-FcHole and an V2-FcKnob-anti-CD3.

Bispecific Antibody	Sequence	SEQ ID NO
V2-U2-LHLH-scDb	MYRMQLLSIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQAPGKGLEWVALI NPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCARSGYYGSDSDWYFD VWGQGTLVTVSSGGGGGGGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDIRNYLN WYQQKPGKAPKLLIYYTSRLESGVPSRFSGSGGTDYTLTISSLQPEDFATYYCQQGNTLPW TFGQGTKVEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGL EWVAVVMPSDGHNTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVY AFDYWGQGTLVTVSSHHHHH	217
V2-U-LHLH-scDb	MYRMQLLSIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGSEVQLQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQSHGKNLEWMGLI NPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCARSGYYGSDSDWYFDV WGAGTTVTVSSGGGGGGGGGGSDIQMTQTSSLSASLGDRVTITCRASQDIRNYLNW YQQKPDGTVKLLIYYTSRLHSGVPSKFSGSGGTDYSLTISNLEQEDIATYFCQQGNTLPWTF AGGTKLEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLE WVAVVMPSDGHNTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYA FDYWGQGTLVTVSSHHHHH	218

U2-V2-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKPG KAPKLLIYYTSRLESGVPSRFSGSGTDYTLTISSLQPEDFATYYCQQGNTLPWTFGGQTKV EIKGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLEWVAVVM PDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYAFDYWGQ GTLVTVSSGGGGGGGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQ QKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISLQPEDFATYYCQQSYYYFRPITFG QGTKVEIKGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSTGYTMNWVRQAPGKGLE WVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCARSGYYGDS SDWYFDVWGQGTLVTVSSHHHHH	219
U-V2-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQTSSLSASLGDRVTISCRASQDIRNYLNWYQQKPDG TVKLLIYYTSRLHSGVPSKFSGSGTDYSLTISNLEQEDIAIFYCQQGNTLPWTFAGGKLE IKGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLEWVAVVM PDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYAFDYWGQ TLVTVSSGGGGGGGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQ KPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISLQPEDFATYYCQQSYYYFRPITFG GTKVEIKGGGSEVQLQQSGPELVKPGASMKISCKASGYSTGYTMNWVKQSHGKNLEW MGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSTSEDSAVYYCARSGYYGDS DWYFDVWGAGTTVTVSSHHHHH	220
V2-U2-HLHL-scDb	MYRMQLLSCIALSLALVTNSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAP GKGLEWVAVVMPSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRAT NIPVYAFDYWGQGTLVTVSSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWY QQKPGKAPKLLIYYTSRLESGVPSRFSGSGTDYTLTISSLQPEDFATYYCQQGNTLPWTFG QGTKVEIKGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSTGYTMNWV RQAPGKGLEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCA RSGYYGDSDWYFDVWGQGTLVTVSSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVN TAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISLQPEDFATYYCQQSY YYFRPITFGQGTKVEIKHHHHHH	221

V2-U-HLHL-scDb	MYRMQLLSCIALSLALVTNSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAP GKGLEWVAVVMPPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRAT NIPVYAFDYWGQGTLVTVSSGGGGSDIQMTQTSSLSASLGDRVITICRASQDIRNYLNWY QQKPDGTVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQEDIAITYFCQQGNTLPWTF GGTKLEIKGGGGGGGGGGSEVQLQQSGPELVKPGASMKISCKASGYSTGYTMNWV KQSHGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCA RSGYYGDSDWYFDVWGAGTTVTVSSGGGGSDIQMTQSPSSLSASVGDRVITICRASQDVN TAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQSY YYFRPITFGQGTKVEIKHHHHH	222
U2-V2-HLHL-scDb	MYRMQLLSCIALSLALVTNSEVQLVESGGGLVQPGGSLRLSCAASGYSTGYTMNWVRQA PGKGLEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCARSG YYGDSDWYFDVWGQGTLVTVSSGGGGSDIQMTQSPSSLSASVGDRVITICRASQDVNTAV AWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQSYYYFR PITFGQGTKVEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDI HWVRQAPGKGLEWVAVVMPPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAV YYCSRATNIPVYAFDYWGQGTLVTVSSGGGGSDIQMTQSPSSLSASVGDRVITICRASQDIR NYLNWYQQKPGKAPKLLIYYTSRLESGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCQQGN TLPWTFGQGTKVEIKHHHHH	223
U-V2-HLHL-scDb	MYRMQLLSCIALSLALVTNSEVQLQQSGPELVKPGASMKISCKASGYSTGYTMNWVKQS HGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCARSG YYGDSDWYFDVWGAGTTVTVSSGGGGSDIQMTQSPSSLSASVGDRVITICRASQDVNTAV AWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQSYYYFR PITFGQGTKVEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDI HWVRQAPGKGLEWVAVVMPPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAV YYCSRATNIPVYAFDYWGQGTLVTVSSGGGGSDIQMTQTSSLSASLGDRVITICRASQDIR NYLNWYQQKPDGTVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQEDIAITYFCQQGN TLPWTFAGGTKLEIKHHHHH	224

V2-diL2K-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGSDVQLVQSGAEVKKPGASVKVSCKASGYTFTRYTMHWVRQAPGQGGLWEWIGYI NPSRGYTNYADSVKGRFTITTDKSTSTAYMELSSLRSEDTATYYCARYYDDHYCLDYWGQ GTTVTVSSGGGGGGGGGGSDIVLTQSPATLSLSPGERATLSCRASQSVSYMNWYQQK PGKAPKRWIYDTSKVASGVPARFSGSGGTDYSLTINSLEAEDAATYYCQQWSSNPLTFGG GTKVEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLEWV AVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYAFD YWGQGTLVTVSSHHHHHH	225
V2-hXR32-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTENTYAMNWVRQAPGKGLEWVARI RSKYNINYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGNFGNSYVSW FAYWGQGTLVTVSSGGGGGGGGGGGSAVVTQEPSLTVSPGGTVTLTCRSSSTGAVTTS NYANWVQQKPGQAPRGLIGGTNKRAPWTPARFSGSLGGKAALTITGAQAEDEADYYCAL WYSNLWVFGGGTKLTVLGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVR QAPGKGLEWVAVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCS RATNIPVYAFDYWGQGTLVTVSSHHHHHH	226
V2-L2K-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPQGGLWEWIGYI NPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWG QGTTLTVSSGGGGGGGGGGGSDIQLTQSPAIMSASPGKEKVTMTCRASSSVSYMNWYQ QKSGTSPKRWIYDTSKVASGVPYRFSGSGGTSLTSSMEAEADAATYYCQQWSSNPLTFG AGTKLELKGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLEW VAVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYAF DYWGQGTLVTVSSHHHHHH	227

V2-OKT3-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPQGQGLEWIGYI NPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSTSEDSAVYYCARYYDDHYCLDYWG QGTTLTVSSGGGGSGGGGGGQIVLTQSPAIMASAPGEKVTMTCSASSSVSYMNNWYQQ KSGTSPKRWIYDTSKLASGVPAHFRGSGGTSYSLTISGMEAEDAATYYCQQQWSSNPFTFGS GTKLEINGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLEWV AVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYAFD YWGQGTLVTVSSHHHHHH	228
V2-28F11-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGSQVQLVESGGGVVQGRSLRLSCAASGFKFSGYGMHWVRQAPGKGLEWVAVI WYDGSKKYYVDSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARQMGYWHFDLWG RGTLVTVSSGGGGSGGGGGGSEIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQ KPGQAPRLLIYDASN RATGIPARFSGSGGTDFTLTISLLEPEDFAVYYCQQRSNWPPLTFGG GTKVEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLEWV AVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYAFD YWGQGTLVTVSSHHHHHH	229
V2-27H5VL1-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGSQVQLVESGGGVVQGRSLRLSCAASGFTFRSYGMHWVRQAPGKGLEWVAII WYDGSKKNYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGTGYNWFDPWG QGTLVTVSSGGGGSGGGGGGSEIVLTQSPRTLSPGERATLSCRASQSVSSYLAWYQ QKPGQAPRLLIYGASSRATGIPDRFSGSGGTDFTLTISRLLDPEDFAVYYCQQYGGSSPITFGQG TRLEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLEWVAV VMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYAFDYW GQGTLVTVSSHHHHHH	230

V2-23F10-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGSQVQLVQSGGGVVQSGRSLRLSCAASGFKFSGYGMHWVRQAPGKGLEWVAV IWYDGSKKYYVDSVKGRFTISRDN SKNTLYLQMNSLRGEDTAVYYCARQMGYWHFDLWG RGTLVTVSSGGGGSGGGGGGSEIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQ KPGQAPRLLIYDASN RATGIPARFSGSGGTDFTLTISLLEPEDFAVYYCQQRSNWPPLTFGG GTKVEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLEWV AVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYAFD YWGQGTLVTVSSHHHHHH	231
V2-15C3VL1-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGSQVQLVQSGGGVVQGRSLRLSCVASGFTFSSYGMHWVRQAPGKGLEWVAI WYNGRKQDYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCTRGTGYNWFDPWG QGTLVTVSSGGGGSGGGGGGSEIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQ KPGQAPRLLIYDASN RATGIPARFSGSGGTDFTLTISLLEPEDFAVYYCQQRSNWPWTFGQ GTKVEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLEWV AVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYAFD YWGQGTLVTVSSHHHHHH	232
V2-15C3VL2-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGSQVQLVQSGGGVVQGRSLRLSCVASGFTFSSYGMHWVRQAPGKGLEWVAI WYNGRKQDYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCTRGTGYNWFDPWG QGTLVTVSSGGGGSGGGGGGSAIQLTQSPSSLSASVGDRVTITCRASQGISSALAWYQQ KPGKAPKLLIYDASSLESGVPSRFSGSGGTDFTLTISSLQPEDFATYYCQQFN SYPITFGQGT RLEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLEWVAV VMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYAFDYW GQGTLVTVSSHHHHHH	233

V2-U212F6-LHLH-scDb	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTSYTMHWVKQRPGQGLEWIGYI NPSSGYTKYNQKFKDKATLTADKSSSTAYMQLSSLTSEDSAVYYCARWQDYDYFDYWG QGTTLTVSSGGGGSGGGGGGQIVLSQSPAILSASPGEKVTMTCRASSSVSYMHWYQQ KPGSSPKPWYATSNLASGVPARFSGSGGTSYSLTISRVEAEDAAATYYCQQWSSNPPTFGG GTKLETKRGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLEW VAVVMPDSGHTNAYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYAF DYWGQGTLVTVSSHHHHH	234
V2-U2-LHHL-BITE	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGSGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPG KGLEWVAVVMPDSGHTNAYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTTLTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSTGYTMNW VRQAPGKGLEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYC ARSGYYGDSDWYFDVWGQGTTLTVSSGGGGSGGGGGSDIQMTQSPSSLSASVGDR VTITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGVPSRFSGSGGTDYTLTISLQPE DFATYYCQQGNTLPWTFGQGTKVEIKHHHHH	235
V2-U-LHHL-BITE	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGSGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPG KGLEWVAVVMPDSGHTNAYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTTLTVSSGGGGSEVQLQQSGPELVKPGASMKISCKASGYSTGYTMNWV KQSHGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCA RSGYYGDSDWYFDVWGAGTTVTVSSGGGGSGGGGGSDIQMTQTTSSLSASLGDRVT ISCRASQDIRNYLNWYQQKPDGTVKLLIYYTSRLHSGVPSKFSGSGGTDYSLTISNLEQEDI ATYFCQQGNTLPWTFAGGTKLEIKHHHHH	236

V2-diL2K-LHHL-BITE	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHVVVRQAPG KGLEWVAVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGGGSDVQLVQSGAEVKKPGASVKVSCKASGYTFTRYTMHW VRQAPGGGLEWIGYINPSRGYTNYADSVKGRFTITTDKSTSTAYMELSSLRSEDATYYCAR YYDDHYCLDYWGQGTITVTVSSGGGGGGGGSDIVLTQSPATLSLSPGERATLSCRA SQSVSYMNWYQQKPKGKAPKRWIYDTSKVASGVPARFSGSGGTDYSLTINSLEAEDAATY YCQQWSSNPLTFGGGTKVEIKHHHHH	237
V2-hXR32-LHHL-BITE	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHVVVRQAPG KGLEWVAVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNW VRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVY YCVRHGNFGNSYVSWFAYWGQGTITVTVSSGGGGGGGGGSGQA VVTQEPSLTVSPG GTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNKRAPWTPARFSGSLGGKAAL TITGAQAEDEADYYCALWYSNLWVFGGGTKLVLHHHHH	238
V2-OKT3-LHHL-BITE	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHVVVRQAPG KGLEWVAVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHW VKQRPGGGLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCA RYDDHYCLDYWGQGTITVTVSSGGGGGGGGGSGQIVLTQSPAIMSASPGEKVTMTC SASSSVSYMNWYQQKSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAT YYCQQWSSNPFTFGSGTKLEINHHHHH	239

V2-U2-LHLH-BITE	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHVVVRQAPG KGLEWVAVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQ KPGKAPKLLIYYTSRLESGVPSRFSGSGGTDYTLTISLQPEDFATYYCQQGNTLPWTFGQG TKVEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSTGYTMNWVRQ APGKGLEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCARS GYYGDSDWYFDVWGQGTLLTVSSHHHHH	240
V2-U-LHLH-BITE	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHVVVRQAPG KGLEWVAVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGGGSDIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQ KPDGTVKLLIYYTSRLHSGVPSKFSGSGTDYSLTISNLEQEDIAFYFCQQGNTLPWTFAGG TKLEIKGGGGGGGGGGSEVQLQQSGPELVKPGASMKISCKASGYSTGYTMNWVKQ SHGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCARS GYYGDSDWYFDVWGAGTTTVTSSHHHHH	241
V2-diL2K-LHLH-BITE	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHVVVRQAPG KGLEWVAVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGGGSDIVLTQSPATLSLSPGERATLSCRASQSVSYMNWYQQ KPGKAPKRWIYDTSKVASGVPARFSGSGGTDYSLTINSLEAEDAATYYCQQWSSNPLTFG GGTKVEIKGGGGGGGGGGSDVQLVQSGAEVKKPGASVKVSCKASGYTFTRYTMHW VRQAPGQGLEWIGYINPSRGYTNYADSVKGRFTITTDKSTSTAYMELSSLRSEDATATYCAR YYDDHYCLDYWGQGTTVTVSSHHHHH	242

V2-hXR32-LHLH-BITE	MYRMLLSIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHVVVRQAPG KGLEWVAVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGGGSQAVVTQEPSLTVSPGGTVTLTCRSSSTGAVTTSNYANW VQKPGQAPRGLIGGTNKRAPWTPARFSGSLLGKAAALTITGAQAEDEADYYCALWYSNL WVFGGGTKLTVLGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFTNTYA MNWVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTED TAVYYCVRHGNFGNSYVSWFAYWGQGLVTVSSHHHHHH	243
V2-OKT3-LHLH-BITE	MYRMLLSIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHVVVRQAPG KGLEWVAVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGGGSQIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQ KSGTSPKRWIYDTSKLASGVPAHFRGSGGTSYSLTISGMEADAATYYCQQWSSNPFTFGS GTKLEINGGGGGGGGGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWV KQRPQGQLEWIGYINPSRGYTNYNQKFKDKATLTIDKSSSTAYMQLSSLTSEDSAVYYCAR YYDDHYCLDYWGQGTTLTVSSHHHHHH	244
V2-U2-LH-LH-DART	MYRMLLSIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNVVRQAPGKGLEW VALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCARSGYYGDSDW YFDVWGQGTLVTVSSGGCGGGEVAALEKEVAALEKEVAALEKEVAALEKEGRGSLTTCG DVEENPGPMYRMQLLSIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDIRNYLN WYQQKPGKAPKLLIYYTSRLESGVPSRFSGSGGTDYTLTISSLPEDFATYYCQQGNTLPW TFGQGTKVEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHVVVRQAPG KGLEWVAVVMPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGCGGGKVAALKEKVAALKEKVAALKEKVAALKEHHHHHH	245

V2-U-LH-LH-DART	MYRMLLSIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGSGGGGEVQLQQSGPELVKPGASMKISCKASGYSTGYTMNWWVKQSHGKNLEW MGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCARSGYYGDSDW YFDVWGAGTTVTVSSGGCGGGEVAALEKEVAALEKEVAALEKEVAALEKEGRGSLLTCG DVEENPGPMYRMQLLSIALSLALVTNSDIQMTQTSSLSASLGDRVTISCRASQDIRNYLN WYQQKPDGTVKLLIYYTSRLHSGVPSKFSGSGGTDYSLTISNLEQEDIAFYFCQQGNTLPW TFAGGTKLEIKGGSGGGGEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPG KGLEWVAVVMPPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGCGGGKVAALKEKVAALKEKVAALKEKVAALKEHHHHH	246
V2-diL2K-LH-LH-DART	MYRMLLSIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGSGGGGDVQLVQSGAEVKKPGASVKVSCKASGYTFTRYTMHWVRQAPGQGLEW IGYINPSRGYTNYADSVKGRFTITTDKSTSTAYMELSSLRSEDATYYCARYYDDHYCLDY WGQGTTVTVSSGGCGGGEVAALEKEVAALEKEVAALEKEVAALEKEGRGSLLTCGDVEE NPGPMYRMQLLSIALSLALVTNSDIVLTQSPATLSLSPGERATLSCRASQSVSYMNWYQQ KPGKAPKRWIYDTSKVASGVPARFSGSGGTDYSLTINSLEAEDAATYYCQQWSSNPLTFG GGTKVEIKGGSGGGGEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKG LEWVAVVMPPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPV YAFDYWGQGTLVTVSSGGCGGGKVAALKEKVAALKEKVAALKEKVAALKEHHHHH	247

V2-hXR32-LH-LH-DART	MYRMLLSIALSLALVTNSDIQMTQSPSSLASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGSGGGEVQLVESGGGLVQPGGSLRLSCAASGFTFTYAMNWVRQAPGKGLEW VARIRSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGNFGNS YVSWFAYWGQGTLLVTVSSGGCGGGEVAALEKEVAALEKEVAALEKEVAALEKEGRGSL TCGDVEENPGPMYRMQLLSIALSLALVTNSQAVVTQEPSLTVSPGGTVTLTCRSSTGAVTT SNYANWVQQKPGQAPRGLIGGTNKRAPWTPARFSGSLGGKAAALTITGAQAEDEADYYCA LWYSNLWVFGGGTKLTVLGGSGGGGEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDI HWVRQAPGKLEWVAVVMPSDGHNTYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAV YYCSRATNIPVYAFDYWGQGTLLVTVSSGGCGGKVAALKEKVAALKEKVAALKEKVAAL KEHHHHHH	248
V2-OKT3-LH-LH-DART	MYRMLLSIALSLALVTNSDIQMTQSPSSLASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGSGGGQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPQGQLEW IGYINPSRGYTNYNQKFKDKATLTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDY WGQGTLLTVSSGGCGGGEVAALEKEVAALEKEVAALEKEVAALEKEGRGSL LTCGDVEEN PGPMYRMQLLSIALSLALVTNSQIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQK SGTSPKRWIYDTSKLASGVPAHFRGSGGTSYSLTISGMEAEADAATYYCQQWSSNPFTFGSG TKLEINGGGSGGGEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWVRQAPGKGLE WVAVVMPSDGHNTYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNIPVYA FDYWGQGTLLVTVSSGGCGGKVAALKEKVAALKEKVAALKEKVAALKEHHHHHH	249
V2-U2-LH-LH-diabody	MYRMLLSIALSLALVTNSDIQMTQSPSSLASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSTGYTMNWVRQAPGKGLEWVALI NPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCARSGYYGDSDWYFD VWGQGTLLVTVSSEGRGSL LTCGDVEENPGPMYRMQLLSIALSLALVTNSDIQMTQSPSSLS ASVGDRVTITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGVPSRFSGSGGTDYTLTI SSLQPEDFATYYCQQGNTLPWTFGQGTKVEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAA SGFNLSYSDIHWVRQAPGKGLEWVAVVMPSDGHNTYADSVKGRFTISADTSKNTAYLQMN SLRAEDTAVYYCSRATNIPVYAFDYWGQGTLLVTVSSHHHHHH	250

V2-U-LH-LH-diabody	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGSEVQLQQSGPELVKPGASMKISCKASGYSTGYTMNWVKQSHGKNLEWMGLI NPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCARSGYYGDSDDWYFDV WGAGTTVTVSSEGRGSLLTCCGDVEENPGPMYRMQLLSCIALSLALVTNSDIQMTQTTSLS ASLGDRVTISCRASQDIRNYLNWYQQKPDGTVKLLIYTSRLHSGVPSKFSGSGGTDYSLTI SNLEQEDIAITYFCQQQNTLPWTFAGGTKLEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAA SGFNLSYSDIHWVRQAPGKGLEWVAVVMPPDSGHTNYADSVKGRFTISADTSKNTAYLQMN SLRAEDTAVYYCSRATNIPVYAFDYWGQGTLVTVSSHHHHH	251
V2-diL2K-LH-LH-diabody	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGSDVQLVQSGAEVKKPGASVKVCKASGYTFTRYTMHWVRQAPGQGLEWIGYI NPSRGYTNYADSVKGRFTITTDKSTSTAYMELSSLRSEDTATYYCARYYDDHYCLDYWGQ GTTVTVSSEGRGSLLTCCGDVEENPGPMYRMQLLSCIALSLALVTNSDIVLTQSPATLSLSPGE RATLSCRASQSVSYMNWYQQKPGKAPKRWIYDTSKVASGVPARFSGSGGTDYSLTINSLE AEDAAITYCQQWSSNPLTFGGGTKVEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAAASGF NLSYSDIHWVRQAPGKGLEWVAVVMPPDSGHTNYADSVKGRFTISADTSKNTAYLQMNSLR AEDTAVYYCSRATNIPVYAFDYWGQGTLVTVSSHHHHH	252
V2-hXR32-LH-LH-diabody	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARI RSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRHGNFGNSYVSW FAYWGQGTLVTVSSEGRGSLLTCCGDVEENPGPMYRMQLLSCIALSLALVTNSQAVVTQEPS LTVSPGGTVTLTCRSSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNKRAPWTPARFSGSLLG GKAALTITGAQAEDEADYYCALWYSNLWVFGGGTKLTVLGGGGSEVQLVESGGGLVQPG GSLRLSCAASGFNLSYSDIHWVRQAPGKGLEWVAVVMPPDSGHTNYADSVKGRFTISADTS KNTAYLQMNSLRAEDTAVYYCSRATNIPVYAFDYWGQGTLVTVSSHHHHH	253

V2-OKT3-LH-LH-diabody	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGSRGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLEWIGYI NPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSTSEDSAVYYCARYYDDHYCLDYWG QGTTLTVSSEGRGSLLTGCDVEENPGPMYRMQLLSCIALSLALVTNSQIVLTQSPAIMASAPG EKVVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGM EAEDAAITYYCCQQWSSNPFTFGSGTKLEINGGGGSEVQLVESGGGLVQPGGSLRLSCAASGF NLSYSDIHWWVRQAPGKGLEWVAVVMPSDGHNTNYADSVKGRFTISADTSKNTAYLQMNSLR AEDTAVYYCSRATNIPVYAFDYWGQGTLVTVSSHHHHH	254
V2-FcHole	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGSRGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGSQVQLQQSGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWWVRQAPG KGLEWVAVVMPSDGHNTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGGGSEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDITLMIS RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDW LNGKEYCKVKVSNKALPAPIAKTISKAKGQPREPQVCTLPPSREEMTKNQVSLSCAVKGFYPS DIAVEWESNGQPENNYKTTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVVFSCSVMHEALHNH YTQKSLSLSPGKHHHHH	255
U2-FcKnob	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKPG KAPKLLIYYSRLESGVPSRFSGSGGTDYTLTISSLQPEDFATYYCQQGNTLPWTFGGQGTKV EIKGGGSQVQLQQSGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSTGYTMNWVRQAPG KGLEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCARSGYY GDSDWYFDVWGQGTLVTVSSGGGGSEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDITL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQ DWLNGKEYCKVKVSNKALPAPIAKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKG FYPSDIAVEWESNGQPENNYKTTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSVMHEAL HNHYTQKSLSLSPGK	256

U-FcKnob	MYRMQLLSIALSLALVTNSDIQMTQTTSLSASLGDRVTISCRASQDIRNYLNWYQQKPDG TVKLLIYTSRLHSGVPSKFSGSGGTDYSLTISNLEQEDIATYFCQQGNTLPWTFAGTKLE IKGGGGGGGGGGSEVQLQQSGPELVKPGASMKISCKASGYSTGYTMNWVKQSHGK NLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCARSGYYG DSDWYFDVWGAGTTVTVSSGGGGSEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDITLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQD WLNKEYKCKVSNKALPAPIAKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGF YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLYSKLTVDKSRWQQGNVFCSCVMHEALH NHYTQKSLSLSPGK	257
V2-FcKnob-U2	MYRMQLLSIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYCYCQQSYYYFRPITFGQGTK VEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHWRQAPG KGLEWVAVVMPSDGHNTYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGGGSEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDITLMIS RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIAKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYP SDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLYSKLTVDKSRWQQGNVFCSCVMHEALHN HYTQKSLSLSPGKASGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSTGYTMNWV RQAPGKGLEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCA RSGYYGDSDWYFDVWGQGTLVTVSSGGGGGGGGGGSDIQMTQSPSSLSASVGDRTVT ITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGVPSRFSGSGSGTDYTLTISSLQPEDF ATYYCQQGNTLPWTFGQGTKVEIK	258

V2-FcKnob-U	MYRMQLLSCIALSLALVTNSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISSLQPEDFATYYCQQSYYYFRPITFGQGTK VEIKGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNLSYSDIHVVVRQAPG KGLEWVAVVMPSD SGHTNYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRATNI PVYAFDYWGQGTLVTVSSGGGGSEPKSSDKTHTCPPCPAPELGGPSVFLFPPKPKDTLMIS RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDW LNGKEYCKVSNKALPAPIAKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYP SDIAVEWESNGQPENNYKTTTPPVLDSDGSEFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGKASGGGGGGSEVQLQQSGPELVKPGASMKISCKASGYSTGYTMNWV KQSHGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCA RSGYYGDSDWYFDVWGAGTTVTVSSGGGGGGGGGGSDIQMTQTTSSLSASLGDRVT ISCRASQDIRNYLNWYQQKPDGTVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQEDI ATYFCQQQGNTLPWTFAGGTKLEIK	259

In sum, 42 recombinant proteins were expressed in HEK293 cells to identify the most effective format and configuration (Fig. 49, A and B). Binding to G12V pHLA-A3 and recombinant CD3 ϵ / δ protein (Fig. 49C) were assessed by ELISAs at various bispecific protein concentrations. While a few formats exhibited weak binding to one or both antigens, most had similar performance characteristics upon ELISA (Fig. 49C). To further compare the formats, T2A3 cells were pulsed with two concentrations of the G12V peptide and co-cultured with T cells and each of the V2 bispecific proteins at two concentrations, followed by measurement of IFN γ release to assess T cell activation (Fig. 49D). Despite their similar performance in ELISA assays, the ability of the bispecific formats to recognize the G12V peptide at low antigen densities on cells was highly variable. scDBs generally performed better than other formats, particularly at lower concentration of antibodies. Switching the order of the heavy and light variable domains in the analogous proteins ("LHLH" to "HLHL") abolished their ability to activate T cells, despite these formats showing equivalent functionality on ELISA (Fig. 49). Similarly, while the bivalent scFv-Fc and trivalent scFv-Fc performed particularly well in the ELISAs, they consistently performed poorly when assessed in peptide-pulsed cells.

In the initial tests of formats and configurations, five different anti-CD3 scFvs were used. Based on the results indicating that the scDb performed best, seven additional anti-CD3 clones were tested (Tables 12 and 13). A total of twelve V2 scDBs were expressed, purified (Fig. 50A), and then tested with ELISA for their ability to bind the CD3 ϵ / δ protein (Fig. 50B). Two target cell lines were used to further assess these V2 scDBs. The first was *KRAS* G12V and *HLA-A3* co-transfected COS-7 and the second was the lung cancer cell line NCI-H441, which expressed endogenous HLA-A3 and mutant *KRAS* G12V. In a co-culture experiment with the COS-7 overexpression system, ten of the scDBs activated T cells as shown by substantial IFN γ release (Fig. 50C). However, with NCI-H441 cells, only the scDBs containing the UCHT1 ("U") and UCHT1.v9 ("U2") anti-CD3 clones activated T cells (Fig. 50D), with the UCHT1-based scDb (Fig. 39A) performing particularly well. Both scDBs (hereafter referred to as V2-U and V2-U2, respectively) (Tables 12 and 13) retained the remarkable specificity of V2 phage toward G12V pHLA-A3, as they failed to interact with pHLA-A3 folded with the other RAS peptides or an unrelated CTNNB peptide (Fig.

39B, Fig. 51A). They also bound to the CD3 ϵ / δ heterodimer (Fig. 2B, Fig. 51A). Both scDBs could simultaneously interact with G12V pHLA-A3 and CD3 ϵ / δ heterodimer, as shown by a “sandwich” ELISA (Fig. 51B), suggesting that the proteins were folded and functioned properly. Given all these data on relative expression, ELISA, and cellular reactivity, the V2-U scDb (N-terminus-L_{V2}-H_U-L_U-H_{V2}-C-terminus) was chosen as a focus (Fig. 39A) for most further studies, though V2-U2 was also used for select assays.

Based off of this work with the G12V clone V2, the Q61 scFv clones H1, L2, and R6 were grafted into the optimized scDb format to generate H1-U, L2-U, and R6-U scDBs. All three scDBs retained binding to their cognate mutant-derived Q61 pHLA-A1 and recombinant CD3 ϵ / δ on ELISA and none exhibited appreciable binding to the Q61WT pHLA or to other control pHLA including an unrelated CMV pHLA (Fig. 39, C to E and fig. 51, C to E, Table 11). This result showed that the format and configuration chosen on the basis of the RAS G12V scFv was generalizable, and applicable to three other scFvs with varying amino acid sequences and independent targets.

scDBs recognize cells pulsed with low nanomolar concentrations of exogenous peptides

To assess the minimal concentration of target antigen required for activating T cells, T2A3 cells were pulsed with G12V or G12WT peptides and then co-cultured with healthy donor T cells in the presence of the V2-U scDb. T cells were activated at G12V peptide concentrations as low as 1 nM, as evidenced by IFN γ and TNF α secretion (Fig. 40A, Fig. 52A). Furthermore, even at low peptide concentrations, the scDb mediated antigen-dependent lysis of the peptide-pulsed T2A3 cells (Fig. 40B). There was no appreciable background cytokine secretion or cell killing when cells were pulsed with the G12WT peptide. Similarly, specific activation was seen with the V2-U2 scDb (Fig. 40, A and B).

To determine the approximate antigen density on peptide-pulsed T2A3 cells, the cells were stained with the V2 scFv and assessed by flow cytometry. Antigen density was determined using QIFIKIT and Quantibrite Beads, which allowed quantitative determination of the number of cell surface antigenic molecules (Fig. 52B). It was found that cells pulsed with 320 nM of G12V peptide expressed 300 to 800 antigenic molecules per cell. This flow cytometric method did not have the sensitivity required to detect cells pulsed with <320 nM of G12V peptide. However, 300-fold lower concentrations of pulsed G12V peptide could

activate T cells (Fig. 40B). Though there may not be a linear relationship between the concentration of the pulsed peptide and the number of peptide antigens displayed on the cell surface, these results suggest that the number of antigenic molecules per cell recognized by the V2-U scDb is far less than 300. An analogous peptide-pulsing experiment was

5 performed with HLA-A3+ monocyte-derived immature dendritic cells (iDCs) to present the peptide and assayed for T cell secretion of IFN γ and TNF α . Both V2-U and V2-U2 scDbs were able to activate T cells when the iDCs were pulsed with G12V peptide at low nanomolar range (Fig. 52, C and D).

Similar experiments were performed on the RAS Q61 targeting scDbs using peptide-pulsed SigM5 cells and iDCs. The L2-U scDb elicited T cell activation as shown by IFN γ secretion and SigM5 cytotoxicity when the target cells were pulsed with the Q61L peptide at concentrations as low as 1 nM, without cross-reactivity to the Q61WT peptide (Fig. 40, C and D). While the H1-U and R6-U scDbs also showed no significant Q61WT peptide cross-reactivity, it required 100-fold more peptide (100 nM) for them to activate T cells (Fig. 52, E

10 to H). Likewise, the L2-U scDb could activate T cells in the presence of iDCs at lower peptide concentrations than could the H1-U and R6-U scDbs (Fig. 52, I to N).

scDbs recognize COS-7 cells overexpressing HLA and mutant RAS genes

COS-7 cells were co-transfected with plasmids encoding HLA-A3 and either mutant or WT full-length KRAS (Fig. 53A), then co-cultured with T cells. As noted in the first

20 section of the results, the COS-7 cells expressed ~100 copies of the G12V pHLA complex per cell. Addition of the V2-U scDb resulted in secretion of both IFN γ and TNF α from T cells in a dose-dependent manner only in the presence of COS-7 cells co-transfected with plasmids encoding HLA-A3 and KRAS G12V (Fig. 41A, Fig. 53B). As little as 10 pM of the V2-U scDb could activate T cells in this experiment (Fig. 41A, Fig. 53B). A similar

25 activation of T cells by transfected COS-7 cells was observed in the presence of 30 pM of the V2-U2 scDb (Fig. 53, C and D). The secretion of both IFN γ and TNF α was highly specific as the target cells expressing HLA-A3 and WT, G12C, or G12D KRAS did not trigger secretion of these cytokines (Fig. 41A, Fig. 53, B to D).

Analogous experiments were performed with the mutant RAS Q61-targeting H1-U,

30 L2-U, and R6-U scDbs. COS-7 cells were co-transfected with plasmids encoding HLA-A1

and full-length WT or mutant HRAS, KRAS, or NRAS to assess whether each scDb was capable of recognizing the cognate mutant peptides derived from each of the RAS proteins. Each scDb elicited T cell responses highly specific for the COS-7 cells expressing the *RAS* gene with the Q61 mutation of interest, regardless of the *RAS* gene assessed (Fig. 41, B to D).

Subnanomolar concentrations of the scDbs were sufficient to activate T cells when co-cultured with COS-7 cells harboring Q61 mutations in any of the three genes. Moreover, no activation of T cells was observed when co-cultured with COS-7 cells expressing *HRAS*, *KRAS*, or *NRAS* genes that were WT or contained the non-cognate Q61 mutations (Fig. 41, B to D).

scDbs activate T cells when exposed to cancer cells harboring endogenous mutant RAS genes

As noted above, the NCI-H441 cancer cell line presents only ~9 copies of mutant-derived KRAS G12V pHLA complexes per cell (Table 10). Despite this extremely low level of the target peptide, T cells could be activated by NCI-H441 cells in the presence of the V2-U scDb, as evidenced by the secretion of IFN γ and cytotoxicity (Fig. 42, A and B). The potency of V2-U was high, with EC₅₀ of 140 pM and 76 pM for IFN γ secretion and cytotoxicity, respectively (Fig. 42, A and B). To rigorously assess the specificity of the V2-U scDb, the *HLA-A3* allele was disrupted in NCI-H441 cells by CRISPR-based technologies and the knock-out (KO) was confirmed via flow cytometry (Fig. 54). KO of the *HLA-A3* allele eliminated the ability of the V2-U scDb to elicit IFN γ secretion or cytotoxicity by T cells upon exposure to the target NCI-H441 cells (Fig. 42, C and D). CRISPR-based technologies were then used to replace ("knock-in"[KI]) *KRAS* G12V with *KRAS* G13D in parental NCI-H441 cells containing their endogenous *HLA-A3* allele (Fig. 55). G12V was replaced with G13D, rather than knocking out the G12V allele, to maintain viability of the cells, which require mutant *KRAS* genes. Two independent NCI-H441 clones with the *KRAS* G13D substitution were tested, and both substantially abrogated the ability of T cells to be activated by V2-U scDb in the presence of NCI-H441 cells (Fig. 42, C and D). Similarly, specific activation of T cells co-cultured with NCI-H441 cells was observed with the V2-U2 scDb, but as expected, the potency of the V2-U2 scDb was not as great as the V2-U scDb (Fig. 56). In all the experiments with endogenous levels of *HLA-A3* and G12V alleles (Fig.

42, A and C, Fig. 56A), IFN γ secretion from T cells was considerably lower than that in T cells activated by the transfected COS-7 cells (Fig. 41A, Fig. 539C), consistent with greater numbers of the pHLA complexes on the transfected COS-7 cells. Even so, the scDb were able to induce efficient NCI-H441 target cell lysis through T cell activation in a KRAS G12V and HLA-dependent fashion (Fig. 42, B and D, Fig. 56B). Moreover, other markers of T cell activation (TNF α , IL-2, granzyme B, and perforin) were released in a dose-dependent manner, demonstrating that the V2-U scDb was capable of inducing a poly-functional T cell response against cells expressing very low levels of antigen (Fig. 57).

To further assess the specificity of the V2-U scDb, a second cell line, NCI-H358, was used. This lung cancer cell line contains the *HLA-A3* allele and a *KRAS* G12C mutation. Using CRISPR, the G12V mutation was introduced in the *KRAS* locus in three independent clones (Fig. 55). All three G12V clones were able to induce cytokine secretion from T cells in the presence of the V2-U scDb, while parental cells or a clone retaining the G12C allele were not (Fig. 42E). Cytotoxicity to the G12V clones was also much greater, especially at subnanomolar scDb concentrations, than to isogenic NCI-H358 cells without the G12V allele (Fig. 42F). Similarly, specific cytokine secretion and cytotoxicity was observed upon co-culture of these cells with T cells in the presence of V2-U2 scDb (Fig. 58). All NCI-H358 variants expressed approximately the same level of HLA-A3 (Fig. 54).

IFN γ secretion in co-cultures of T cells with several other HLA-A3⁺ cancer cell lines without *RAS* G12V mutations was assessed. These lines included A-427 (lung adenocarcinoma), COLO 741 (melanoma), Hs 578T (breast invasive ductal carcinoma), Jurkat (acute T cell leukemia), SK-MES-1 (lung squamous cell carcinoma), and SW780 (bladder transitional cell carcinoma). CFPAC-1, the *KRAS* G12V and HLA-A3⁺ pancreatic adenocarcinoma cell line that presents an average of only ~3 copies of the G12V peptide per cell were also assessed (Table 10). Expression of HLA-A3 in all these cell lines was confirmed via flow cytometry (Fig. 54). Neither V2-U nor V2-U2 scDb resulted in appreciable IFN γ release from T cells co-cultured with the cancer cell lines without the *KRAS* G12V mutation (Fig. 59). V2-U scDb induced a low but significantly higher level of IFN γ release with CFPAC-1 cells (Fig. 59A); however, there was no statistically significant difference when V2-U2 was used (Fig. 59B).

To study the ability of the L2-U scDb to induce T cell activation, co-culture with a panel of cell lines that differed in *RAS* mutation status and HLA-A1 expression were employed (Fig. 43A, Fig. 60). Substantial dose-dependent IFN γ secretion by T cells was only observed with the one cell line (HL-60) containing both HLA-A1 and RAS Q61L alleles (Fig. 43A). As noted above, HL-60 presents an average of four Q61L pHLA complexes per cell. In a titration experiment, the L2-U scDb was able to induce IFN γ release from T cells with an EC₅₀ of 60 pM (Fig. 43B) and HL-60 cell death with an EC₅₀ of 34 pM (Fig. 43C), despite the very low level of Q61L peptides per cell. Other markers of T cell activation were similarly released in a dose-dependent manner, showing that the L2-U scDb, like the V2-U scDb, was capable of inducing a poly-functional T cell response (Fig. 61). To assess the specificity of the L2-U scDb antigenic determinants in HL-60 cells, *NRAS* Q61H-KI, Q61R-KI, and *HLA-A1*-KO variants of these cells were generated (Fig. 55). These experiments confirmed that IFN γ secretion (Fig. 43D) and target cell cytotoxicity (Fig. 43E) were dependent on the co-presence of *NRAS* Q61L and *HLA-A1* genes in HL-60 cells.

15 *Assessing potential cross reactivity to other putative HLA-A1 and HLA-A3-binding peptides*

To investigate whether the V2 scFv could bind to similar peptides derived from other proteins, a protein BLAST (BLASTp) search of the human RefSeq proteome was performed with the amino acid sequences of the G12V peptide or its G12WT, G12C, or G12D counterparts. Thirty-two proteins containing similar peptides were identified through this search. Of these, NetMHC v4.0 predicted that 17 peptides had strong or weak binding of HLA-A3 (Table 14). Each of these 17 peptides were synthesized and used to pulse T2A3 cells. While the majority of these peptides bind to HLA-A3, as assessed by GAP.A3 antibody staining, V2 phage only recognized the peptide IIVGAIGVGK ("Blast2"; SEQ ID NO:260), a peptide derived from the protein Rab-7b (Fig. 62A, Table 14).

Table 14. RAS G12 BLAST peptides. BLASTp was used to query the human RefSeq proteome for peptides similar to the G12V, G12WT, G12C, and G12D [7-16] peptides. These similar peptides were extended with the flanking amino acids from the source protein to produce the full peptide sequence. These peptides were analyzed by NetMHCv4.0 for predicted weak and strong binders to HLA*A03:01. These predicted binders are listed as BLAST peptides.

Source Protein	Full Peptide Sequence	SEQ ID NO	BLAST Peptide (predicted HLA*A*03:01 binding peptide)	SEQ ID NO	Predicted HLA- A*03:01 Affinity	BLAST Peptide Name
RAS-related protein Rab-7b isoform a [Homo sapiens]	LIIVGAIGVGKT	261	IVGAIGVGK	262	187.94	Blast1
			IIVGAIGVGK	260	215.14	Blast2
PREDICTED: multidrug resistance- associated protein 6 isoform X1 [Homo sapiens]	LAVVGPVGAGK	263	VVGPVGAGK	264	324.7	Blast3
			AVVGPVGAGK	265	149.9	Blast4
PREDICTED: protein TANC1 isoform X2 [Homo sapiens]	VVVGNVGFGK	266	VVGNVGFGK	267	167.47	Blast5
			VVVGNVGFGK	266	264.35	Blast6
GTPase ERas precursor [Homo sapiens]	VVVGASGVGK	268	VVGASGVGK	269	216.26	Blast7
			VVVGASGVGK	268	355.27	Blast8
PREDICTED: mitochondrial Rho GTPase 2 isoform X1 [Homo sapiens]	KVVGARGVGK	270	VVGARGVGK	271	140.14	Blast9
			KVVGARGVGK	270	80.98	Blast10
glycogen debranching enzyme isoform 1 [Homo sapiens]	LGSVQLCGVGKFP SL	272	SVQLCGVGK	273	576.87	Blast11
mitogen-activated protein kinase kinase kinase MLT isoform 1 [Homo sapiens]	SRNVVIAADGVLKI	274	VIAADGVLK	275	127.74	Blast12
			VVIAADGVLK	276	157.48	Blast13
RAS-related protein M-Ras isoform 1 precursor [Homo sapiens]	VVVGDDGGVGK	277	VVVGDDGGVGK	277	718.71	Blast14

inactive hydroxysteroid dehydrogenase-like protein 1 isoform a [Homo sapiens]	WAVVSGATDGIGK	278	VSGATDGIGK	279	954.98	Blast15
60S acidic ribosomal protein P0 [Homo sapiens]	KCFIVGADNVGSK	280	IVGADNVGSK	281	505.97	Blast16
rho-related GTP-binding protein RhoJ precursor [Homo sapiens]	KCVVVGDGAVGKT	282	VVVGDGAVGK	283	468.81	Blast17
OTU domain-containing protein 5 isoform a [Homo sapiens]	VVVGVGGAVGVG	284	none			
PREDICTED: traB domain-containing protein isoform X1 [Homo sapiens]	VVVGVVGMGHVP	285	none			
PREDICTED: multidrug resistance-associated protein 6 isoform X1 [Homo sapiens]	AGEKVGVGRTGAG	286	none			
multidrug resistance-associated protein 4 isoform 1 [Homo sapiens]	LQVVGVVSVAVAVIP	287	none			
MAGUK p55 subfamily member 7 [Homo sapiens]	RLVVVLVGPVGVGLNE	288	none			
CD151 antigen [Homo sapiens]	LRVIGAVGIGIAC	289	none			
PREDICTED: protein TANC1 isoform X2 [Homo sapiens]	AENRGAVVVGNVG	290	none			
trimethylguanosine synthase isoform 1 [Homo sapiens]	DVVVDAFCGVGG	291	none			
keratin, type II cytoskeletal 5 [Homo sapiens]	VSLAGACGVGGYGS	292	none			

PREDICTED: uncharacterized protein LOC105376341 [Homo sapiens]	HSLPGACGVGPPRA	293	none			
disks large homolog 5 [Homo sapiens]	ETEVGPCVGEAS	294	none			
solute carrier family 22 member 17 isoform a [Homo sapiens]	TLGLVGPCGVGGA	295	none			
solute carrier family 22 member 17 isoform a [Homo sapiens]	LQHVVLAACALLC	296	none			
rho GTPase-activating protein 5 isoform a [Homo sapiens]	EKDKGNCGVGKS	297	none			
olfactory receptor 9I1 [Homo sapiens]	WSLVVGAYVCGVSG	298	none			
receptor-type tyrosine-protein phosphatase beta isoform a [Homo sapiens]	KVVVGSCNRTIQN	299	none			
WD repeat-containing protein 90 [Homo sapiens]	SDSGALLCGVGKDH	300	none			
ribokinase isoform 1 [Homo sapiens]	VVVVGSCMTDLVS	301	none			
dedicator of cytokinesis protein 3 [Homo sapiens]	QQVVGACKPCSDPN	302	none			
PREDICTED: fibroin heavy chain-like isoform X2 [Homo sapiens]	GVDAGVGADVGTGV D	303	none			
translation initiation factor eIF-2B subunit alpha [Homo sapiens]	DLVIVGAEGVVENG	304	none			
PREDICTED: mitochondrial Rho GTPase 2 isoform X1 [Homo sapiens]	LCEVGTDGLLATSL	305	none			

methylothioribose-1-phosphate isomerase isoform 1 [Homo sapiens]	AVVVGADRVVANG	306	none			
collagen alpha-1(XVIII) chain isoform 3 preproprotein [Homo sapiens]	KGEVGADGVPGFPGL	307	none			
collagen alpha-1(XVIII) chain isoform 3 preproprotein [Homo sapiens]	GDPGKDGVGQPGLP	308	none			
protocadherin gamma-A3 isoform 1 precursor [Homo sapiens]	SHFVGADGVRAFL	309	none			

Rab-7b is a RAS-related protein expressed in monocytic cells and keratinocytes. To investigate whether this peptide represents an authentic alternative target and thus could cause off-target toxicity of the V2-U scDb, T cells first co-cultured with peripheral blood mononuclear cells (PBMC), monocytes, iDCs, and mature dendritic cells (mDC) prepared from an HLA-A3+ donor (Fig. 62, B and C). None of these cells were able to activate T cells significantly above background as assessed by IFN γ secretion in the presence of the V2-U scDb (Fig. 62C). Importantly, these cells have the ability to activate T cells when pulsed with the G12V peptide (Fig. 62C). Next, V2-U scDb was tested against a skin derived cell line, Hs 695T, which highly expresses Rab-7b (Fig. 62B). As these cells do not express the HLA-A3 allele, they were transfected with either *HLA-A3* or *HLA-A2* and co-cultured with T cells. Despite high levels of expression of endogenous Rab-7b, Hs 695T cells did not activate T cells in the presence of V2-U scDb. (Fig. 62D). As a positive control, the same experiment was performed after pulsing Hs 695 T cells with the G12V peptide, and under these circumstances, robust activation of T cells was achieved (Fig. 62D).

As a final assessment of the potential cross-reactivity with the Rab-7b peptide, plasmids encoding full length Rab-7b, KRAS WT, or KRAS G12V, in combination with HLA-A3, were transfected into COS-7. Cells overexpressing the mutant KRAS induced robust T cell activation in the presence of V2-U scDb (Fig. 62E), whereas COS-7 cells expressing control proteins, KRAS WT and Rab-7b, showed only marginal, non-dose dependent activation of the same T cells (Fig. 62, B and E). This experiment was repeated in HCT 116 cells transfected with the same genes and again found that T cells were activated by KRAS in a mutant-dependent fashion but no activation was observed in Rab-7b-expressing cells (Fig. 62, B and F).

To further assay for potential cross-reactivity of the V2-U and L2-U scDbs, their binding to libraries of positional scanning variant peptides was evaluated. The library of peptides was generated by systematically substituting each amino acid of the original peptides with the other 19 amino acids. This resulted in 190 variants for each of the G12V and Q61L peptides for the V2 and L2 scDbs. The variant peptides were pulsed on to T2A3 cells (for V2-U scDb) or SigM5 cells (for L2-U scDb) and co-cultured with T cells. Recognition of the variant peptides was evaluated through IFN γ release (Fig. 44, A and B,

Fig. 63, A and B). For both scDBs, amino acid positions in the C-terminal half of the peptides, where the mutant residue resided, demonstrated greater specificity. Most changes of amino acids at these positions abolished recognition by the scDBs. On the other hand, amino acids at the N-terminus of the peptides could in many cases be substituted without substantially changing interaction with the scDBs. These recognition patterns are also illustrated as Seq2Logo graphs (Fig. 44, C and D). Next, using a 20% cognate peptide reactivity as a cutoff for permissive amino acids at each position, decamer binding motifs were generated for each candidate target peptide (Materials and Methods). A search of these motifs in the UniProtKB human protein database using ScanProsite yielded 162 peptides (including Rab-7b IIVGAIGVGK (SEQ ID NO:260)) that could potentially bind to V2 and 232 peptides that could potentially bind to L2 (Tables 15 and 16). Comparing these peptides to an extensive database of peptides actually presented by HLA as assessed by mass spectrometry, it was found that none of the 162 peptides and only one of the 232 peptides were known to be presented by the cognate HLA. However, when this one peptide (DTELQGMNEY (SEQ ID NO:310), from chromodomain-helicase-DNA-binding protein 4 [CHD4]) was pulsed on SigM5 cells, which was then co-cultured with T cells and L2-U scDb, it did not elicit IFN γ release (Fig. 63C), demonstrating that L2-U scDb did not bind the CHD4 peptide.

Table 15. Human peptides matching the V2 binding motif. Using a 20% cognate peptide reactivity as a cutoff for permissive amino acids at each position based on the results of the peptide library analysis, a decamer binding motif for V2 was generated. These motifs were searched in the UniProtKB human protein database using ScanProsite to identify peptides matching the motif.

UniProt Accession Number (Entry Name)	Start position	End position	Peptide Sequence	SEQ ID NO
sp Q8N412 STPG2_HUMAN	115	124	ACDSTLGPAY	311
sp Q05469 LIPS_HUMAN	351	360	ALGRLLGVAH	312
sp O43151 TET3_HUMAN	1188	1197	ALHNSLSPAY	313
sp P14550 AK1A1_HUMAN	59	68	ALKEDVGPGK	314
sp Q7RTX9 MOT14_HUMAN	207	216	ALMRPLSPGK	315
sp P78363 ABCA4_HUMAN	1626	1635	ALVSFLNVAH	316
sp Q6PIV7 S2534_HUMAN	266	275	ALYKGLGPAY	317
sp P28838 AMPL_HUMAN	244	253	AMGSFLSVAK	318

sp P17302 CXA1_HUMAN	249	258	ATSGALSPAK	319
sp O15197 EPHB6_HUMAN	545	554	ATVTQLSPGH	320
sp Q96JB1 DYH8_HUMAN	200	209	AVINVLNVAH	321
sp P14550 AK1A1_HUMAN	32	41	AVKYALSVGY	322
sp Q8N104 D106A_HUMAN	9	18	AVLFFLTPAK	323
sp Q86WI1 PKHL1_HUMAN	1294	1303	CLLPKLSPGK	324
sp Q9Y6T7 DGKB_HUMAN	261	270	CLNMLIGVGK	325
sp Q8IXW5 RPAP2_HUMAN	77	86	ECGRFITPAH	326
sp Q8WWQ8 STAB2_HUMAN	1866	1875	ELLFDLGVAY	327
sp Q9H3U7 SMOC2_HUMAN	408	417	ELMGCLGVAK	328
sp Q16653 MOG_HUMAN	50	59	ELPCRISPGK	329
sp O75366 AVIL_HUMAN	771	780	ELPEDVNPAK	330
sp Q5VUG0 SMBT2_HUMAN	550	559	ELPQSVGPGK	331
sp Q8IWI9 MGAP_HUMAN	2357	2366	ELSEEINVAH	332
sp Q8NGI7 O10V1_HUMAN	270	279	EMGRVVSVAH	333
sp A7E2V4 ZSWM8_HUMAN	1738	1747	ETLQRLSPA	334
sp Q9HB07 MYG1_HUMAN	114	123	ETMSSLSPGK	335
sp Q9NRY4 RHG35_HUMAN	441	450	ETSPFITPGK	336
sp Q01094 E2F1_HUMAN	301	310	ETVGGISPGK	337
sp Q8NEB7 ACRBP_HUMAN	513	522	ETYSALSPGK	338
sp P36382 CXA5_HUMAN	322	331	EVPNGVSPGH	339
sp P07942 LAMB1_HUMAN	1429	1438	GCGGLVTVAH	340
sp O15353 FOXN1_HUMAN	343	352	GCLWALNPAK	341
sp Q9C0H6 KLHL4_HUMAN	295	304	GCTELLNVAH	342
sp Q14332 FZD2_HUMAN	285	294	GCYTMVSVAY	343
sp Q9ULJ3 ZBT21_HUMAN	3	12	GLLHYINPAH	344
sp Q96Q06 PLIN4_HUMAN	689	698	GLMGAVNVAK	345
sp Q96Q06 PLIN4_HUMAN	161	170	GLTGAVNVAK	346
sp Q96Q06 PLIN4_HUMAN	491	500	GLTGAVNVAK	346
sp Q96Q06 PLIN4_HUMAN	590	599	GLVGAVNVAK	347
sp Q9BT76 UPK3B_HUMAN	67	76	GTPTPVSVAH	348
sp Q9BW71 HIRP3_HUMAN	119	128	GVAAEVSPA	349
sp Q95460 HMR1_HUMAN	40	49	GVPEFISVGY	350
sp Q96Q06 PLIN4_HUMAN	920	929	GVTGAVNVAK	351
sp Q96Q06 PLIN4_HUMAN	953	962	GVTGAVNVAK	351
sp Q96Q06 PLIN4_HUMAN	524	533	GVTSAVNVAK	352
sp Q96Q06 PLIN4_HUMAN	623	632	GVTSAVNVAK	352
sp Q96Q06 PLIN4_HUMAN	656	665	GVTSAVNVAK	352
sp Q8N594 MPND_HUMAN	130	139	HCKKLVNPAK	353

sp Q3B8N5 PROX2_HUMAN	435	444	HIQEGLNPGH	354
sp P51003 PAPOA_HUMAN	311	320	HLMPIITPAY	355
sp Q9NRJ5 PAPOB_HUMAN	311	320	HLMPIITPAY	355
sp Q9BWT3 PAPOG_HUMAN	310	319	HLMPIITPAY	355
sp Q02040 AK17A_HUMAN	451	460	HTHDELGVAH	356
sp O60384 ZN861_HUMAN	40	49	HVCGEVGVG Y	357
sp O94915 FRYL_HUMAN	426	435	IIFDLLSVGK	358
sp Q96AH8 RAB7B_HUMAN	12	21	IIVGAIGVGK	260
sp Q8N109 KI2LA_HUMAN	80	89	ILMGPVTPAH	359
sp Q8NHK3 KI2LB_HUMAN	80	89	ILMGPVTPAH	359
sp Q68DQ2 CRBG3_HUMAN	2098	2107	ILSSEVSPGH	360
sp Q92621 NU205_HUMAN	475	484	IMGSYLGVAH	361
sp Q99550 MPP9_HUMAN	974	983	IMLTPVTVAY	362
sp Q9Y3R5 DOP2_HUMAN	1355	1364	IMMQLVSVAK	363
sp Q9P2P6 STAR9_HUMAN	356	365	IMVATVSPA H	364
sp A8TX70 CO6A5_HUMAN	946	955	ITIFAVGVGK	365
sp O00370 LORF2_HUMAN	983	992	ITIQDIGVGK	366
sp O00339 MATN2_HUMAN	787	796	ITMYAVGVGK	367
sp O95460 MATN4_HUMAN	518	527	IVMYAVGVGK	378
sp Q9BZG1 RAB34_HUMAN	56	65	IVVGDL SVGK	369
sp O60645 EXOC3_HUMAN	733	742	IVVPSLNVAK	370
sp P19838 NFKB1_HUMAN	85	94	KICNYVGPAK	371
sp Q8TE73 DYH5_HUMAN	2739	2748	KIFGVIGVGH	372
sp Q5PT55 NTCP5_HUMAN	68	77	KILQMVNVAK	373
sp P43004 EAA2_HUMAN	148	157	KLKKQLGPGK	374
sp Q96Q15 SMG1_HUMAN	2054	2063	KLKTPLNPAK	375
sp Q8N1V2 CFA52_HUMAN	237	246	KLLTDVGPAK	376
sp Q14204 DYHC1_HUMAN	447	456	KMVWRINPAH	377
sp O00206 TLR4_HUMAN	150	159	KTLKELNVAH	378
sp Q8WTR2 DUS19_HUMAN	91	100	KVTHILNVAY	379
sp Q9BRR8 GPTC1_HUMAN	143	152	LLDDLITPAK	380
sp Q9NP78 ABCB9_HUMAN	61	70	LLGATIGVAK	381
sp Q8N4A0 GALT4_HUMAN	13	22	LLLAFLT VAY	382
sp Q86VR7 VS10L_HUMAN	456	465	LLLPAVGPGH	383
sp Q9P2N4 ATS9_HUMAN	849	858	LLLQVLSVGK	384
sp Q15155 NOMO1_HUMAN	21	30	LLLSGVGPAH	385
sp Q5JPE7 NOMO2_HUMAN	21	30	LLLSGVGPAH	385
sp P69849 NOMO3_HUMAN	21	30	LLLSGVGPAH	385
sp Q8TCY9 URGCP_HUMAN	278	287	LLNAVLSPGH	386

sp Q9UQL6 HDAC5_HUMAN	854	863	LLQQKLNVGK	387
sp Q8TDG4 HELQ_HUMAN	294	303	LLSEEINVAK	388
sp Q9NX45 SOLH2_HUMAN	22	31	LLVGDVTVGY	389
sp Q99698 LYST_HUMAN	2747	2756	LLVHILSPA	390
sp Q9Y3R4 NEUR2_HUMAN	142	151	LTDAAGPAY	391
sp Q8TE58 ATS15_HUMAN	779	788	LTVEVLSVGK	392
sp P57729 RAB38_HUMAN	13	22	LVIGDLGVGK	393
sp Q13637 RAB32_HUMAN	29	38	LVIGELGVGK	394
sp Q9UN74 PCDA4_HUMAN	580	589	LVPWSVGVGH	395
sp Q9Y2L1 RRP44_HUMAN	667	676	MLLANISVAK	396
sp Q9BXL7 CAR11_HUMAN	32	41	MLSRYPNPAK	397
sp O76082 S22A5_HUMAN	349	358	MLWMTISVGY	398
sp Q8NA54 IQUB_HUMAN	318	327	MTDKLVTPGK	399
sp Q6ZMS4 ZN852_HUMAN	494	503	NLMNVLSVGK	400
sp Q4VNC0 AT135_HUMAN	722	731	NLQTAITVAK	401
sp Q9Y4K4 M4K5_HUMAN	562	571	NTLMSLSVGK	402
sp Q99502 EYA1_HUMAN	451	460	NVGGLLGPAK	403
sp O95677 EYA4_HUMAN	498	507	NVGGLLGPAK	403
sp Q9ULU8 CAPS1_HUMAN	868	877	NVGRLITPAK	404
sp P98155 VLDLR_HUMAN	314	323	NVNQCLGPGK	405
sp Q9NP78 ABCB9_HUMAN	524	533	NVSFSLSPGK	406
sp Q00688 FKBP3_HUMAN	161	170	PLSFKVGVGK	407
sp Q9NS98 SEM3G_HUMAN	141	150	PTCALITVGH	408
sp Q6UXF1 TM108_HUMAN	266	275	PTTTS LGPAK	409
sp Q9BRQ3 NUD22_HUMAN	20	29	QIQAE LSPA	410
sp Q96B70 LENG9_HUMAN	443	452	QLHPHLTVAK	411
sp Q96CT2 KLH29_HUMAN	294	303	QMLRTIGVGK	412
sp Q07157 ZO1_HUMAN	1515	1524	QTQKTVTPAY	413
sp Q13702 RAPSN_HUMAN	285	294	QVQALLGVAK	414
sp Q9BWT7 CAR10_HUMAN	37	46	RLARALNPAK	415
sp P56975 NRG3_HUMAN	501	510	RLGGIVGPAY	416
sp Q03001 DYST_HUMAN	546	555	RLTPSVTPAY	417
sp Q13769 THOC5_HUMAN	518	527	RLVKWVTVAH	418
sp Q96PU9 ODF3A_HUMAN	78	87	RTGKDLGPAY	418
sp O75192 PX11A_HUMAN	230	239	SIAGMITVAY	420
sp O60284 ST18_HUMAN	23	32	SLIQELSVAY	421
sp Q12797 ASPH_HUMAN	455	464	SLKNDLGVGY	422
sp Q9P0R6 GSKIP_HUMAN	109	118	SLLDTLSPAY	423
sp Q8NDA8 MROH1_HUMAN	168	177	SLLPVLGVAK	424

sp Q96LD8 SENP8_HUMAN	185	194	SLLQLLTPAY	425
sp Q8N1T3 MYO1H_HUMAN	992	1001	SLQFFISPGK	426
sp Q9UKZ4 TEN1_HUMAN	1923	1932	SLQTMLSVGY	427
sp Q15911 ZFH3_HUMAN	353	362	STANLIGPGH	428
sp Q86X29 LSR_HUMAN	326	335	STYHLSPAK	429
sp A2RUS2 DEND3_HUMAN	728	737	SVKTNLGVGK	430
sp Q13330 MTA1_HUMAN	558	567	SVLSSLTPAK	431
sp Q15788 NCOA1_HUMAN	389	398	SVNPSISPAH	432
sp P98168 ZXDA_HUMAN	743	752	SVSELLTPAK	434
sp Q6V0I7 FAT4_HUMAN	2206	2215	SVTGAITVAK	435
sp P21912 SDHB_HUMAN	252	261	TCPKGLNPGK	436
sp Q9Y6D6 BIG1_HUMAN	1025	1034	TIKTLITVAH	437
sp Q9Y6D5 BIG2_HUMAN	970	979	TIKTLITVAH	437
sp P27816 MAP4_HUMAN	565	574	TLANNVTPAK	438
sp Q9Y251 HPSE_HUMAN	82	91	TLARGLSPAY	439
sp Q96T83 SL9A7_HUMAN	154	163	TLKGEISPGK	440
sp Q6P1M9 ARMX5_HUMAN	332	341	TMIMGISPAY	441
sp Q17RG1 KCD19_HUMAN	264	273	TTCSPSPGK	442
sp O94851 MICA2_HUMAN	945	954	TVHPQLTVGK	443
sp P28332 ADH6_HUMAN	151	160	TVIKEISVAK	444
sp Q14973 NTCP_HUMAN	203	212	TVLSAINVGK	445
sp O15014 ZN609_HUMAN	716	725	TVNPALTPAK	446
sp Q9BSJ5 CQ080_HUMAN	588	597	TVPPCIGVAK	447
sp P11766 ADHX_HUMAN	150	159	TVVADISVAK	448
sp Q9UPT5 EXOC7_HUMAN	321	330	VCAADISPGH	449
sp Q8IV61 GRP3_HUMAN	221	230	VITKFINVAK	450
sp P27695 APEX1_HUMAN	206	215	VLCGDLNVAH	451
sp P01266 THYG_HUMAN	1156	1165	VLSRRVSPGY	452
sp Q96DY7 MTBP_HUMAN	633	642	VLTPELSPGK	453
sp Q13423 NNTM_HUMAN	1040	1049	VMKRSLGVGY	454
sp P10809 CH60_HUMAN	73	82	VTKDGVTVAK	455
sp O43598 DNPH1_HUMAN	94	103	VTQPSLGVGY	456
sp Q15772 SPEG_HUMAN	2927	2936	VTVQSLSPAK	457
sp P49281 NRAM2_HUMAN	509	518	VVAAVVSVAY	458
sp Q9NYS0 KBRS1_HUMAN	8	17	VVCGLLSVGK	459
sp O15091 MRPP3_HUMAN	406	415	VVIDGLNVAK	460
sp Q8N441 FGRL1_HUMAN	115	124	VVLDDISPGK	461

Table 16. Human peptides matching the L2 binding motif. Using a 20% cognate peptide reactivity as a cutoff for permissive amino acids at each position based on the results of the peptide library analysis, a decamer binding motif for L2 was generated. These motifs were searched in the UniProtKB human protein database using ScanProsite to identify peptides matching the motif.

UniProt Accession Number (Entry Name)	Start position	End position	Peptide Sequence	SEQ ID NO
sp O15018 PDZD2_HUMAN	2787	2796	AAEQAGIIEA	462
sp P23276 KELL_HUMAN	598	607	ACDNHALQEA	463
sp Q9P2V4 LRIT1_HUMAN	121	130	AFPPWAALRDA	464
sp Q9NVI7 ATD3A_HUMAN	135	144	AGECCALQEY	465
sp P15884 ITF2_HUMAN	651	660	AGPHPGMGDA	466
sp A0JNW5 UH1BL_HUMAN	1438	1447	AKAKMALAEA	467
sp O75533 SF3B1_HUMAN	706	715	ALAIAALAEA	468
sp P20711 DDC_HUMAN	216	225	AMRASALQEA	469
sp Q86Y56 DAAF5_HUMAN	592	601	AQSGPALGEA	470
sp P14679 TYRO_HUMAN	348	357	ASPLTGIADA	471
sp Q92508 PIEZ1_HUMAN	164	173	ASPTAGLQEA	472
sp Q9NVR2 INT10_HUMAN	500	509	ATCHFALGEY	473
sp Q8NFY4 SEM6D_HUMAN	369	378	CCAKHGLAEA	474
sp Q9UGU0 TCF20_HUMAN	1869	1878	CGRLYGLQEA	475
sp Q14674 ESPL1_HUMAN	647	656	CSALDAIREA	476
sp Q8IZJ3 CPMD8_HUMAN	1420	1429	CVALQALAEY	477
sp Q86X53 ERIC1_HUMAN	359	368	DAASAALADA	478
sp P35443 TSP4_HUMAN	497	506	DADRDGIGDA	479
sp Q99459 CDC5L_HUMAN	519	528	DARKQAIRDA	480
sp Q14674 ESPL1_HUMAN	206	215	DASGHGLNEA	481
sp P35442 TSP2_HUMAN	730	739	DFDKDGIGDA	482
sp Q6F5E8 CARL2_HUMAN	615	624	DISGNAMGDA	483
sp Q49MG5 MAP9_HUMAN	594	603	DKDKQAINCY	484
sp Q63HN8 RN213_HUMAN	2947	2956	DKEFFGLRDY	485
sp P04035 HMDH_HUMAN	115	124	DKELTGLNEA	486
sp Q8IWE5 PKHM2_HUMAN	976	985	DLPHTAIQEA	487
sp Q9C0D3 ZY11B_HUMAN	4	13	DQAGAAMEEA	488
sp P49747 COMP_HUMAN	397	406	DSDGDGIGDA	489
sp P21283 VATC1_HUMAN	343	352	DSSAAAIIDA	490
sp Q14839 CHD4_HUMAN	1267	1276	DTELQGMNEY	310
sp Q7Z3K3 POGZ_HUMAN	706	715	DTPPSALQEA	491

sp Q14686 NCOA6_HUMAN	934	943	DTRPAGLEEA	492
sp P49747 COMP_HUMAN	302	311	DVDRDGIGDA	493
sp P42684 ABL2_HUMAN	83	92	DVEPQALNEA	494
sp O43633 CHM2A_HUMAN	199	208	EAAASALADA	495
sp Q8TE73 DYH5_HUMAN	3285	3294	EAAKPALEEA	496
sp Q96JB1 DYH8_HUMAN	3149	3158	EAAKPALEEA	496
sp Q14CN2 CLCA4_HUMAN	463	472	EAQNNGLIDA	497
sp Q969T3 SNX21_HUMAN	344	353	EARLQALQEA	498
sp Q13415 ORC1_HUMAN	784	793	EFRRSGLEEA	499
sp Q96FB5 RRNAD_HUMAN	316	325	EGACHALEEY	500
sp P07711 CATL1_HUMAN	176	185	EGCNGGLMDY	501
sp P51649 SSDH_HUMAN	515	524	EGSKYGIDEY	502
sp P30876 RPB2_HUMAN	555	564	EISPAAIADA	503
sp Q0VDF9 HSP7E_HUMAN	149	158	EKQKNALGEA	504
sp Q96S94 CCNL2_HUMAN	299	308	EKRKHAIEEA	505
sp P10589 COT1_HUMAN	346	355	EKSQCALEEY	506
sp P24468 COT2_HUMAN	339	348	EKSQCALEEY	506
sp Q9P2L0 WDR35_HUMAN	800	809	EQANNAIGDY	507
sp Q8NF91 SYNE1_HUMAN	991	1000	EQEQQGLQEA	508
sp Q7Z5Q1 CPEB2_HUMAN	385	394	ESSVQALIDA	509
sp Q8NE35 CPEB3_HUMAN	494	503	ESSVQALIDA	509
sp Q17RY0 CPEB4_HUMAN	525	534	ESSVQALIDA	509
sp Q9UGU0 TCF20_HUMAN	1217	1226	ETDGHGLAEA	510
sp Q9BVI0 PHF20_HUMAN	858	867	ETRGSALDDA	511
sp Q96IS3 RAX2_HUMAN	155	164	FADGFALEEA	512
sp Q86YC2 PALB2_HUMAN	1016	1025	FAEVQGMQEA	513
sp Q17RP2 TIGD6_HUMAN	491	500	FGQLNGIDEY	514
sp Q9HBE1 PATZ1_HUMAN	280	289	FGSPGGLREA	515
sp Q53GS7 GLE1_HUMAN	538	547	FKEGMALEDY	516
sp Q8N998 CCD89_HUMAN	39	48	FKELDGLREA	517
sp Q9Y5W7 SNX14_HUMAN	217	226	FLQQAALIEY	518
sp Q9C0E2 XPO4_HUMAN	68	77	FQAATAIMEA	519
sp Q5T011 SZT2_HUMAN	2376	2385	GAARQALADA	520
sp A6NGH8 ANR61_HUMAN	191	200	GADVNAINEA	521
sp Q8IX07 FOG1_HUMAN	807	816	GAPAPALADY	522
sp Q9Y275 TN13B_HUMAN	96	105	GAPKAGLEEA	523
sp Q02080 MEF2B_HUMAN	151	160	GCDPSGLGEA	524
sp O15357 SHIP2_HUMAN	1190	1199	GGRASGLGEA	525
sp P00742 FA10_HUMAN	99	108	GKCKDGLGEY	526

sp Q9UJX5 APC4_HUMAN	380	389	GLDAAGIEEA	527
sp Q5SRE5 NU188_HUMAN	891	900	GNDAAAIRDA	528
sp Q8N7H1 CL061_HUMAN	114	123	GNSRSALQEA	529
sp O94761 RECQ4_HUMAN	1113	1122	GQEPGGMEDA	530
sp Q8IYF1 ELOA2_HUMAN	291	300	GQRVPALEEA	531
sp Q9NR16 C163B_HUMAN	1290	1299	GSALAALRDA	532
sp O94921 CDK14_HUMAN	173	182	GTPFTAIREA	533
sp Q96JH8 RADIL_HUMAN	194	203	GTPTPALGDA	534
sp O94933 SLIK3_HUMAN	781	790	GTQPPGMGEA	535
sp Q96Q40 CDK15_HUMAN	141	150	GVPFTAIREA	536
sp Q9ULL4 PLXB3_HUMAN	1278	1287	HKSKQALRDY	537
sp Q92539 LPIN2_HUMAN	363	372	HLPNAALAEA	538
sp O95613 PCNT_HUMAN	1152	1161	HSERGALQDA	539
sp Q9BQS2 SYT15_HUMAN	112	121	HTSSGGLGDA	540
sp P78562 PHEX_HUMAN	644	653	IADNGGLREA	541
sp Q68CZ6 HAUS3_HUMAN	71	80	ILEGAALDEA	542
sp P05120 PAI2_HUMAN	323	332	ILRSMGMEDA	543
sp Q96PC5 MIA2_HUMAN	41	50	INRVSAMRDY	544
sp Q8NF91 SYNE1_HUMAN	654	663	IQQHTAMNDA	545
sp Q93073 SBP2L_HUMAN	746	755	IQSKGGLDEA	546
sp Q9H9A5 CNO10_HUMAN	204	213	KAESGALIEA	547
sp P46821 MAP1B_HUMAN	1217	1226	KFSRSALRDA	548
sp P24071 FCAR_HUMAN	46	55	KIQCQAIREA	549
sp A0AUZ9 KAL1L_HUMAN	964	973	KKQSDGMEEY	550
sp Q96CP6 ASTRA_HUMAN	513	522	KNSWSGIEDY	551
sp Q8IY33 MILK2_HUMAN	457	466	KQALSALEEA	552
sp Q9BXF6 RFIP5_HUMAN	506	515	KSSWFGLREA	553
sp Q86WX3 AROS_HUMAN	54	63	KVPKSALDEY	554
sp P23610 HAP40_HUMAN	178	187	LAALQALGEA	555
sp Q9H8S5 CNTD2_HUMAN	59	68	LARPPGLEEA	556
sp A6NK58 LIPT2_HUMAN	126	135	LCELQGLQDA	557
sp Q13946 PDE7A_HUMAN	177	186	LFSLHGLIEY	558
sp Q6ZW49 PAXI1_HUMAN	607	616	LGCVFAIADY	559
sp Q12882 DPYD_HUMAN	67	76	LGERGALREA	560
sp A3KFT3 OR2M5_HUMAN	151	160	LGSMDAIIDA	561
sp Q6P0Q8 MAST2_HUMAN	1583	1592	LGSPQAIEEA	562
sp Q96R28 OR2M2_HUMAN	151	160	LGSTDGIIDA	563
sp Q8NG81 OR2M7_HUMAN	151	160	LGSTDGIIDA	563
sp Q96AY4 TTC28_HUMAN	1680	1689	LKASAALGEA	564

sp Q9UID3 VPS51_HUMAN	385	394	LLAAAGLADA	565
sp A6NKF1 SAC31_HUMAN	327	336	LLALDGLREA	566
sp Q6ZQQ6 WDR87_HUMAN	485	494	LLCSYGMDDY	567
sp Q99727 TIMP4_HUMAN	20	29	LLRPPGLGEA	568
sp Q5TF39 MFS4B_HUMAN	428	437	LLSSSGLNEY	569
sp P45379 TNNT2_HUMAN	109	118	LNELQALIEA	570
sp P34897 GLYM_HUMAN	197	206	LNPKTGLIDY	571
sp O75461 E2F6_HUMAN	147	156	LSDLSAMEDA	572
sp Q8N393 ZN786_HUMAN	770	779	LSQLFAMIEA	573
sp O95620 DUS4L_HUMAN	301	310	LSSTSIIIDY	574
sp P01303 NPY_HUMAN	19	28	LVCLGALAEA	575
sp Q9NU02 ANKE1_HUMAN	577	586	LVESGALIDA	576
sp P0C1H6 H2BFM_HUMAN	1	10	MAAASAMAEA	577
sp Q9H2Z4 NKX24_HUMAN	163	172	MGSLTGIADA	578
sp Q9BVS5 TR61B_HUMAN	210	219	MLRRPALEDY	579
sp Q9BRK5 CAB45_HUMAN	310	319	MNEYNALNEA	580
sp O43681 ASNA_HUMAN	136	145	MSAFPGIDEA	581
sp P48200 IREB2_HUMAN	96	105	MVDFAAMREA	582
sp Q8IZY2 ABCA7_HUMAN	1703	1712	MVRNQAMADA	583
sp A6NIE6 RN3P2_HUMAN	233	242	NASRQGIEDA	584
sp Q9NYV6 RRN3_HUMAN	257	266	NASRQGIEDA	584
sp Q8WZ42 TITIN_HUMAN	21672	21681	NFRISAINDA	585
sp Q96A65 EXOC4_HUMAN	818	827	NKDISAIEEA	586
sp O95359 TACC2_HUMAN	991	1000	NKSQQALADA	587
sp O43895 XPP2_HUMAN	201	210	NQPIYALQEA	588
sp O14647 CHD2_HUMAN	1179	1188	NSCVSAMQEY	589
sp P53597 SUCA_HUMAN	123	132	PFAAAAINEA	590
sp Q96EP1 CHFR_HUMAN	451	460	PGAPQALGDA	591
sp O15067 PUR4_HUMAN	775	784	PGEGAALADA	592
sp Q5M9Q1 NKAPL_HUMAN	302	311	PGEGAAMAAY	593
sp Q8N5F7 NKAP_HUMAN	315	324	PGEGAAMAAY	593
sp Q9H0D2 ZN541_HUMAN	462	471	PGPGGGLEDA	594
sp Q9HBR0 S38AA_HUMAN	561	570	PGQAQALEEA	595
sp Q01955 CO4A3_HUMAN	1203	1212	PGRKGAMGDA	596
sp Q9Y4K3 TRAF6_HUMAN	71	80	PICLMALREA	597
sp Q5VWJ9 SNX30_HUMAN	239	248	PLEFAAIGDY	598
sp Q63HN8 RN213_HUMAN	173	182	PLQAQALGEA	599
sp P0C2L3 F163B_HUMAN	146	155	PNRLSAMREA	600
sp Q9NSE4 SYIM_HUMAN	247	256	PSSRTALAEA	601

sp Q9H7M6 ZSWM4_HUMAN	242	251	PTAGAGIEDA	602
sp O00159 MYO1C_HUMAN	834	843	PTPPPALREA	603
sp Q9P1Y6 PHRF1_HUMAN	1217	1226	PTRLPALGEA	604
sp Q96RL7 VP13A_HUMAN	1176	1185	QAAKQALAEA	605
sp Q9Y623 MYH4_HUMAN	896	905	QAEADALADA	606
sp Q8TF72 SHRM3_HUMAN	1080	1089	QFQQSALADY	607
sp Q8N205 SYNE4_HUMAN	282	291	QGRGQGLEEA	608
sp Q6ZR54 YN009_HUMAN	171	180	QGSALAALGEA	609
sp Q8N7Z5 ANR31_HUMAN	1252	1261	QKQKSALDEA	610
sp Q4G0P3 HYDIN_HUMAN	2481	2490	QLPPAGMEEA	611
sp O95479 G6PE_HUMAN	127	136	QLQHAGLREA	612
sp Q6WRI0 IGS10_HUMAN	697	706	QLRTSALMEA	613
sp Q9HAV4 XPO5_HUMAN	642	651	QMEKCALMEA	614
sp Q9H254 SPTN4_HUMAN	1600	1609	QSAWAGLREA	615
sp A8K7I4 CLCA1_HUMAN	462	471	QVQNNGLIDA	616
sp Q8WZ42 TITIN_HUMAN	9235	9244	QVRKAGMNDA	617
sp Q9P225 DYH2_HUMAN	83	92	RAALTGLADA	618
sp P57082 TBX4_HUMAN	15	24	RAPGPALGEA	619
sp O96024 B3GT4_HUMAN	91	100	RASWGGLREA	620
sp Q8IUA7 ABCA9_HUMAN	768	777	RCSNQGIEDY	621
sp Q7Z3C6 ATG9A_HUMAN	231	240	RFRLPGLGEA	622
sp Q8WUT4 LRRN4_HUMAN	184	193	RGAQGGIAEA	623
sp Q8IUL8 CILP2_HUMAN	966	975	RGQLYGLRDA	624
sp O43157 PLXB1_HUMAN	1513	1522	RKSKQALRDY	625
sp Q5TH69 BIG3_HUMAN	1605	1614	RLACCALQDA	626
sp Q9C0G6 DYH6_HUMAN	1294	1303	RLCKAAIADY	627
sp Q9Y6D6 BIG1_HUMAN	208	217	RMENQALQEA	628
sp P30679 GNA15_HUMAN	186	195	RMPTTGINEY	629
sp Q96NK8 NDF6_HUMAN	104	113	RNRMHGLNDA	630
sp O75154 RFIP3_HUMAN	645	654	RSSSMGLQEY	631
sp Q9HD90 NDF4_HUMAN	97	106	RTRMHGLNDA	632
sp Q96RL1 UIMC1_HUMAN	650	659	RVSPSPGMEEA	633
sp P29992 GNA11_HUMAN	183	192	RVPTTGIIIEY	634
sp O95837 GNA14_HUMAN	179	188	RVPTTGIIIEY	634
sp P50148 GNAQ_HUMAN	183	192	RVPTTGIIIEY	634
sp P84243 H33_HUMAN	87	96	SAAIGALQEA	635
sp P0DPK5 H3X_HUMAN	87	96	SAAIGALQEA	635
sp P0DPK2 H3Y_HUMAN	87	96	SAAIGALQEA	635
sp Q6NXT2 H3C_HUMAN	86	95	SAAVGALQEA	636

sp Q96JG9 ZN469_HUMAN	3262	3271	SASATALADA	637
sp Q2NKKX8 ERC6L_HUMAN	922	931	SASHSALQDA	638
sp P54750 PDE1A_HUMAN	161	170	SFDVFALNEA	639
sp Q8WXE1 ATRIP_HUMAN	369	378	SFSLSALREA	640
sp P39059 COFA1_HUMAN	243	252	SGETSGLQEA	641
sp Q9ULT8 HECD1_HUMAN	372	381	SKDTDALIDA	642
sp Q96H22 CENPN_HUMAN	308	317	SLAPAGIADA	643
sp Q6ZS17 RIPR1_HUMAN	1005	1014	SLRQPGLAEA	644
sp P15172 MYOD1_HUMAN	204	213	SNCSDGMMMDY	645
sp Q16695 H31T_HUMAN	87	96	SSAVMALQEA	646
sp P68431 H31_HUMAN	87	96	SSAVMALQEA	646
sp Q71DI3 H32_HUMAN	87	96	SSAVMALQEA	646
sp O95428 PPN_HUMAN	391	400	SSDGAGIQEA	647
sp Q96BP3 PPWD1_HUMAN	240	249	SSDKSGMIEY	648
sp Q9BR61 ACBD6_HUMAN	105	114	SSPSQAMQEY	649
sp O75694 NU155_HUMAN	13	22	STSAAALQEA	650
sp O00750 P3C2B_HUMAN	225	234	SVDYDGINDA	651
sp Q9HCU0 CD248_HUMAN	665	674	TAAPTALGEA	652
sp Q9NSE4 SYIM_HUMAN	393	402	TAPAHGMEDY	653
sp P37837 TALDO_HUMAN	28	37	TGDFHAIDEY	654
sp O15050 TRNK1_HUMAN	1979	1988	TGQLSGIAEA	655
sp Q9H0X9 OSBL5_HUMAN	667	676	TQEKFALEEA	656
sp Q58EX7 PKHG4_HUMAN	885	894	TQELSALREA	657
sp A6NMB9 FIGL2_HUMAN	535	544	TSRPAALDEA	658
sp Q7Z6P3 RAB44_HUMAN	133	142	TTSFPALEEA	659
sp Q9BTD8 RBM42_HUMAN	253	262	VAAAAGLEEA	660
sp Q6ZVN8 RGMC_HUMAN	375	384	VAAQAALEDA	661
sp Q6UXB0 F131A_HUMAN	171	180	VAEQFAIAEA	662
sp Q9C026 TRIM9_HUMAN	317	326	VAQCDALIDA	663
sp O95450 ATS2_HUMAN	158	167	VGDVAGLAEA	664
sp Q99437 VATO_HUMAN	158	167	VGSGAALADA	665
sp Q9BXW9 FACD2_HUMAN	680	689	VKALYGLEEY	666
sp Q8IVU9 CBCO1_HUMAN	59	68	VKQANAIIIDY	667
sp Q9UIV8 SPB13_HUMAN	299	308	VLAAMGMGDA	668
sp Q13342 SP140_HUMAN	248	257	VLESNGMIDA	669
sp Q9BZJ7 GPR62_HUMAN	39	48	VL RTPGLRDA	670
sp O94967 WDR47_HUMAN	118	127	VQCLHALEEY	671
sp Q9UBC5 MYO1A_HUMAN	232	241	VSRVDGMDDA	672
sp Q8NCK7 MOT11_HUMAN	282	291	VVAVAAMGDA	673

sp P21399 ACOC_HUMAN	94	103	VVDFAAMRDA	674
sp Q9NTG1 PKDRE_HUMAN	782	791	WQANQALQEY	675
sp Q9NUU6 OTULL_HUMAN	210	219	WTEFNGIRDY	676
sp Q8N239 KLH34_HUMAN	405	414	WTEVPAMREA	678
sp Q9BXL5 HEMGN_HUMAN	371	380	YQEIPGLEEY	679
sp Q9Y646 CBPQ_HUMAN	213	222	YSPHTGIQEY	680

Evaluation of scDb in mouse models

To determine whether the L2-U scDb could control tumor growth *in vivo*, HL-60 leukemia cells (*NRAS* Q61L/WT) and human T cells were intravenously injected into NSG mice to establish widespread leukemic infiltrates. As controls, HL-60 cells engineered to harbor Q61H instead of Q61L/WT alleles were used in a separate group of mice. Bioluminescence established tumor uptake, and mice were randomized to receive the L2-U scDb or a control scDb through intraperitoneal 14-day infusion pumps. The L2-U scDb slowed the growth of the Q61L leukemic cells (Fig. 64C), but not the HL-60 Q61H cells (Fig. 64D). Though the tumor growth retardation was significant ($p < 0.05$, multiple t-tests with Bonferroni-Dunn correction), the effect size was modest. Therapeutic effects were not assessed at later time points because of the lifetime of the pumps. No substantial changes in body weight were noted in any of the V2-U or L2-U scDb treated mice (Fig. 65).

15

Together these results demonstrate that highly specific bispecific antibodies can be generated against pHLA complexes resulting from common mutations occurring in cancer cells. The format and configuration of the bispecific antibodies developed here are highly specific and sensitive scDbs against protein products containing mutations occurring in cancer cells.

20

Materials and Methods

Study Design

The objective of this study was to generate therapeutic agents that target common mutations in *RAS* genes. This was accomplished by using phage display to identify scFvs specific to MANAs that had been confirmed to be presented via mass spectrometry. These

25

scFvs were grafted into an optimized bispecific antibody format, the scDb. The scDbs were then shown to mediate MANA-specific T cell activation and target cell cytotoxicity in overexpression and endogenous-level expression model systems. All data presented were representative of data collected during this study. All experiments were performed in triplicate with three technical replicates unless otherwise noted. All experiments were performed in a way to minimize confounding variables, such as plate layout effects.

Plasmids

Plasmids encoding KRAS (isoform B), HRAS (isoform 1), and NRAS variants (WT and mutant) and HLA class I alleles A*01:01 and A*03:01 were synthesized and cloned into pcDNA3.1 by GeneArt (Thermo Fisher Scientific, Waltham, MA) or synthesized into gBlocks (IDT, Coralville, Iowa) and assembled into pcDNA3.4 using NEBuilder® HiFi DNA Assembly Cloning Kit (NEB, Ipswich, MA).

Cell Lines and Primary Cells

All cells were grown at 37°C under 5% CO₂. HEK293F cells (Thermo Fisher Scientific) were cultured in FreeStyle Expression media. T2A3 cells (a kind gift from Eric Lutz and Elizabeth Jaffee, JHU) were cultured in RPMI-1640 (ATCC, Manassas, VA) with 10% HyClone FBS (GE Healthcare, Chicago, IL), 1% Penicillin-Streptomycin (Thermo Fisher Scientific), 500 µg/mL Geneticin (Thermo Fisher Scientific), and 1X Non-Essential Amino Acids (Thermo Fisher Scientific). COS-7, NCI-H441, CFPAC-1, NCI-H358, and HCT 116 cells (all from ATCC, Manassas, VA) were cultured in McCoy's 5A (Modified) (Thermo Fisher Scientific) with 10% HyClone FBS and 1% Penicillin-Streptomycin. Jurkat (Clone E6-1, ATCC), COLO 741 (Sigma-Aldrich, St. Louis, MO), and SW780 (ATCC) cells were cultured in RPMI-1640 with 10% HyClone FBS and 1% Penicillin-Streptomycin. KMS-21-BM (JCRB Cell Bank, Osaka, Japan) cells were cultured in RPMI-1640 with 20% HyClone FBS and 1% Penicillin-Streptomycin. A-427, Hep G2, Hs 695T, SK-MES-1 (all from ATCC) cells were cultured in Eagle's Minimum Essential Medium (ATCC) with 10% HyClone FBS and 1% Penicillin-Streptomycin. SigM5 (DSMZ) and HL-60 (ATCC) cells were cultured in Iscove's Modified Dulbecco's Medium (ATCC) with 20% HyClone FBS and 1% Penicillin-Streptomycin. RD (ATCC) cells were cultured in Dulbecco's Modified Eagle's Medium (ATCC) with 10% HyClone FBS and 1% Penicillin-Streptomycin. Hs 578T

(ATCC) cells were cultured in Dulbecco's Modified Eagle's Medium (ATCC) with 10% HyClone FBS, 1% Penicillin-Streptomycin, and 0.01 mg/ml bovine insulin (Sigma-Aldrich).

Peripheral blood cells were obtained from healthy volunteer donors or purchased as leukapheresis samples (Stem Cell Technologies, Vancouver, BC). PMBCs were purified by
5 density gradient centrifugation with Ficoll Paque Plus (GE Healthcare). T cells were expanded from PBMCs with addition of the anti-human CD3 antibody (clone OKT3, BioLegend, San Diego, CA) at 15 ng/mL, or with Human T-Activator CD3/CD28 Dynabeads (Thermo Fisher Scientific) at a 1:5 bead:cell ratio for three days, after which beads were removed with a magnet and the medium was changed. T cells were cultured in
10 RPMI-1640 with 10% HyClone FBS, 1% Penicillin-Streptomycin, 100 IU/mL recombinant human IL-2 (aldesleukin, Prometheus Therapeutics and Diagnostics, San Diego, CA), and 5 ng/mL recombinant human IL-7 (BioLegend). The culture medium was changed every 3-4 days and cells were maintained at ~1 million cells/mL.

To generate dendritic cells, monocytes were negatively isolated from PBMCs using
15 microbeads (Miltenyi) and cultured in Mo-DC differentiation media (Miltenyi) for 5 days to induce differentiation into immature dendritic cells. To generate mature dendritic cells, immature dendritic cells were cultured with 0.5 mg/mL CD40 ligand oligomer (Enzo) in Mo-DC differentiation media for 2 more days.

Phage Display Library Construction

20 All cloning was modeled in SnapGene (GSL Biotech LLC, San Diego, CA). Oligonucleotides were synthesized by GeneArt (Thermo Fisher Scientific) using trinucleotide mutagenesis (TRIM) technology. The oligonucleotides were incorporated into the pADL-10b phagemid (Antibody Design Labs, San Diego, CA) (Fig. 66).

Ten ng of the ligation product was mixed on ice with 10 µL of electrocompetent
25 SS320 cells (Lucigen, Middleton, WI) and 14 µL of double-distilled water. This mixture was electroporated (200 ohms, 25 microFarads, 1.8 kV) using a Gene Pulser electroporation system (Bio-Rad, Hercules, CA) and allowed to recover in Recovery Media (Lucigen) for 45 minutes at 37°C. Cells transformed with 60 ng of ligation product were pooled and plated on a 24-cm x 24-cm plate containing 2xYT medium supplemented with carbenicillin (100
30 µg/mL) and 2% glucose. Cells were grown at 37°C for 6 hours and placed at 4°C overnight.

To determine the transformation efficiency for each series of electroporations, aliquots were titered by serial dilution. Cells grown on plates were scraped into 850 mL of 2xYT medium with carbenicillin (100 µg/mL) plus 2% glucose for a final OD₆₀₀ of 5-15. Two mL of the 850 mL culture were taken and diluted ~1:200 to reach a final OD₆₀₀ of 0.05-0.07. To the
 5 remaining culture, 150 mL of sterile glycerol was added before snap freezing to produce glycerol stocks. The diluted bacteria were grown to an OD₆₀₀ of 0.3-0.5, transduced with M13K07 helper phage at an MOI of 4 (Antibody Design Labs) and shaken at 37°C for 1 hour. The culture was centrifuged and the cells were re-suspended in 2xYT medium with carbenicillin (100 µg/mL), kanamycin (50 µg/mL), and IPTG (50 mM, Thermo Fisher
 10 Scientific) and grown overnight at 30°C for phage production. The following morning, the bacterial culture was aliquoted into 50 mL Falcon tubes and pelleted twice at high speed to obtain clarified supernatant. The phage-laden supernatant was precipitated on ice for 40 minutes with a 20% PEG-8000 / 2.5 M NaCl solution at a 1:4 ratio of PEG/NaCl:supernatant. After precipitation, phage were centrifuged at 12,000 g for 40 minutes and re-suspended in
 15 1x TBS (25 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 2 mM EDTA. Phage from multiple tubes were pooled and re-precipitated to achieve a higher concentration. Final phage were re-suspended in 1x TBS, 2 mM EDTA, and 1x Complete Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). The total number of transformants obtained was determined to be 3.6×10^{10} . The library was aliquoted and stored in 15% glycerol at -80°C
 20 and in 50% glycerol at -20°C.

DNA from the library was amplified using the following primers (Forward: CGACGTAAAACGACGGCCAGTNNNNNNNNNNNNNNNNNNCGTGCAGAGGATACAGC
 AGTG (SEQ ID NO:681), Reverse:
 CACACAGGAAACAGCTATGACCATGCTAACGGTAACCAGGGTGCCCTG (SEQ
 25 ID NO:682)) which flank the CDR-H3 region. (All oligonucleotide sequences listed in this manuscript begin with the most 5' nucleotide.) The sequences at the 5'-ends of these primers incorporated molecular barcodes to facilitate unambiguous enumeration of distinct phage sequences as well as universal primer sites. The protocols for PCR-amplification and sequencing were described elsewhere (Kinde et al., *Proc Natl Acad Sci U S A* 108:9530-9535
 30 (2011)). Sequences were processed and translated using a custom SQL database and both the nucleotide sequences and amino acid translations were assessed.

Peptides and pHLAs

Peptides (Table 11) were synthesized at a purity of >90% by Peptide 2.0 (Chantilly, VA) or ELIM Biopharm (Hayward, CA), with the exception of the crude peptides that were used for the positional scanning library. Peptides were re-suspended in DMF at 10 mg/mL and stored at -80°C. pHLAs were synthesized by refolding recombinant HLA-A*01:01 (HLA-A1) or HLA-A*03:01 (HLA-A3) with peptide and beta-2 microglobulin, purified by gel-filtration, and biotinylated (Fred Hutchinson Immune Monitoring Lab, Seattle, WA; or Baylor MHC Tetramer Production Lab, Houston, TX). These pHLA were confirmed to be folded prior to selection via ELISA using the W6/32 antibody (BioLegend, San Diego, CA), which recognizes only folded HLA. Blast peptides (Table 14) were synthesized as described above, re-suspended in DMF at 10 mg/mL and stored at -80°C. Cognate peptide reactivity search of the UniProtKB human protein database using ScanProsite was performed using binding motifs with a 20% parental peptide IFN γ value as a cutoff. The V2 motif was {FWDY}-{ILMVTC}-{RE}-{ILV}-x-[ILV]-[GNST]-[VP]-[AG]-[HKY] (SEQ ID NO:683). The L2 motif was x-{PWRHDEY}-{APRDEQSC}-{DE}-{AMFPGHDNQSTYC}-[AG]-[ILM]-[AIMGRDENQ]-[DE]-[AY] (SEQ ID NO:684).

Selection of mutant pHLA-specific phage clones

scFv-bearing phage specific to the RAS G12V[7-16] “G12V” pHLA-A3 and RAS Q61H, Q61L, Q61R, referred to collectively as “Q61X”, in pHLA-A1 were identified using methods similar to those described elsewhere (Skora et al., *Proc Natl Acad Sci U S A* 112:9967-9972 (2015); and Miller et al., *J Biol Chem* 294:19322-19334 (2019)). The phage display library was regrown within a week of starting the selection process. A colony of phage-competent SS320 cells (Lucigen, Middleton, WI) was inoculated in 2xYT medium (Sigma-Aldrich, St. Louis, MO) supplemented with tetracycline (20 μ g/mL) and cultured at 37°C overnight, then grown to 2 L of mid-log phase (OD₆₀₀ of 0.3-0.5) bacteria. Bacteria were transduced with the phage library at an MOI of 0.5 and M13K07 helper phage (Antibody Design Labs, San Diego, CA) at an MOI of 4 along with the addition of 2% (W/V) glucose (Sigma-Aldrich, St. Louis, MO) and shaken, not stirred, for 1 hour at 37°C. The cells were pelleted and re-suspended in 2xYT medium with carbenicillin (100 μ g/mL), kanamycin (50 μ g/mL), and 50 μ M IPTG and subsequently shaken and grown overnight at

30°C for phage production. The following morning, the bacterial culture was aliquoted into 50 mL Falcon tubes and pelleted twice by centrifugation at 12,000 x g to obtain clarified supernatant. The phage-laden supernatant was precipitated on ice for 40 minutes with a 20% PEG-8000 / 2.5 M NaCl solution at a 1:4 ratio of PEG/NaCl:supernatant. After precipitation, phage were pelleted by centrifugation at 12,000 x g for 40 minutes and re-suspended in 1 mL of 1x TBS with 2 mM EDTA, 0.1% sodium azide, and 1x Complete Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO).

For the G12V pHLA-A3 target MANA, the selection scheme involved an enrichment phase (one round), a competition phase (up to three rounds), and a final selection phase (two rounds). The biotinylated pHLA were incubated with 25 µL of M-280 streptavidin Dynabeads (Invitrogen, Thermo Fisher Scientific) or 100 µL of streptavidin-coated agarose beads (Novagen EMD Millipore, Burlington, MA) per 1 µg of pHLA in blocking buffer (phosphate-buffered saline or PBS, 0.5% BSA, 0.1% sodium azide) for 1 hour at room temperature (RT). After the initial incubation, the complexes were washed and re-suspended in 100 µL of blocking buffer. During the enrichment phase (Round 1), approximately 4 x 10¹² phage, representing ~100-fold coverage of the library, were negatively selected overnight at 4°C against 500 µL unconjugated washed Dynabeads, 500 µg free streptavidin protein (RayBiotech, Norcross, GA), and 3 µg heat-denatured, allele-matched HLA conjugated to Dynabeads. This step was designed to remove phage recognizing Dynabeads, streptavidin or denatured HLA-A3. After negative selection, beads were isolated with a DynaMag-2 magnet (Life Technologies, Carlsbad, CA) and the supernatant containing unbound phage was used for positive selection by incubation with 0.5 µg G12V pHLA-A3 conjugated to Dynabeads for 1 hour at RT. The beads were washed 10 times with 1x TBS with 0.5% Tween-20 using the DynaMag-2 magnet, and the phage were eluted from the beads by re-suspension in 1 mL of 0.2 M glycine, pH 2.2. After a 10-minute incubation, the solution was pH-neutralized by the addition of 150 µL of 1 M Tris, pH 9.0. This neutralized solution was used to transduce 10 mL cultures of mid-log-phase SS320s to which M13K07 helper phage (MOI of 4) and 2% glucose was added. Bacteria were then incubated as described above and the phage precipitated the next morning with PEG/NaCl.

During the competitive phase (Rounds 2-4), the amount of input phage used in each round was decreased to 5%, 1%, and 0.1% of the total precipitated phage from the previous

round, respectively. These phage were subjected to negative selection against 1 μ g heat-denatured HLA-A3, 1 μ g total of unrelated pHLA-A3, and 20 μ g free streptavidin for 1 hour at RT. After negative selection, beads were isolated with a DynaMag-2 magnet and the unbound phage were used for positive selection. This was accomplished by simultaneously
5 co-incubating phage with 0.5 μ g G12V pHLA-A3 conjugated to the magnetic Dynabeads and corresponding 1 μ g G12WT pHLA-A3 conjugated to streptavidin-coated agarose beads as competitor. Prior to elution, beads were washed 10 times in 1 mL 1x TBS with 0.5% Tween-20. Phage were eluted from magnetic Dynabeads and used to transduce mid-log phase SS320 cells as described above.

10 During the final selection phase, phage resulting from rounds 2, 3, and 4 were separately subjected to additional, more stringent rounds of selection. 0.1% of the precipitated phage from these rounds underwent two negative selections against 0.5 μ g G12WT pHLA-A3, followed by a positive selection against 0.5 μ g of G12V pHLA-A3. The beads were washed 10 times in 1 mL 1x TBS with 0.5% Tween-20, and phage were eluted
15 and used to transduce mid-log phase SS320 cells as described above. The final selection steps described above was repeated a second time, thus the phage underwent a total of four to six total rounds of negative/positive selection.

scFv-bearing phage specific to RAS Q61X-HLA-A1 MANA targets were selected as described above with the following differences. The Q61X pHLA-A1 panning scheme
20 involved one round of enrichment and four rounds of more stringent selection. In the enrichment round, $\sim 2.6 \times 10^{13}$ phage, representing ~ 720 -fold coverage of the library, were negatively selected against 1 mL unconjugated washed Dynabeads and 1 mg free streptavidin protein. This was followed by positive selection of the unbound phage using 2 μ g of the mutant Q61X pHLA-A1. For the four subsequent selection rounds, 10%, 1%, 0.1%, and
25 0.02% of the phage from the previous round were used as input for panning, respectively. In each of these rounds, phage were negatively selected using 2 μ g Q61WT pHLA-A1, 2 μ g unrelated pHLA-A1, and 5×10^8 cells from HLA-A1+ cell lines lacking the *RAS* mutation of interest. Unbound phage were used for positive selection with 1 μ g, 0.5 μ g, 0.5 μ g, and 0.25 μ g Q61X pHLA-A1 in the four sequential rounds, respectively.

30 To obtain monoclonal phage, individual colonies of SS320 cells transduced with a limiting dilution of phage were inoculated into 200 μ L of 2xYT medium containing 100

μg/mL carbenicillin and 2% glucose and grown for 3 hours at 37°C. The cells were then transduced with 1.6×10^7 M13K07 helper phage and incubated for 1 hour at 37°C with shaking. The cells were pelleted, re-suspended in 300 μL of 2xYT medium containing carbenicillin (100 μg/mL), kanamycin (50 μg/mL), and 50 μM IPTG, and grown overnight at
 5 30°C for phage production. Cells were pelleted and the phage-laden supernatant was used for downstream analysis.

PCR and Sanger Sequencing

Monoclonal phage DNA was PCR amplified using 1 μL of monoclonal phage supernatant in a reaction with primers flanking the CDRs
 10 (Forward: GGCCATGGCAGATATTCAGA (SEQ ID NO:198), Reverse: CCGGGCCTTTATCATCATC (SEQ ID NO:199)) and Q5 Hot Start High-Fidelity 2X Master Mix (New England BioLabs). The PCR product was Sanger-sequenced by Genewiz (South Plainfield, NJ). Sequences flanking the CDRs were trimmed using DNA Baser Sequence Assembler v4 (Arges, Romania) and the sequences spanning the CDRs were
 15 clustered using the CD-HIT Suite. Colonies containing unique phage clones were selected for further assays.

ELISAs

Streptavidin-coated, 96-well plates (R&D Systems, Minneapolis, MN) were coated with 50 ng of biotinylated pHLA-A3 or pHLA-A1 (unless otherwise specified) or 25 ng of
 20 biotinylated recombinant heterodimeric CD3ε/δ (Abcam, Cambridge, MA) in 50 μL of blocking buffer (PBS with 0.5% BSA, 2 mM EDTA, and 0.1% sodium azide) at 4°C overnight. Plates were washed with 1x TBST (1x TBS + 0.05% Tween-20) using a BioTek 405 TS plate washer (BioTek, Winooski, VT).

The phage clones resulting from the RAS G12V pHLA-A3 panning were
 25 characterized via monoclonal ELISA, where individual monoclonal phage clones were separately interrogated for their binding to G12V pHLA-A3 and G12WT pHLA-A3 via ELISA. 50 μL of phage supernatant was added to washed streptavidin ELISA plates coated with either G12V or G12WT pHLA-A3. Plates were incubated for 2 hours at RT and then washed 6 times. The bound phage were then incubated with 50 μL of rabbit anti-fd/M13
 30 bacteriophage antibody (Novus Biologicals, Abingdon, UK) diluted 1:3000 in 1x TBST for 1

hour at RT, followed by washing and incubation with 50 μ L of goat anti-rabbit HRP (Thermo Fisher Scientific) diluted 1:10,000 in 1x TBST for 1 hour at RT. After washing, 50 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (BioLegend) was added to each well and the reaction was quenched with 1 N sulfuric acid (Fisher Scientific, Thermo Fisher Scientific). Absorbance at 450 nm was measured with a Synergy H1 Multi-Mode Reader (BioTek).

ELISA with purified scFvs, scDBs, and other bispecific antibody formats was performed essentially as above, with serial dilutions of the recombinant protein of interest incubated for 1 hour at RT, followed by incubation with 1 μ g/mL recombinant protein L (Pierce, Thermo Fisher) for 1 hour at RT, followed by incubation with anti-protein L HRP (Abcam). Plates were washed, exposed, and read as described above. pHLA titration ELISAs assessing the binding of the scFvs (at a fixed concentration) to the mutant and WT pHLA (at varying concentrations) were performed by AxioMx Inc (Abcam).

Sandwich ELISAs were performed by coating biotinylated pHLA-A3 on a streptavidin plate and incubating with scDBs as described above, followed by incubation with recombinant heterodimeric CD3 ϵ / δ protein containing a human Fc domain at 1 μ g/mL for 1 hour at RT, followed by detection with anti-human Fc HRP (Abcam) at 1:10,000 for 1 hour at RT. Plates were washed, exposed, and read as described above.

Flow Cytometry

For peptide pulsing of cells, cells were washed once with PBS and once with RPMI-1640 containing 1% Penicillin-Streptomycin without serum. The cells were then incubated at $5 \times 10^5 - 1 \times 10^6$ cells per mL in serum-free RPMI-1640 containing 50 μ g/mL or specified concentration of peptide and 10 μ g/mL human beta-2 microglobulin (ProSpec, East Brunswick, NJ) for 4 hours at 37°C. Prior to staining, cells were spun and re-suspended in cold stain buffer (PBS containing 0.5% BSA, 2 mM EDTA, and 0.1% sodium azide).

The phage clones resulting from the RAS Q61X pHLA-A1 selection were characterized via flow cytometry, where individual monoclonal phage clones were separately interrogated for their binding to mutant Q61X and Q61WT peptide-pulsed SigM5 cells. Monoclonal phage were grown and sequenced as described above. Phage supernatant from representative wells of each unique clone was selected for flow cytometry analysis. In each

well of a deep 96-well 2 mL plate, 50 μ L of monoclonal phage was incubated with 2.5×10^5 peptide-pulsed SigM5 cells in 50 μ L of stain buffer. Plates were incubated on ice for 1 hour, followed by washing with 1 mL of stain buffer. Cells were then stained with 1 μ g of rabbit anti-M13 antibody (Novus Biologicals), washed, stained with anti-rabbit-PE (BioLegend),
 5 incubated with an additional 100 μ L of LIVE/DEAD Fixable Near-IR dye diluted 1:1000 in PBS for 10 minutes at RT in the dark, followed by washing in stain buffer before analysis. Stained cells were analyzed using an Intellicyt iQue3 flow cytometer (Sartorius, Göttingen, Germany).

Peptide-pulsed T2A3 phage staining assays were performed by incubating $5 \times 10^5 - 1$
 10 $\times 10^6$ cells with 1×10^{10} phage in 100 μ L stain buffer on ice for 1 hour, followed by one wash in stain buffer. Cells were then stained with 1 μ g of rabbit anti-M13 antibody (Novus Biologicals), washed, stained with anti-rabbit-PE (BioLegend), incubated with an additional 500 μ L of LIVE/DEAD Fixable Near-IR dye diluted 1:1000 in PBS for 10 minutes at RT in the dark, and washed in stain buffer before analysis. V2 scFv staining was
 15 performed using 0.33 μ g of V2 scFv premixed with 1 μ g anti-FLAG-PE antibody (BioLegend) overnight at 4°C followed by incubation with peptide-pulsed T2A3 cells as described above. Anti-HLA-A3 staining was performed by staining 5×10^5 cells with 0.125 μ g clone GAP.A3-PE (eBioscience, Thermo Fisher Scientific) or mouse isotype IgG2a-PE (BioLegend). Anti-HLA-A1 staining was performed by staining 5×10^5 cells with 0.5 μ g
 20 anti-HLA-A1/A11/A26 antibody clone 8.L.101 (Abcam) or mouse isotype IgM (Thermo Fisher) followed by 0.25 μ g anti-mouse-PE (BioLegend). Stained cells were analyzed using an LSRII flow cytometer (Becton Dickinson, Mansfield, MA).

Quantification of cell surface-bound G12V peptide was performed using two commercial kits: PE Quantibrite Beads (BD, Franklin Lakes, NJ) and QIFIKIT (Agilent,
 25 Santa Clara, CA). For Quantibrite-based quantification, peptide-pulsed T2A3s were stained with 0.5 μ g V2 scFv preconjugated to 1.5 μ g clone L5 anti-FLAG-PE (BioLegend), followed by flow cytometry and quantification according to the manufacturer's instructions. For QIFIKIT-based quantification, peptide-pulsed T2A3s were stained with 0.5 μ g V2 scFv preconjugated to 1.5 μ g clone M2 anti-FLAG (Sigma Aldrich), followed by staining with
 30 anti-mouse-PE (BioLegend), flow cytometry and quantification according to the manufacturer's instructions.

Recombinant scFv and bispecific antibody production

Recombinant scFv proteins were produced and purified by AxioMx Inc (Abcam). In brief, the V2, H1, L2, and R6 scFv sequences were subcloned into a vector containing a periplasmic localization sequence, and C-terminal Flag and His tags. ScFvs were expressed
5 in *E. coli* and purified via nickel chromatography.

Bispecific antibodies were produced after subcloning gBlocks (IDT, Coralville, Iowa) encoding each of the variants with an IL-2 signal sequence and C-terminal 6xHIS tag into the pcDNA3.4 vector (Thermo Fisher Scientific). Bispecific antibodies were produced by the Eukaryotic Tissue Culture Core Facility of Johns Hopkins University. In brief, 1 mg of
10 plasmid was transfected using polyethylenimine (PEI) into HEK293F cells which were then grown as suspension culture at a density of 2×10^6 cells/mL in FreeStyle 293 expression media (Thermo Fisher Scientific) at 37°C, 170 rpm, and 5% CO₂. Protein was expressed for 5 days, after which cells were harvested by centrifugation, and the resulting supernatant filtered using a 0.22 µm filter. To each 1 L of supernatant, 2 mL of Ni-NTA His-Bind
15 (Millipore Sigma) resin slurry was added and allowed to incubate at 4°C overnight on an orbital shaker. The supernatant containing the slurry was passed through a centrifuge column (Pierce, Thermo Fisher Scientific), whereby the slurry was washed with 20 mM imidazole in PBS, and eluted in 50 mM, 100 mM, and 250 mM imidazole fractions. Protein fractions were run on mini-PROTEAN TGX stain-free gels (Bio-Rad, Hercules, California) and
20 appropriate fractions were combined for desalting into PBS, pH 7.4 or 20 mM Tris, 150 mM NaCl, pH 9 using 7k MWCO Zeba Spin desalting columns (Thermo Fisher Scientific). Proteins were quantified via stain free gel and BCA protein assay (Pierce, Thermo Fisher Scientific). For scDb western blots, protein was transferred from the stain-free gel to a Trans-Blot Turbo Mini 0.2 µm PVDF membrane (Bio-Rad) using the Trans-Blot Turbo
25 Transfer System (Bio-Rad). Membrane was blocked with blocking buffer (5% Bio-Rad Blotting-Grade Blocker in 1x TBST) on an orbital shaker (VWR) for 1 hour at RT, followed by incubation with anti-6x His tag antibody clone ab9108 at 1:1000 in blocking buffer at 4°C overnight, washing in 1x TBST, and incubation with anti-rabbit-HRP (Thermo Fisher Scientific) at 1:10,000 in blocking buffer for 1 hour at RT. Membrane was washed in 1x
30 TBST followed by ddH₂O, then imaged using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) on a ChemiDoc XRS+ imager (Bio-

Rad). Proteins were stored at 4°C for short term storage or snap frozen with the addition of 7% glycerol and stored at -80°C for long term storage. Alternatively, the V2-U scDb and L2-U scDb proteins were produced by GeneArt in Expi293s, purified with a HisTrap column followed by size exclusion chromatography using a HiLoad Superdex 200 26/600 column.

5 *SPR*

RAS G12V pHLA-A3, G12WT pHLA-A3, and V2 scFv binding experiments were performed at 25°C using a Biacore T200 SPR instrument (GE Healthcare, Chicago, IL). Approximately 130-140 response units (RU) of biotinylated pHLA-A3 were captured in flow cells (Fc) 2 and 4, respectively, using a streptavidin chip. Single-cycle kinetics were
 10 performed by injecting increasing concentrations (1.56, 6.25, 25, 100, 400 nM) of purified V2 scFv flowed over Fc 1-4. Binding responses for kinetic analysis were both blank and reference subtracted. Both binding curves were fit with a 1:1 binding model using Biacore Insight evaluation software. Measurements for L2-U scDb were done similarly, using biotinylated Q61L pHLA-A1 and Q61WT pHLA-A1.

15 *pHLA Immunoprecipitation and Mass Spectrometry*

pHLA immunoprecipitation and mass spectrometry were performed as described elsewhere (Wang et al., *Cancer Immunol Res* 7:1748-1754 (2019)). Briefly, COS-7 cells seeded into 24.5 x 24.5 cm² plates were transfected at 95% confluency using Lipofectamine 3000 Reagent (Thermo Fisher Scientific). For each plate, 125 µg of plasmids (50 µg of HLA
 20 plasmid and 75 µg of mutant or WT protein plasmid) were mixed with 200 µL of Lipofectamine P3000 in 6 mL of Opti-MEM (Thermo Fisher Scientific). In a separate tube, 200 µL of Lipofectamine 3000 Reagent was mixed with 6 mL of Opti-MEM. The contents of the two tubes were mixed and allowed to complex for 10 minutes. Medium bathing cells were removed and 50 mL of fresh complete medium was added followed by the
 25 Lipofectamine-DNA mixture. Cells were harvested 48 hours post-transfection. The transfection efficiency of COS-7 was >90% as assessed by GFP+ cell fraction on flow cytometry (BD LSRII).

Cells (transfected or untransfected) were grown to near confluency in 24.5 x 24.5 cm² plates. Cultured cells were washed with PBS two times, followed by another wash with PBS
 30 pre-chilled at 4°C containing 1x protease inhibitor. Cells were scraped and collected in a

500-mL centrifuge bottle. The bottle was centrifuged at 1,000 g for 5 minutes and the supernatant discarded. Cell pellets were snap frozen in liquid nitrogen and stored at -80°C for future experiments.

Neoantigen-expressing cells were processed as described elsewhere (Wang et al.,
 5 *Cancer Immunol Res* 7:1748-1754 (2019)). In brief, a total of 500 million cells were lysed and pHLA complexes were immunoprecipitated using Protein G Dynal Magnetic Beads (Thermo Fisher Scientific) pre-conjugated with anti-human HLA-A, B, C antibody clone W6/32 (Bio-X-Cell). After elution, dissociation, and filtration, peptides were lyophilized before further analysis. HPLC fractionation and a Dual-Reduction procedure were then
 10 performed (Wang et al., *Cancer Immunol Res* 7:1748-1754 (2019)). Controls for detection of the RAS G12V [7-16] and [8-16] peptides were established using AQUA™ heavy isotope labeled peptides of the same sequence (Sigma-Aldrich). These AQUA peptides were added to the cell lysates in every experiment. Transition parameters were manually examined and curated to exclude ions with excessive noise due to co-elution with impurities. Absolute
 15 copy numbers of peptides presented on the cell surface were calculated based on the MANA-SRM quantification using the AQUA™ heavy isotope labeled peptides and the recovery ratios of the pipeline, as described elsewhere (Wang et al., *Cancer Immunol Res* 7:1748-1754 (2019)).

CRISPR on Cell Lines

20 The Alt-R CRISPR system (Integrated DNA Technologies, IDT) was used to modify the *HLA* alleles, the *KRAS* mutation status of the NCI-H358 and NCI-H441 cell lines, and the *NRAS* mutation status of the HL-60 cell line. Alt-R® CRISPR Cas9 crRNAs (IDT) and Alt-R® CRISPR-Cas9 tracrRNA (IDT) were re-suspended at 100 µM with Nuclease-Free Duplex Buffer (IDT). The crRNAs and tracrRNA were mixed at a 1:1 molar ratio and
 25 denatured for 5 minutes at 95°C, followed by slow cooling to room temperature to duplex prior to mixing with Cas9 Nuclease (IDT) at a 1.2:1 molar ratio for 15 minutes. To knock out the *HLA-A3* allele in NCI-H441 cells, 40 pmoles of the Cas9 ribonucleoprotein (RNP) containing tracrRNA/*HLA-A* crRNA (GCTGCGACGTGGGGTTCGGAC; SEQ ID NO:685) duplex were mixed with 2×10^5 NCI-H441 cells in 20 µL of OptiMEM. This mixture was
 30 loaded into a 0.1 cm cuvette (Bio Rad) and electroporated at 150 V for 10 ms using an ECM

2001 (BTX). *HLA-A1* was similarly knocked out in HL-60 cells by mixing Cas9 RNP containing tracrRNA/*HLA-A1* crRNA (CAGACTGACCGAGCGAACCTG; SEQ ID NO:686) duplex with 5×10^5 HL-60 cells, and electroporated at 120V for 16 ms. Cells were immediately transferred to complete growth medium and cultured for 10 days.

5 To change ("KI") the *KRAS* mutation status of NCI-H358 from G12C/WT to G12V and NCI-H441 from G12V/WT to G13D/WT, Cas9 RNPs were co-electroporated with single-strand DNA homology directed repair templates. The G12V repair template was ATTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCAACGGCGCCGACAACGACG AGTTTATATTCAGTCATTTTCAGCAGGCCTTATAA (SEQ ID NO:687) for *KRAS*-
10 G12V crRNA (AATGACTGAATATAAACTTG; SEQ ID NO:688). The G13D repair template was AACAAGATTTACCTCTATTGTTGGATCATATTCGTCCACAAAATGATTCTGAATT AGCTGTATCGTCAAGGCACTCTTGCCTACGTCACCAGCTCCAACCTACCACAAGTT TATATTCAGTCATTTTC (SEQ ID NO:689) for *KRAS*-G13D-1 crRNA
15 (CTTGTGGTAGTTGGAGCTGT; SEQ ID NO:690). Both repair templates were obtained as Ultramer® Oligos from IDT. To improve rates of homology directed repair, NCI-H441 cells were pre-treated with 200 ng/mL of nocodazole (Sigma Aldrich) and 1 μ M of SCR7 pyrazine (Sigma Aldrich) for 17 hours prior to electroporation. The electroporation mixture contained 40 pmoles of Cas9 RNP, 20 pmoles of repair template, and 2×10^5 cells in 20 μ L
20 of OptiMEM. NCI-H441 cells were electroporated at 150 V for 10 ms, while NCI-H358 cells were electroporated at 120 V for 16 ms. Both cell types were transferred to complete growth media containing 1 μ M SCR7 for 72 hours following electroporation. Cells were grown in culture for 5 to 10 more days before use.

The *NRAS* mutation status in HL-60 was modified similarly. 5×10^5 HL-60 cells
25 were co-electroporated at 120 V for 16 ms with Cas9 RNPs containing tracrRNA/*NRAS* crRNA (CCTCATGTATTGGTCTCTCATGG; SEQ ID NO:691) duplex and either the repair template for Q61H (AAACCTGTTTGTGTTGGACATACTGGATACAGCTGGACATGAGGAATATTCTGCAA TGAGAGACCAATACATGAGGACAGGCGAAGGCTTCCT; SEQ ID NO:692) or Q61R
30 (AAACCTGTTTGTGTTGGACATACTGGATACAGCTGGAAGAGAGGAATATTCTGCAA TGAGAGACCAATACATGAGGACAGGCGAAGGCTTCCT; SEQ ID NO:693).

HLA-A and *RAS* modified polyclonal pools were plated at a density of 0.5 to 2 cells per well in 96 well plates and cultured for 3 weeks. Single colonies were transferred into 2 or 3 replica 96-well plates. For *HLA-A* modified NCI-H441 cells, two replica plates were used. One plate was used to stain cells with the *HLA-A3* specific antibody GAP.A3-PE and the other with anti-*HLA-A2* specific antibody BB7.2-PE (BioLegend). Comparison of the staining allowed identification of clones with only the *HLA-A3* allele knocked out, as NCI-H441 normally expresses both *HLA-A2* and *HLA-A3*. HL-60 cells were stained with anti-*HLA-A1* (clone 8.L.101) to select clones with *HLA-A1* knocked out. For the *RAS* modified cells, genomic DNA was harvested from one of the plates using the Quick-DNA™ 96 Kit (Zymo Research), PCR amplified using Q5® Hot Start High-Fidelity 2X Master Mix (New England BioLabs), and Sanger sequenced to identify clones with the desired *RAS* mutation status. Targeted next generation sequencing was performed to confirm the mutation status of selected clones.

Co-cultures

COS-7 cells were plated in a T25 flask and transfected at 70% confluency. For each flask, 10 µg of plasmid DNA (1:1 ratio of *HLA* plasmid:*RAS* plasmid, or control plasmid DNA, was mixed with 20 µL of P3000 Reagent in 250 µL of Opti-MEM (Thermo Fisher Scientific). In a separate tube, 20 µL of Lipofectamine 3000 Reagent was mixed with 250 µL of Opti-MEM. The contents of the two tubes were mixed and allowed to complex for 10 minutes at room temperature. Existing medium on pre-plated COS-7 cells was removed and fresh medium was added followed by the Lipofectamine-DNA mixture. Cells were harvested 24 hour post-transfection for co-culture after washing once with PBS, adding 1 mL 0.05% trypsin (Thermo Fisher Scientific) and incubation at 37°C for 5-10 minutes. Trypsin was quenched with serum-containing media and cells were counted. The co-culture was set up in 96-well flat-bottom tissue culture treated plates. To each well, the following components were combined: 50 µL of antibody diluted to the desired concentration in complete RPMI-1640 with a final IL-2 concentration of 100 IU/mL, 1×10^4 COS-7s in 100 µL complete RPMI-1640, 5×10^4 human T cells in 50 µL complete RPMI-1640. The co-cultures were incubated for 24 hours at 37°C. The resultant supernatant was assayed for

cytokines using a Human IFN γ Quantikine and Human TNF α Quantikine ELISA Kits (both R&D Systems Bio-technie, Minneapolis, MN) according to the manufacturer's instructions.

For co-cultures with pulsed cells, cells were peptide-pulsed in a 96-well plate by combining the specified number of target cells in 50 μ L of serum-free RPMI-1640 media (for T2A3, SigM5, Hs 695T) or serum-containing RPMI-1640 media (for PBMCs, monocytes, iDCs, mDCs) with 50 μ L of serially diluted peptide in serum-free RPMI-1640 media. Cells were incubated for 4 hours at 37°C, after which 5×10^4 human T cells and antibodies were added in an additional 100 μ L of serum-containing RPMI-1640 medium, with a final IL-2 concentration of 100 IU/mL. The co-cultures were incubated for 24 hours at 37°C, the cells pelleted by centrifugation at 500 g, and the cell-free supernatant was assayed for cytokines as described above. Cell viability was assayed by CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to the manufacturer's instructions. Cytotoxicity was calculated by taking the luciferase signal of a given well, subtracting the luciferase signal of the T cell only wells, and normalizing to the luciferase signal of the wells without scDb.

Co-cultures with other target cell lines were set up similarly, with target cells and human T cells combined as specified in the figure legends. For Luminex assays, IFN γ , IL-2, TNF α , granzyme B, and perforin were measured using MILLIPLEX panels (MilliporeSigma, Burlington, MA).

20 *Western Blots*

COS-7 cells were transfected as above and harvested after 24 hours. Cells were pelleted, snap frozen, and re-suspended in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific). 25 μ g of cell lysate or approximately 0.5-1.5 μ g of recombinant protein was run on mini-PROTEAN TGX stain-free gels (Bio-Rad, Hercules, California). Protein was transferred from the stain-free gel to a Trans-Blot Turbo Mini 0.2 μ m PVDF membrane (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). Membrane was blocked with blocking buffer (5% Bio-Rad Blotting-Grade Blocker Bio-Rad in 1x TBST) on an orbital shaker (VWR) for 1 hour at RT. All antibody incubations were also performed using this blocking buffer. All incubations with primary antibodies were done at 4°C overnight (except for β -Actin as noted below). All incubations with secondary

antibodies were done for 1 hour at RT. All washes were performed with 1x TBST. KRAS was detected with mouse monoclonal antibody (mAb) F234 (Santa Cruz Biotechnology, Dallas, TX, at 1:500), followed by anti-mouse-HRP (Thermo Fisher Scientific, at 1:10k). HLA-A3 was detected with mouse mAb 7g7h8 (Abcam, at 1:500), followed by anti-mouse-HRP (Thermo Fisher Scientific, at 1:10k). Rab-7b was detected with rabbit anti-Rab7b mAb ab193360 (Abcam, at 1:500), followed by anti-rabbit-HRP (Thermo Fisher Scientific, at 1:10k). β -Actin was detected with rabbit mAb 13E5 conjugated to HRP (Cell Signaling Technology, Danvers, MA, at 1:3000) for 1 hour at RT. Recombinant proteins with 6xHIS tags were detected with anti-6x HIS tag rabbit polyclonal antibody ab9108 (Abcam, 1:1000), followed by anti-rabbit-HRP (Thermo Fisher Scientific, at 1:10k). Prior to imaging, the membranes were washed with ddH₂O, then imaged using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) on a ChemiDoc XRS+ imager (Bio-Rad).

Viral transduction of cell lines

To generate luciferase-expressing NCI-H358 and HL-60 cell lines for *in vivo* experiments, NCI-H358 and HL-60 cells were transduced with RediFect Red-Fluc-GFP lentiviral particles (Perkin Elmer, Waltham, MA). Non-tissue culture-treated 48-well plates were coated with 250 μ L of 20 μ g/mL RetroNectin (Clontech) per well for 2 hours at RT, then blocked with 10% FBS for 1 hour at RT. Viral particles and 2×10^5 target cells were added to each well in a total volume of 275 μ L of cell culture media and subjected to centrifugation at 1200 x g for 1 hour at 20°C. Cell culture volumes were brought up to 500 μ L with complete media. Cells were then incubated at 37°C for 3 days before exchanging media. Transduced cells were sorted based on the presence of GFP using FACS Aria Fusion (BD Biosciences) 18 days after transduction.

Mouse xenograft model

Female NOD.*Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* (NSG) mice at 6-10 weeks were acquired from the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center Animal Resources Core and treated in compliance with a research protocol approved by the Johns Hopkins University Animal Care and Use Committee. Mice were maintained on an irradiated Uniprim rodent diet (Envigo, Indianapolis, IN). Littermate controls were used for all experiments.

For the NCI-H358 intrasplenic model, 5×10^5 luciferase-expressing NCI-H358 cells (CRISPR *KRAS* G12V-KI clone 1) or isogenic parental NCI-H358 clone (*KRAS* G12C/WT) were inoculated into mouse spleens on day zero using sterile surgical techniques.

Intraoperatively, two-week micro-osmotic pumps (model 1002, ALZET, Cupertino, CA)

5 filled with V2-U scDb or isotype control (L2-U scDb) were placed intraperitoneally.

Successful tumor inoculation was ensured by bioluminescence imaging one day later, followed by intravenous injection of 1×10^7 human T cells via lateral tail vein.

Bioluminescent imaging (IVIS imaging system) was performed using RediJect D-luciferin Ultra (Perkin Elmer) according to the manufacturer's instructions (Perkin Elmer). Image

10 analysis was performed using Living Image software. Individual luminescence measurements were normalized to the average fluorescence of the injection marker dye ($745^{\text{ex}}/800^{\text{em}}$) in the thoracic region.

For the HL-60 leukemia cell model, mice were inoculated intravenously with 1×10^7 human T cells and 5×10^5 luciferase-expressing parental HL-60 (*NRAS* WT/Q61L) or

15 isogenic control HL-60 (*NRAS* Q61H/Q61H) via lateral tail vein injection. On day 1, mice were randomized based on luminescence to ensure similar pretreatment tumor burden. Two-week micro-osmotic pumps filled with L2-U scDb or isotype control scDb (V2-U scDb) that had been primed in 1 mL PBS overnight at 37°C were then placed intraperitoneally using sterile surgical techniques. Tumor growth was serially monitored by bioluminescent

20 imaging.

Statistics

Statistical analyses were performed with Prism 8 (GraphPad Software, La Jolla, CA).

Unless otherwise indicated, error bars represent the standard deviation of three technical replicates that were independently assembled. Error bars smaller than the symbols used to
25 represent the mean of these replicates are not shown. Percent cytotoxicity of target cells for *in vitro* experiments was calculated as described above. For *in vivo* experiments, statistical significance was performed with an unpaired, two-tailed t-test with Bonferroni-Dunn correction for multiple comparisons.

Example 4: Targeting a neoantigen derived from a common TP53 mutation

This Example describes an immunotherapeutic agent that targets a common *TP53* mutation. Results from Example 2 were reanalyzed with additional samples and included in the following.

5 Results

The p53^{R175H} neoantigen is presented on the surface of cancer cells

The p53^{R175H} (aa 168-176, HMTEVVRHC; SEQ ID NO:1) and p53^{WT} (HMTEVVRRC; SEQ ID NO:135) peptides were predicted on the NetMHCpan 4.0 server to bind HLA-A*02:01 at 5177.6 nM (rank 9.7%) and 7121.5 nM (11.6%), respectively. To provide experimental evidence of, and to quantify such presentation, peptides eluted from HLA molecules were analyzed in four different cell culture systems using a mass spectrometry (MS)-based method. First, the human HLA-A*02:01 and either full-length p53^{R175H} or p53^{WT} were co-expressed in monkey COS-7 cells. MS analysis of the peptides immunopurified with an anti-HLA antibody detected the p53^{R175H} peptide at approximately 700 copies per cell (Fig. 73A, Table 17). Though relatively abundant amounts of the p53^{R175H} peptide were detected in pHLA complexes, the p53^{WT} peptide was not observed in pHLA complexes in transfected cells, despite equivalent amounts of p53^{WT} and p53^{R175H} total protein expression as assessed by Western blotting (Fig. 73B). Second, MS analysis was performed on three human cancer cell lines, KMS26, TYK-nu, and KLE, all of which harbor the p53^{R175H} mutation and carry an HLA-A*02:01 allele. The p53^{R175H} peptide was detected on all three cell lines, and at much lower levels than in the COS-7 cells in which the mutant *TP53* and HLA genes were exogenously introduced (Fig. 73C, Table 17). Based on comparisons with heavy isotope labeled controls, it was estimated that there were 2.4, 1.3, and 1.5 copies of cell-surface p53^{R175H}/HLA-A*02:01 complexes on the cell surfaces of KMS26, TYK-nu, and KLE cell lines, respectively (Table 17).

Table 17. Quantitative assessment of the p53^{R175H} neoantigen peptide. The amount of p53^{R175H} neoantigen peptide (HMTEVVRHC) present in COS-7 cells transfected with HLA-A*02:01 and p53^{R175H} or p53^{WT}, as well as cells lines that endogenously express HLA-A*02:01 and p53^{R175H}, were quantified using mass spectrometry. *Corrected for peptide recovery (all cell lines) and transfection efficiency (COS-7).

Cell line	HLA and p53 status	No. cells (millions) used for analysis	Abundance (femtomole)	Copy no. / cell *
COS-7	Exogenous HLA-A*02:01/p53 ^{R175H}	375.0	32.43	703.5
COS-7	Exogenous HLA-A*02:01/p53 ^{WT}	375.0	Not detectable	Not detectable
KMS26	Endogenous HLA-A*02:01/p53 ^{R175H}	157.0	0.48	2.4
KLE	Endogenous HLA-A*02:01/p53 ^{R175H}	92.4	0.18	1.5
TYK-nu	Endogenous HLA-A*02:01/p53 ^{R175H}	168.3	0.28	1.3

*Identification of scFv-expressing phage clones specific for the HLA-A*02:01-restricted p53^{R175H} peptide and conversion to scDb format*

To identify TCRm single-chain variable fragments (scFvs) selectively targeting mutant pHLA complexes, an scFv-displaying phage library was screened with an estimated complexity $>1 \times 10^{10}$. Positive selection against HLA-A*02:01 pHLA monomers containing the p53^{R175H} peptide were combined with negative selection against pHLA monomers containing the p53^{WT} and irrelevant peptides. Selected phage clones were amplified and assessed for binding to T2 cells presenting the mutant or wild-type (WT) peptide via flow cytometry (Fig. 74A).

Twenty-three phage clones with median fluorescence intensity (MFI) ratios of p53^{R175H} to p53^{WT} >4 were then converted to T cell-retargeting bispecific antibodies. This was achieved through linking each individual scFv to an anti-CD3 ϵ scFv (UCHT1) in a single-chain diabody (scDb) format (Fig. 74B). The scDb format was chosen after evaluating several previously described bispecific antibody formats, such as bispecific T-cell engager (BiTE), dual-affinity re-targeting antibody (DART), and diabody in pilot experiments assessing protein expression and in vitro T cell activation. The ability of scDbs to activate T cells was assessed by interferon- γ (IFN- γ) release after co-incubation with COS-7 cells overexpressing HLA-A*02:01 and either full-length p53^{R175H} or p53^{WT} proteins. Two

scDb clones, named H2-scDb and H20-scDb and derived from phage clones H2 and H20, respectively, showed the most potent and specific T-cell activation in the presence of p53^{R175H}/HLA-A*02:01 (Fig. 74C, Table 18). The specificity of these scDbs was further evaluated by titration enzyme-linked immunosorbent assay (ELISA). Both H2- and H20-
5 scDb bound to p53^{R175H}/HLA-A*02:01 at low concentrations. At high concentrations, H20-scDb also bound to p53^{WT}/HLA-A*02:01, while H2-scDb did not bind to the WT pHLA complex even at very high concentrations of the scDb (Fig. 67A, Fig. 74D). H2-scDb was therefore chosen for further analysis. As assessed by surface plasmon resonance (SPR), the H2-scDb bound to p53^{R175H}/HLA-A*02:01 with a $K_D=86$ nM, a k_{on} of $1.76 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, and
10 a k_{off} of $1.48 \times 10^{-2} \text{ s}^{-1}$ (Fig. 67B). The k_{on} of $1.76 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ suggested a lack of overall conformational change of the p53^{R175H}/HLA-A*02:01 upon binding. No detectable binding of the H2-scDb to p53^{WT}/HLA-A*02:01 was observed in the SPR experiments (Fig. 67B).

Table 18. Sequences of the top scFv clones from phage library selection.

scFv	VL	SEQ ID NO	VH	SEQ ID NO
H2	DIQMTQSPSSLSASVGDRVTITCRASQDVNT AWAYQQKPGKAPKLLIYSAYFLYSGVPSRF SGSRSGTDFTLTISSLQPEDFATYYCQQYSRY SPVTFGQGGTKVEIK	729	EVQLVESGGGLVQPGGSLRLSCAASGFN VYASGMHWVRQAPGKGLEWVAKIYPDS DYTYADSVKGRFTISADTSKNTAYLQM NSLRAEDTAVYYCSRDSSTFYVYAMDY WGQGTLVTVSS	730
H20	DIQMTQSPSSLSASVGDRVTITCRASQDVNT AWAYQQKPGKAPKLLIYSASFLEYSGVPSRF SGSRSGTDFTLTISSLQPEDFATYYCQQSNAY PITFGQGGTKVEIK	731	EVQLVESGGGLVQPGGSLRLSCAASGFN LNSYYMHWVRQAPGKGLEWVAMIIPGY GYTNYADSVKGRFTISADTSKNTAYLQM NSLRAEDTAVYYCSRSYMYMDYWGQ GTLVTVSS	732

Next, it was examined whether anti-CD3 arms of the scDb other than the original UCHT1, could influence the ability of H2 to induce T-cell activation. The H2-scFv was
 5 linked to a panel of commonly used anti-CD3 ϵ scFvs, including UCHT1, UCHT1v9, L2K-07, OKT3, and hXR32 (Fig. 75A, B). It was found that, among the anti-CD3 ϵ scFvs tested, UCHT1, which has the highest reported affinity (Table 19), activated T cells at the lowest p53^{R175H} peptide concentration when linked to the H2-scFv (Fig. 75C, Fig. 67C). H2-UCHT1-scDb (hereafter H2-scDb) was thus used for further experiments. Thermal stability
 10 of H2-scDb as measured by differential scanning fluorimetry showed a single melting temperature (T_m) at 69 °C, suggesting it being a stable molecule (Fig. 75D, E).

Table 19. Reported affinity of the anti-human CD3 ϵ scFvs used in the study.

anti-CD3 ϵ scFv clone	Reported affinity (K_D , nM)	Format in which affinity was measured
UCHT1	1.8	Full-length antibody
UCHT1v9	4.7	Full-length bispecific antibody
L2K-07	110	BiTE
OKT3	2730	Fab
hXR32	21.2	Fc-bearing DART

15 *H2-scDb specifically recognizes cancer cells expressing the p53^{R175H} neoantigen*

The ability of H2-scDb to recognize cancer cell lines expressing various levels of HLA-A*02:01 and having different p53 mutation status was next evaluated. H2-scDb elicited T-cell responses in a dose-dependent manner when T cells were co-cultured with three cell lines that expressed moderate to high levels of HLA-A*02:01 and harboring
 20 p53^{R175H} (KMS26, KLE, TYK-nu, as well as the cisplatin-resistant variant of TYK-nu) (Fig. 68A, B, Fig. 76A). This activation was noted even at very low (sub-nanomolar) concentrations of the bispecific antibody and the reactivity was strictly T cell and H2-scDb-dependent (Fig. 76B, C). The T-cell responses were polyfunctional, as indicated by the release of cytotoxic granule proteins granzyme B and perforin, cytotoxicity, and the
 25 production of cytokines IFN- γ , tumor necrosis factor α (TNF- α), interleukin-2 (IL-2), and others (Fig. 68C, Fig. 76D-F). Clustering of T cells around tumor cells, leading to their lysis

in the presence of H2-scDb, was also visualized by real-time live-cell imaging (Fig. 68D). The specificity of the bispecific antibody for both the p53^{R175H} peptide and HLA-A*02:01 was evident from the observation that much lower levels of IFN- γ were induced by cells harboring a p53^{R175H} mutation but low levels of expression of HLA-A*02:01 (AU565 or SK-
 5 BR3) or by cells without p53^{R175H} but relatively high levels of HLA-A*02:01 expression (Fig. 68B, Fig. 77A).

The specificity of H2-scDb was validated using nine pairs of isogenic cell lines that differed with respect to HLA-A*02:01 expression or p53^{R175H} mutation (Fig. 69A). First, human HEK293FT (*TP53*^{WT}/HLA-A*02:01) or Saos-2 (*TP53*^{null}/HLA-A*02:01) cells were
 10 transfected with plasmids expressing either full-length p53^{R175H} or p53^{WT}. H2-scDb induced robust T-cell activation when co-cultured with both cell lines overexpressing p53^{R175H} but not with p53^{WT}-overexpressing or parental cells (Fig. 69B). Second, HLA-A*02:01-encoding retrovirus was transduced into four cell lines (AU565, SK-BR-3, HuCCT1, CCRF-CEM) that harbored the p53^{R175H} mutation but had low levels of HLA-A*02:01 expression (Fig. 77B).
 15 Exogenous expression of HLA-A*02:01 in all four lines conferred T-cell activation by H2-scDb (Fig. 69C). Third, *TP53* in KMS26, TYK-nu, and KLE cancer cell lines that carry endogenous p53^{R175H} were genetically disrupted using a CRISPR-based technology (Fig. 78A). T-cell activation, as assessed by IFN- γ secretion, was reduced to control levels when *TP53* was knocked out in all three cell lines (Fig. 69D). The cytotoxicity mediated by H2-
 20 scDb was similarly mitigated by *TP53* knock-out (KO) in these cells (Fig. 69E, Fig. 78B).

*Overall structure of the H2-Fab–p53R175H/HLA-A*02:01 ternary complex*

To understand the structural basis for the high specificity of the H2 clone for p53^{R175H}/HLA-A*02:01, H2 was converted into full-length IgG (H2-IgG) and confirmed that binding specificity was preserved in this format (Fig. 79A). The H2-IgG was then digested
 25 into an antigen-binding fragment (H2-Fab) with papain (Fig. 79B). The H2-Fab–p53^{R175H}/HLA-A*02:01 complex was purified (Fig. 79C, D) and its crystal structure was determined by molecular replacement and refined to 3.5 Å resolution (PDB ID 6W51, Table 20). There were four H2-Fab and four p53^{R175H}/HLA-A*02:01 per asymmetric unit (Fig. 70A, B). All four H2-Fab were firmly positioned on the p53^{R175H}/HLA-A*02:01 with a
 30 pairwise root-mean-square deviation (rmsd) range of 0.27 to 0.45 Å for 382 to 419 C^{alpha}

carbon's, as calculated by PyMOL (Table 21). The total buried surface area of the H2-Fab–
p53^{R175H}/HLA-A*02:01 interface was 1173 Å², with roughly equal contributions from heavy
and light chains (644 Å² and 529 Å², respectively, Table 22). Although the entire structure
was refined to a resolution of 3.5 Å, particularly clear electron densities were observed for
5 the p53^{R175H} neoantigen, the complementarity-determining regions (CDRs) of the H2-Fab,
and the HLA-A*02:01 (Fig. 70C, D).

Table 20. X-ray Crystallography data collection and refinement statistics.

p53 ^{R175H} /HLA-A*02:01–Fab H2 (PDB ID 6W51)	
Data Collection	
Diffraction source	NSLS-II X17-ID-2
Wavelength (Å)	0.979321
Temperature (K)	100
Detector	Dectris EIGER X 16M
Space group	P12 ₁ 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	113.3, 123.7, 136.9
α , β , γ (°)	90, 100.4, 90
Resolution range (Å)	30.37–3.53 (3.66–3.53)
Total no. of reflections	104,474 (9,774)
No. of unique reflections	43,734 (4,273)
Completeness (%)	95.3 (89.2)
Redundancy	2.4 (2.3)
$\langle I/\sigma(I) \rangle$	3.6 (1.4)
<i>R</i> _{merge}	0.25 (0.76)
<i>R</i> _{meas}	0.29 (0.89)
<i>R</i> _{pim}	0.18 (0.58)
CC _{1/2}	0.94 (0.54)
Refinement	
Resolution range (Å)	30.38–3.53 (3.62–3.53)
No. of reflections, working set	41,530
<i>R</i> _{work} / <i>R</i> _{free}	0.20/0.28 (0.30/0.35)
<i>No. of non-H atoms</i>	
MHC (HLA-A*02:01 + β2m)	3,078
p53 ^{R175H} peptide	75
Fab H2 Heavy Chain	1,667
Fab H2 Light Chain	1,652
Total of non-H atoms	6,472
<i>R.m.s. deviations</i>	
Bonds (Å)	0.009
Angles (°)	1.66
Wilson B-factor (Å ²)	62

<i>Average B factors (Å²)</i>	
MHC (HLA-A*02:01 + β2m)	74
p53 ^{R175H} peptide	56
Fab H2 Heavy Chain	59
Fab H2 Light Chain	63
Total average B factor	63
<i>Ramachandran (%)</i>	
Favorable	95.2
Allowed	3.8
Outlier	1.0

*Values in parentheses are for highest-resolution shell. All atoms refer to non-H atoms.

Table 21. Pairwise rmsd of the four H2-Fab in the asymmetric unit. The root-mean square deviation (rmsd) and number of C^{alpha} carbons were calculated by PyMOL (v2.2.3,

5 Schrödinger, LLC, New York, NY). For chain reference see PDB ID 6W51.

	Chains M,N (Å/#C ^{alpha})	Chains S,T (Å/#C ^{alpha})	Chains Q,R (Å/#C ^{alpha})
Chains O,P	0.32/382	0.36/414	0.42/413
Chains Q,R	0.45/419	0.27/386	0
Chains S,T	0.34/408	0	—

Table 22. Structural comparison of H2-Fab–p53^{R175H}/ HLA-A*02:01 with various TCR and Fab antibody-pHLA. Total bonds were calculated using a 4 Å cutoff which includes both hydrogen bonds and van der Waals interactions as calculated using CONTACTS in the CCP4 suite. PDB, Protein Data Bank; BSA, buried surface area; α, TCRα chain; β, TCRβ chain; H, V_H domain; L, V_L domain; pep, HLA presented peptide.

10

	H2-Fab	MAGE-A3 TCR	NY-ESO-1 Fab (3M4E4)	ESK-1 Fab
Affinity (K _D)	86 nM	7.1 nM	95 nM	13.2 nM
PDB	6W51	5BRZ	3HAE	4WUU
Angle of rotation (°)	36°	57°	138°	122°
Total bonds	115	114	167	183
Peptide bonds (bold ≥10)	4, 5, 6, 7, 8	1, 4, 5, 7, 8	1, 2, 4, 5 , 6, 7, 8, 9	1 , 4
Peptide bonds	36 (31%)	16 (14%)	89 (53%)	19 (10%)
Bonds from β/H	21	10	47	10
Bonds from α/L	15	6	42	9

HLA bonds	79	98	78	164
Bonds from β/H	31	20	47	130
Bonds from α/L	48	78	31	34
HLA ≥5 bonds (bold ≥10)	61, 65, 72, 80, 146, 155	66, 154, 155, 157, 158, 163	65, 66, 72, 73	58, 62, 63, 65, 66, 155, 161, 162, 166, 167, 169, 170
BSA				
BSA total	1173	1027	1366	1084
BSA β/H pep	253	158	256	72
BSA α/L pep	102	112	260	93
BSA β/H HLA	391	247	523	601
BSA α/L HLA	427	510	327	318

*Binding of the p53^{R175H} peptide to HLA-A*02:01*

The p53^{R175H} peptide (HMTEVVRHC; SEQ ID NO:1) occupied the binding cleft α1-α2 of HLA-A*02:01, burying a solvent accessible surface area of 870 Å², slightly larger than other peptide/HLA-A*02:01 complexes (Fig. 71A, B, Fig. 80A) and with the C-terminal arginine at position 7 (Arg174) and mutant histidine at position 8 (His175) pointing up, out of the groove. In contrast, the N-terminus of the peptide is situated deep within the peptide binding cleft, anchored by multiple residues in the HLA-A*02:01 (Fig. 71A, B, Fig. 80A).

The anchor residues of the peptide, a methionine at position 2 (P2, Met169) and a cysteine residue at position 9 (P9, Cys176) (Fig. 80B), departed from the canonical anchor residues—leucine at P2 and valine or leucine at P9. Peptides that bind to HLA-A*02:01 through either a methionine at P2 or a cysteine at P9 have been reported, but not both (Webb et al., *J. Biol. Chem.* 279:23438-23446 (2004); and Ataie et al., *J. Mol. Biol.* 428:194-205 (2016)). Based on alignments with structures of other HLA-A*02:01 peptides in complex with TCR or TCRm, the unconventional anchoring of p53^{R175H} did not result in drastic peptide conformational change or positioning (Fig. 80C, D).

*Structural basis for the recognition of p53^{R175H}/HLA-A*02:01 by the H2-Fab*

The recognition of the HLA-A*02:01 by the H2-Fab was mediated by all six CDRs. There were a total of 79 contacts, with a cutoff of 4 Å, between the H2-Fab CDRs and the α1

and $\alpha 2$ of HLA-A*02:01, with the light chain contributing to 61% of those contacts (Table 22). The H2-Fab buried a solvent accessible surface area of $818 \text{ \AA}^2$ within the HLA, of which $427 \text{ \AA}^2$ were contributed by the light chain and $391 \text{ \AA}^2$ by the heavy chain (Table 22). In contrast, only four of the six H2-Fab CDRs (H1, H2, H3 and L3) interacted with the p53^{R175H} peptide. Overall, the H2-Fab made 36 contacts with the p53^{R175H} neoantigen, including five hydrogen bonds and numerous van der Waals interactions. His175 made 47% of all direct contacts with the H2-Fab. The CDR-H1, H2, and H3 of the heavy chain and CDR-L3 of the light chain formed a cage-like configuration around the C-terminus of the p53^{R175H} peptide, trapping Arg174 and His175 into position by providing a stable interaction (Fig. 71C). The imidazole side chain of His175 was anchored by a hydrogen bonding network with Asp54 (CDR-H2) and Tyr94 (CDR-L3) (Fig. 71C, Fig. 81). Tyr52 (CDR-H2) acted as a ceiling and capped the cage-like structure around His175 by forming π - π interactions (Fig. 71C, Fig. 81).

Viewed from the axis of the C-terminus to the N-terminus of the p53^{R175H} peptide, the CDRs were arranged in the order H2, H1, L3, H3, L1, L2 (Fig. 70E, F, G). The docking angle of the H2-Fab to the peptide within the HLA groove is 36° (Fig. 70G, H). This orientation angle was quite different from that of most previously described TCRs or TCRm antibodies to pHLA complexes, in which the axis of the peptide is almost perpendicular to the axis defined between the disulfide bonds of the V_L/ α to V_H/ β chains (Fig. 82).

Assessing candidate cross-reactive peptides

One of the major challenges confronting new immunotherapeutic antibodies is off-target binding, which can result in toxicity to normal cells. Scanning mutagenesis was employed to identify peptides in the human proteome to which H2-scDb might cross-react. A peptide library was generated by systemically substituting amino acids at each position of the target p53^{R175H} peptide (HMTEVVRHC; SEQ ID NO:1) with each of the remaining 19 common amino acids. T2 cells loaded with each of the 171 variant peptides were then used to assess T-cell activation by measuring IFN- γ release following incubation with T cells and H2-scDb (Fig. 71D). In congruence with the X-ray structural analysis, any changes in P8, where the mutant histidine residue lies, and any change in P7, which is encased with P8 by the CDR loops, abolished recognition of the peptide. Peptides with substitutions at these

positions retained their ability to bind to HLA-A*02:01 (Fig. 83A), but not to the H2-scDb. Other non-anchor residues at positions 3-6 also highly favored the parental amino acids present in the target peptide. This recognition pattern is illustrated as a Seq2Logo graph (Fig. 71E).

5 Next, a nonamer binding motif, x-[AILMVNQTC]-[ST]-[DE]-[IV]-[IMVST]-R-H-[AILVGHSTYC] (SEQ ID NO:197), was generated using 20% target peptide reactivity as a cutoff for permissive amino acids at each position (Fig. 83B). A search of this motif in the UniProtKB human protein database using ScanProsite yielded 3 homologous peptides from STAT2 (PLTEIIRHY; SEQ ID NO:185), VPS13A (LQSEVIRHY; SEQ ID NO:186), and
10 ZFP3 (QNSEIIRHI; SEQ ID NO:187) (Table 9). None of these 3 peptides were predicted to be potent binders of HLA-A*02:01 by NetMHCpan 4.0 (% rank all >2.0) and had lower predicted binding affinity than the parental p53^{R175H} peptide (Table 9). However, to experimentally exclude the possibility of cross-reactivity, T2 cells were pulsed with each of these peptides. H2-scDb activated T cells only in the presence of T2 pulsed with the
15 p53^{R175H} peptide (Fig. 71F). Additionally, COS-7 cells were co-transfected with expression plasmids for HLA-A*02:01 and full-length STAT2 or ZFP3; VPS13A was not tested due to its large size (>3000 aa). Again, no T-cell activation was detected in the co-culture assay with COS-7 cells expressing the two proteins containing the candidate cross-reactive peptides (Fig. 83C, D).

20 *Antitumor activity of the H2-scDb in vivo*

To determine whether H2-scDb could control tumor growth *in vivo*, KMS26 multiple myeloma cells were engrafted into NOD-SCID-*Il2rg*^{-/-} (NSG) mice through intravenous injection, establishing widespread, actively growing cancers throughout the body. Two models were used to assess the effects of the H2-scDb in combination with human T cells
25 engrafted in these mice (Fig. 84A). In an early treatment model, mice were randomized based on luminescence quantification of tumor burden and H2-scDb was subsequently administered through continuous intraperitoneal infusion pumps at 0.3 mg/kg/day, starting one day after tumor inoculation. The pumps were able to maintain significant plasma concentrations of scDb for two weeks (Fig. 84B). An irrelevant isotype scDb was
30 administered to mice in parallel as control. H2-scDb markedly suppressed the growth of

parental KMS26 tumors (Fig. 72A). In contrast, the H2-scDb had no effect on KMS26 tumors in which the *TP53* gene had been disrupted using CRISPR (Fig. 72A). In the second model, mice were randomized 6 days after tumor inoculation. The H2-scDb was administered at two doses (0.15 and 0.3 mg/kg/day). Both doses resulted in major tumor regressions and were well-tolerated as assessed by the absence of significant changes in body weight (Fig. 72B, Fig. 84C). No treatment effect of H2-scDb was observed in the absence of human T cells, supporting the T cell-dependent nature of H2-scDb (Fig. 84D).

Together these results demonstrate that highly specific bispecific antibodies can be generated against pHLA complexes resulting from common mutations occurring in cancer cells. The format and configuration of the bispecific antibodies developed here are highly specific and sensitive scDbs against protein products containing mutations occurring in cancer cells.

Materials and Methods

Cell lines and primary T cells

COS-7, RPMI 6666, T2 (174 x CEM.T2), Raji, HH, AU565, SK-BR-3, KLE, HCT116, SW480, NCI-H441, Saos-2, and CCRF-CEM cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). KMS26, TYK-nu, and HuCCT1 were purchased from Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan). SigM5 was obtained from DSMZ (Braunschweig, Germany). HEK293FT was obtained from Invitrogen (Thermo Fisher Scientific, Waltham, MA). T2, Raji, Jurkat, HH, AU565, NCI-H441, TOV-112D, CCRF-CEM, KMS26, TYK-nu, TYK-nu.CP-r and HuCCT1 were cultured in RPMI-1640 (ATCC, 30-2001) with 10% FBS (GE Healthcare, SH30070.03) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, 15140163). RPMI 6666 was cultured in RPMI-1640 with 20% FBS and 1% Penicillin-Streptomycin. COS-7, SK-BR-3, HCT116, SW480, and Saos-2 were cultured in McCoy's 5A modified media (Thermo Fisher Scientific, 16600108) with 10% FBS and 1% Penicillin-Streptomycin. SigM5 was cultured in IMDM (Thermo Fisher Scientific, 12440061) with 20% FBS and 1% Penicillin-Streptomycin. HEK293FT was cultured in DMEM (high glucose, pyruvate, Thermo Fisher Scientific, 11995065) with 10% FBS, additional 2 mM GlutaMAX (Thermo

Fisher Scientific, 35050061), 0.1 mM MEM non-essential amino acids (Thermo Fisher Scientific, 11140050), 1% Penicillin-Streptomycin, and 500 µg/mL Geneticin (Thermo Fisher Scientific, 10131027). PBMCs were isolated from leukapheresis samples (Stem Cell Technologies, Vancouver, BC) by standard density gradient centrifugation with Ficoll Paque Plus (GE Healthcare, 17-1440-03). T cells were expanded from PBMCs with addition of the anti-human CD3 antibody (OKT3, BioLegend, San Diego, 317347) at 15 ng/mL for three days. T cells were cultured in RPMI-1640 with 10% FBS, 1% Penicillin-Streptomycin, 100 IU/mL recombinant human IL-2 (aldesleukin, Prometheus Therapeutics and Diagnostics, San Diego, CA), and 5 ng/mL recombinant human IL-7 (BioLegend, 581908). In general, T cells from at least two different donors were tested in *in vitro* assays. All cells were grown at 37°C in 5% CO₂ with humidification.

Detection of neoantigen peptide

HLA-A*02:01 restricted p53^{R175H} peptide was directly detected and quantified in human cancer cells carrying p53^{R175H} mutations through MANA-SRM in COS-7 cells transfected with HLA-A*02:01 and p53^{R175H} and in human cancer cells carrying p53^{R175H} mutations and expressing HLA-A*02:01. In particular, the dual-reduction approach described in MANA-SRM was critical for this detection because a cysteine and a methionine coexist in the p53^{R175H} peptide. One hundred femtomole heavy-isotope labeled p53^{R175H} peptide HMTEVVRHC (SEQ ID NO:1) and p53^{WT} peptide HMTEVVRRC (SEQ ID NO:135; New England Peptide Inc, Gardner, MA) were spiked into each sample before the assay. The MANA-SRM assays were performed at Complete Omics Inc. (Baltimore, Maryland).

Peptides and monomers

All peptides were synthesized at a purity of >90% by Peptide 2.0 (Chantilly, VA) or ELIM Biopharm (Hayward, CA), except for the positional scanning library, where crude peptides were used. Peptides were resuspended in dimethylformamide at 10 mg/mL and stored at -20°C. Biotinylated pHLA monomers were synthesized by Fred Hutchinson Cancer Research Center Immune Monitoring Lab (Seattle, WA). Monomers were confirmed to be folded prior to selection by performing an ELISA using W6/32 antibody (BioLegend, 311402), which recognizes only folded HLA.

Phage display library construction

The scFv-bearing phage library used in this study has been described elsewhere (see, e.g., Miller et al., *J. Biol. Chem.* 294:19322-19334 (2019)). Briefly, oligonucleotides were synthesized by GeneArt (Thermo Fisher Scientific) using trinucleotide mutagenesis (TRIM) technology to diversify complementarity-determining region (CDR)-L2, CDR-L3, CDR-H1, CDR-H2, and CDR-H3. A FLAG (DYKDDDDK; SEQ ID NO:190) epitope tag was placed immediately downstream of the scFv, which was followed in-frame by the full-length M13 pIII coat protein sequence. The total number of transformants obtained was determined to be 3.6×10^{10} .

Selection of mutant pHLA specific phage clone

Phage clones bearing scFvs specific to p53^{R175H}/HLA-A*02:01 pHLA were identified using an approach described elsewhere (see, e.g., Skora *et al*, 2015 *PNAS*.112:9967-72). One μ g of biotinylated HLA-A*02:01 pHLA monomer complexes were conjugated to 25 μ L of M-280 streptavidin magnetic Dynabeads (Thermo Fisher Scientific, 11206D). During the enrichment phase (Round 1), phages were negatively selected with a mixture of unconjugated Dynabeads and free streptavidin protein (RayBiotech, Norcross, GA, 228-11469). After negative selection, supernatant containing unbound phages were transferred for positive selection using 1 μ g of p53^{R175H}/HLA-A*02:01 pHLA. Beads were then washed and phages were eluted to infect mid-log-phase SS320 bacteria, with the addition of M13K07 helper phages (multiplicity of infection of 4). Bacteria were then grown overnight at 30°C for phage production and the phages were precipitated the next morning with PEG/NaCl.

During the selection phase (Rounds 2-5), phages from the previous round were subjected to two stages of negative selection: 1) against cell lines without p53^{R175H}/HLA-A*02:01 (RPMI 6666, Jurkat, Raji, SigM5, HH, T2, and NCI-H441) and 2) against p53^{WT}/HLA-A*02:01 pHLA, unrelated HLA-A*02:01 pHLA, and free streptavidin. For negative selection using cell lines, phages were incubated with a total number of $0.5-1 \times 10^7$ of cells at 4°C overnight. After negative selection, beads were isolated and unbound phages were transferred for positive selection by incubating with 1 μ g (Round 2), 0.5 μ g (Round 3), or 0.25 μ g (Round 4, 5) of p53^{R175H}/HLA-A*02:01 pHLA. Phages were then eluted and amplified by infecting SS320 as described above.

After five rounds of selection, SS320 cells were infected with a limiting dilution of the enriched phages. A total of 190 individual colonies of SS320 were picked and phage DNA was PCR amplified by primers flanking the CDRs (Forward:

GGCCATGGCAGATATTCAGA (SEQ ID NO:198), Reverse:

- 5 CCGGGCCTTTATCATCATC (SEQ ID NO:199)) using Q5 Hot Start High-Fidelity 2X Master Mix (New England BioLabs, M0494L) and Sanger sequenced by GENEWIZ (South Plainfield, NJ). Sequences flanking the CDRs were trimmed using DNA Baser Sequence Assembler v4 (Arges, Romania) and the sequences spanning the CDRs were clustered using the CD-HIT Suite. Colonies containing unique phage clones were selected and grown
10 overnight in 400 μ L of media in deep 96-well plates (Thermo Fisher Scientific, 278743) with the addition of M13K07 helper phages. Bacteria were pelleted the next day and the phage-laden supernatants were used for downstream analysis.

Peptide pulsing

- For peptide pulsing, T2 cells were washed with serum-free RPMI-1640 media before
15 incubation at $0.5-1 \times 10^6$ cells per mL in serum-free RPMI-1640 containing peptides at the specified concentration of 2 hours at 37°C. For experiments assessed using flow cytometry, human β 2M (ProSpec, East Brunswick, NJ, PRO-337) at 10 μ g/mL was added with the peptides and specified in the figure legends of such experiments.

Flow cytometry

- 20 Phage staining of peptide-pulsed T2 cells was performed with 50 μ L phage supernatant on ice for 1 hour, followed by staining with 1 μ g of rabbit anti-M13 antibody (Novus Biologicals, NB100-1633), and anti-rabbit-PE (BioLegend, 406421). HLA-A*02 staining was performed by staining cells with fluorescently labeled anti-human HLA-A*02 (BB7.2, BioLegend, 343308) or mouse isotype IgG2b, κ (BioLegend, 402206). Stained cells
25 were analyzed using an LSRII flow cytometer (Becton Dickinson, Mansfield, MA) or an iQue Screener (IntelliCyt, Albuquerque, NM).

ELISAs

Streptavidin-coated, 96-well plates (R&D Systems, Minneapolis, MN, CP004) were coated with 50 ng of biotinylated HLA-A*02:01 pHLA monomers in 50 μ L of blocking

buffer (PBS with 0.5% BSA, 2 mM EDTA, and 0.1% sodium azide) or 25 ng of recombinant human CD3 ϵ/δ (Acro Biosystems, DE, CDD-H52W4) at 4°C overnight. Plates were washed with 1X TBST (TBS + 0.05% Tween-20) using a BioTek 405 TS plate washer (BioTek, Winooski, VT). Serial dilutions of scDb or IgG was incubated on the plate for 1 hour at RT and washed. For scDbs, the plate was then incubated with 1 μ g/mL recombinant protein L (Thermo Fisher Scientific, 77679) for 1 hour at RT, washed, followed by incubation with anti-protein L HRP (1:10000, Abcam, ab63506) for 1 hour at RT. For IgG, the plate was incubated with anti-human IgG HRP (1:1000, Thermo Fisher Scientific 62-8420) for 1 hour at RT. Plates were washed, 50 μ L of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (BioLegend, 4211101) was added to each well, and the reaction was quenched with 50 μ L 2N sulfuric acid (Thermo Fisher Scientific). Absorbance at 450 nm was measured with a Synergy H1 Multi-Mode Reader (BioTek).

scDb production

scDbs were produced by cloning gBlocks (IDT, Coralville, Iowa) encoding each of the variants in the format (from N- to C-terminus): IL-2 signal sequence, anti-pHLA variable light chain (V_L), GGGGS (SEQ ID NO:200) short linker, anti-CD3 variable heavy chain (V_H), (GGGGS)₃ (SEQ ID NO:201) long linker, anti-CD3 V_L, GGGGS (SEQ ID NO:200) short linker, anti-pHLA V_H, and 6 x HIS tag into linearized pcDNA3.4 vector (Thermo Fisher Scientific, A14697). The proteins were expressed by the Eukaryotic Tissue Culture Core Facility of Johns Hopkins University. Briefly, 1 mg of plasmid DNA was transfected with polyethylenimine (PEI) at a ratio of 1:3 into 1 L of FreeStyle 293-F cells at a concentration of 2–2.5 x 10⁶ cells per mL and the transfected cells incubated at 37°C. Five days after transfection, culture media was collected and filtered through a 0.22- μ m unit. The scDbs were purified using HisPur Ni-NTA Resin (Thermo Fisher Scientific, 88222) and desalted into PBS pH 7.4 or 20 mM Tris pH 9.0, 150 mM NaCl using 7k MWCO Zeba Spin desalting columns (Thermo Fisher Scientific, 89890). Proteins were quantified using a 4 – 15% Mini-PROTEAN TGX gel (Bio-Rad, Hercules, CA, 4568085) and/or NanoDrop (Thermo Fisher Scientific). Alternatively, the scDb proteins were produced by GeneArt (Thermo Fisher Scientific) in Expi293s, purified with a HisTrap column (GE Healthcare, 17-5255-01) followed by size exclusion chromatography with a HiLoad Superdex 200 26/600

column (GE Healthcare, 28989335). Analytic chromatography was performed using TSKgel G3000SWxl column (TOSOH Bioscience, Tokyo, Japan) using a running buffer of 50 mM sodium phosphate and 300 mM sodium chloride at pH 7, at a flow rate of 1.0 mL/minute.

*Surface plasmon resonance affinity measurements of p53^{R175H}/HLA-A*02:01 and H2-scDb interaction*

Biotinylated p53^{R175H}/HLA-A*02:01, p53^{WT}/HLA-A*02:01, and H2-scDb binding experiments were performed at 25°C using a Biacore T200 SPR instrument (GE Healthcare). Approximately 100-110 response units (RU) of biotinylated p53^{R175H}/HLA-A*02:01 and p53^{WT}/HLA-A*02:01 were captured in flow cells (Fc) 2 and 4, respectively, using a streptavidin chip. Single-cycle kinetics were performed by injecting increasing concentrations (3, 12, 50, 200, and 800 nM) of purified H2-scDb which was flowed over Fc 1-4. Binding responses for kinetic analysis were both blank- and reference-subtracted. Both binding curves were fit with a 1:1 binding model using Biacore Insight evaluation software.

Differential Scanning Fluorimetry

Thermal stability of the H2-scDb was evaluated by a differential scanning fluorimetry (DSF) assay which monitor the fluorescence of a dye that binds to the hydrophobic region of a protein as it becomes exposed upon temperature induced denaturation. Reaction mixture (20 µL) was set up in a white low-profile 96-well, unskirted polymerase chain reaction plate (BioRad, MLL9651) by mixing 2 µL of purified H2-scDb at a concentration of 1 mg/mL (final concentration ~2 µg) with 2 µL of 50X SYPRO orange dye (Invitrogen, S6650, 5X final concentration) in PBS, pH 7.4. The plate was sealed with an optical transparent film and centrifuged for 1,000 x g for 30 seconds. Thermal scanning was performed from 25 to 100 °C (1 °C/minute temperature gradient) using a CFX9 Connect real-time polymerase chain reaction instrument (BioRad). Protein unfolding/melting temperature T_m was calculated from the maximum value of the negative first derivative of the melt curve using CFX Manager software (BioRad).

CRISPR-mediated knockout of TP53

The Alt-R CRISPR system (IDT) was used to knock out the *TP53* gene from KMS26, TYK-nu, and KLE cell lines. CRISPR-Cas9 crRNAs targeting *TP53* exon 3 (p53-5:

CCCCGGACGATATTGAACAA (SEQ ID NO:191) or p53-6:

CCCCTTGCCGTCCCAAGCAA (SEQ ID NO:202)) as well as CRISPR-Cas9 tracrRNA

were resuspended at 100 μ M with Nuclease-Free Duplex Buffer. The crRNAs and tracrRNA were duplexed at a 1:1 molar ratio for 5 minutes at 95°C followed by cooling down slowly to

RT according to the manufacturer's instructions. The duplexed RNA was then mixed with

Cas9 Nuclease at a 1.2:1 molar ratio for 15 minutes. A total of 40 pmols of the Cas9 RNP

complexed with *TP53* gRNA were mixed with 2×10^5 cells in 20 μ L of OptiMEM. This

mixture was loaded into a 0.1 cm cuvette (Bio-Rad, 1652089) and electroporated at 120V

and 16 ms using an ECM 2001 (BTX, Holliston, MA). Cells were transferred to complete

growth medium and cultured for 7 days. Single cell clones were established by limiting

dilution and genomic DNA was harvested using a Quick-DNA 96 Kit (Zymo Research,

Irvine, CA, D3012). A region flanking the CRISPR cut site was PCR amplified (forward

primer: GCTGCCCTGGTAGGTTTTCT (SEQ ID NO:203), reverse primer:

GAGACCTGTGGGAAGCGAAA (SEQ ID NO:204)) and Sanger sequenced to select for

clones with the desired *TP53* status.

Immunoblotting analysis

Cells were lysed in cold RIPA buffer (Thermo Fisher Scientific, 89901)

supplemented with protease inhibitor cocktail (Thermo Fisher Scientific, 87785). Protein

concentration was determined using a BCA assay (Thermo Fisher Scientific, 23227). Equal

amounts of total protein (20-50 μ g) were loaded in each lane of a 4-15% Mini-PROTEAN

TGX gel (Bio-Rad, 4568085) and transferred to polyvinylidene difluoride membranes after

electrophoresis. The membranes were incubated with appropriate primary antibodies (anti-

6x His tag, 1:2000, Abcam, ab9108; p53 [DO-1], 1:1000, Santa Cruz, sc-126; STAT2,

1:1000, Thermo Fisher Scientific, 44-362G; ZFP3, 1:1000, Thermo Fisher Scientific, PA5-

62726; β -actin [13E5], 1:1000, Cell Signaling Technology, 5125S; β -actin [8H10D10],

1:1000, Cell Signaling Technology, 3700S) and species-specific HRP-conjugated secondary

antibodies (1:5000-10000). Signal was detected by a ChemiDoc MP chemiluminescence

system (Bio-Rad).

Transfection of cell lines

gBlocks (IDT) encoding HLA and target proteins were cloned into pcDNA3.1 or pcDNA3.4 vectors (Thermo Fisher Scientific, V79020, A14697). COS-7, HEK293FT, and Saos-2 cells were transfected at 70-80% confluency using Lipofectamine 3000 (Thermo Fisher Scientific, L3000015) and incubated at 37°C overnight. A total of 15 µg and 30 µg plasmid (1:1 ratio of HLA plasmid/target protein plasmid in co-transfections) was used for T25 and T75 flasks, respectively.

Viral transduction of cell lines

HLA-A*02:01-encoding retrovirus was produced using the MSCV retroviral expression system (Clontech, Mountain View, CA, 634401). In brief, a gBlock encoding HLA-A*02:01-T2A-GFP (IDT) was cloned into the pMSCVpuro retroviral vector by HiFi DNA assembly (New England Biolabs, Ipswich, MA, E2621L). The pMSCVpuro-HLA-A*02:01-T2A-GFP plasmid was then co-transfected with a pVSV-G envelope vector into the GP2-293 packaging cell line. Viral supernatant was harvested 48 hours after transfection and concentrated 20-fold using Retro-X Concentrator (Clontech, 631456). RediFect Red-Fluc-GFP lentivirus particles (Perkin Elmer, Waltham, MA, CLS960003) was used for generating luciferase-expressing cell lines. NucLight green lentivirus (Essen Bioscience, Ann Arbor, MI, 4624) was used to generate TYK-nu cell lines with nuclear GFP expression.

For transduction, non-tissue culture-treated 48-well plates were coated with 200 µL of 10 µg/mL RetroNectin (Clontech, T100B) per well overnight at 4°C and blocked with 10% FBS for 1 hour at RT. Viral particles and 2×10^5 target cells were added to each well in a total volume of 500 µL cell culture media and spun at 2000 x g for 1 hour then incubated at 37°C. Selection with 1 µg/mL puromycin (Thermo Fisher Scientific, A1113803) began three days later. Transduced cells were sorted based on presence of GFP using FACS Aria Fusion (BD Biosciences, San Jose, CA) 10-14 days after transduction.

In vitro scDb co-incubation assays

To each well of a 96-well flat-bottom plate, the following components were combined in a final volume of 100 µL RPMI-1640 with 10% FBS, 1% Penicillin-Streptomycin, and 100 IU/mL IL-2: scDb diluted to the specified concentration, 5×10^4 human T cells, and $1-5 \times 10^4$ target cells (COS-7, T2, or other tumor cell lines). The effector

to target cell ratio is specified in the figure legend for each experiment. The co-culture plate was incubated for 20 hour at 37°C and conditioned media was assayed for cytokine and cytotoxic granule protein secretion using the Human IFN- γ Quantikine Kit (R&D Systems, Minneapolis, MN, SIF50), Human IFN- γ Flex Set Cytometric Bead Array (BD, 558269), or the MILLIPLEX Luminex assays (Millipore Sigma, HSTCMAG28SPMX13, HCD8MAG-15K) read on the Bioplex 200 platform (Bio-Rad). Cytotoxicity was assayed by CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, G7571), Bio-Glo Luciferase Assay (Promega, G7941), or Steady-Glo Luciferase Assay (Promega, E2510) per manufacturer's instructions. For CellTiter-Glo assays, percent cytotoxicity was calculated by subtracting the luminescence signal from the average of the T cell only wells and normalizing to the no scDb condition: $1 - (\text{scDb well} - \text{T cell only}) / (\text{no scDb well} - \text{T cell only}) \times 100$. For Bio-Glo assays, percent cytotoxicity was calculated by normalizing luminescence signal to the no scDb condition: $1 - (\text{scDb well}) / (\text{no scDb well}) \times 100$.

Real-time live-cell imaging

A total of 1×10^4 NucLight Green-labeled target cells were plated in each well of a 96-well flat bottom plate and allowed to attach for 4 hours before adding 2×10^4 T cells and scDb at the indicated concentrations. Each condition was plated in triplicate. Plates were imaged every 6 hours using the IncuCyte ZOOM Live-Cell analysis system (Essen Bioscience) for a total of 120 hours. Four images per well at 10X zoom were collected at each time point. The number of GFP positive objects per mm^2 in each well was quantified using the green fluorescence channel.

*Expression, purification and refolding of p53^{R175H}/HLA-A*02:01*

Plasmids for HLA-A*02:01 and $\beta 2\text{M}$ were received from the NIH Tetramer Facility (Atlanta, GA) and separately transformed into BL21(DE3) cells. Each was expressed in inclusion bodies using auto-induction media. Purification of the HLA-A*02:01 and $\beta 2\text{M}$ inclusion bodies was achieved with a series of detergent washes followed by solubilization with 8 M urea. Refolding of the HLA-A*02:01, $\beta 2\text{M}$, and mutant p53^{R175H} peptide was performed. Briefly, solubilized HLA-A*02:01 and $\beta 2\text{M}$ were combined in a refolding buffer containing 100 mM Tris pH 8.3, 400 mM L-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 2 mM PMSF, and 30 mg of the mutant p53^{R175H}

peptide (aa 168-176, HMTEVVRHC; SEQ ID NO:1) dissolved in 1 mL of DMSO. The resultant solution was stirred at 4°C for 2 days, with two further additions of HLA-A*02:01 on day 2, concentrated to 10 mL and purified by size exclusion chromatography on a HiLoad 26/60 Superdex 75 Prep grade column (GE Healthcare, 28989334). For incubation with the H2-Fab, purified pHLA-A*02:01 was concentrated to ~1-3 mg/mL and stored at -80°C until use.

Production of the H2-Fab antibody fragment

The light chain (LC) and heavy chain (HC) variable region sequences of H2 scFv were grafted onto the respective constant chains of trastuzumab and separately cloned into a pcDNA3.4 vector (Thermo Fisher Scientific, A14697). Both chains were preceded by a mouse IgKVIII signal peptide. Before large-scale expression of full-length antibody, optimization of the LC:HC DNA ratio for transfection was performed to determine optimal recombinant protein yields. For a 1 L expression, a total of 50 µg of purified plasmids (1:1 LC:HC ratio) were transfected with PEI at a ratio of 1:3 into Freestyle 293-F cells at a concentration of 2–2.5 x 10⁶ cells per mL and incubated at 37°C for 7 days. The media was harvested via centrifugation, filtered through a 0.22-µm unit and the full-length antibody was purified via protein A affinity chromatography on a HiTrap MabSelectTM SuReTM column (GE Healthcare, 29-0491-04). Full-length antibody was eluted using a linear gradient of 0–100 mM sodium citrate, pH 3.5. The protein A fractions containing pure H2 antibody were pooled, quantified by SDS-PAGE gel electrophoresis and dialyzed into 20 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA.

For generation of H2-Fab fragments, ~1–3 mg of full-length antibody was mixed with 0.5 mL of a 50 % Immobilized Papain slurry (Thermo Fisher Scientific, 20341) pre-activated with digestion buffer (20 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA) containing 20 mM cysteine-HCl. The mixture was incubated at 37°C overnight with constant shaking at 200 rpm. The H2 antibody digest was separated from the immobilized resin by a gravity resin separator and washed with 10 mM Tris-HCl, pH 7.5. Newly generated H2-Fab fragments were further purified by cation-exchange chromatography using a Mono-S column (GE Healthcare, 17516801) and eluted using a linear gradient of 0–500 mM NaCl.

The H2-Fab fragments were concentrated, mixed with equimolar p53^{R175H}/HLA-A*02:01 and incubated at 4°C overnight. The H2-Fab–p53^{R175H}/HLA-A*02:01 mixture was evaluated by size exclusion chromatography on a Superdex™ 200 Increase 10/300 column (GE Healthcare, 28990944). The fractions of ~ 98% pure pHLA-A*02:01–H2-Fab complex
 5 were pooled, concentrated to 12.6 mg/mL and exchanged into a buffer containing 25 mM HEPES, pH 7.0, 200 mM NaCl.

Crystallization, data collection and structure determination

Crystals of the ternary complex H2-Fab–p53^{R175H}/HLA-A*02:01 were grown by vapor diffusion in hanging drops set up with a TTP mosquito robot with a reservoir solution
 10 of 0.2 M ammonium chloride and 20% (w/v) PEG 3350 MME. Crystals were flash-cooled in mother liquor. Data were collected at National Synchrotron Light Source-II at beamlines 17-ID-1(AMX) on a Dectris EIGER X 16M detector. The dataset was indexed, integrated and scaled using fastdp, XDS, and aimless. Monoclinic crystals of H2-Fab–p53^{R175H}/HLA-A*02:01 diffracted to 3.5 Å. The structure for the H2-Fab–p53^{R175H}/HLA-A*02:01 complex
 15 was determined by molecular replacement with PHASER using PDB ID 6O4Y and 6UJ9 as the search models. The data were refined to a final resolution of 3.5 Å using iterative rounds of refinement with REFMAC5 and manual rebuilding in Coot. Structures were validated using Coot and PDB Deposition tools. The model has 95.2% of the residues in preferred and 3.8% in allowed regions according to Ramachandran statistics (Table 20). Figures were
 20 rendered in PyMOL (v2.2.3, Schrödinger, LLC, New York, NY). Buried areas were calculated with PDBePISA. The docking angle that determines the relative orientation between the pHLA and the Fab/TCR was calculated by the web server TCR3d.

Mouse xenograft model

Female NOD.*Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* (NSG) mice at 6-10 weeks were acquired
 25 from the Jackson Laboratory (Bar Harbor, Maine, 005557) and treated in compliance with the institutional Animal Care and Use Committee approved protocol. In the early treatment model, mice were inoculated intravenously with 1 x 10⁶ luciferase-expressing KMS26 or KMS26-TP53 KO cells and 1 x 10⁷ *in vitro* expanded human T cells via lateral tail vein injection on day 0. On day 1, mice were randomized based on luminescence quantification
 30 using the IVIS imaging system and Living Image software (Perkin Elmer) to ensure similar

pretreatment tumor burden. Prior to imaging, mice received intraperitoneal injection of luciferin (150 μ L, RediJect D-Luciferin Ultra Bioluminescent Substrate, PerkinElmer, 770505) were anesthetized using inhaled isoflurane in an induction chamber for 5 minutes. After randomization, two-week micro-osmotic pumps (ALZET, Cupertino, CA, 1002) filled
5 with H2-scDb, isotype control scDb (scFv against an irrelevant pHLA linked with UCHL1 scFv), or vehicle only that had been primed in 1 mL PBS overnight at 37°C were placed intraperitoneally using sterile surgical technique. Tumor growth was serially monitored by bioluminescent imaging. In the established tumor model, mice were inoculated with 3.5×10^5 or 5×10^5 luciferase-expressing KMS26 cells and 1×10^7 human T cells via lateral tail
10 vein injection on day 0. On day 6, H2-scDb or isotype control scDb was administered similarly as in the early treatment model.

For mouse blood-based analysis, 200 μ L blood was collected in EDTA-treated microvettes (Sarstedt, Nümbrecht, Germany, 20.1278.100) by cheek bleed, followed by centrifugation at 1000 $\times$ g for 3 minutes. Plasma was collected and stored at -80 °C until
15 analysis. The blood cell pellet was resuspended with 100 μ L PBS, followed by two 5-minute incubations with 1 mL ACK lysis buffer (Thermo Fisher Scientific, A1049201) with one PBS wash in between, and resuspended in flow stain buffer with TruStain FcX (anti-mouse CD16/32) antibody (BioLegend, 101320) and cell-surface staining antibodies. For scDb quantification, plasma was thawed and incubated in biotinylated recombinant human CD3 ϵ / δ
20 coated streptavidin plate and detected as described in “ELISA.”

Statistical analysis

Data are presented as means $\pm$ SD unless otherwise specified. Statistical analyses were carried out using specific tests indicated in the figure legends. A *P* value of < 0.05 was used to denote statistical significance. All analyses were performed using Prism version 8.0
25 (GraphPad, San Diego, CA). In all figures, NS, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Example 5: Exemplary bispecific molecules targeting a TP53 mutation

Table 23. Amino acid sequences of bispecific molecules targeting a TP53 mutation

	Sequence	SEQ ID NO
H2-UCHT1-scDb	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGK APKLLIYSAYFLYSGVPSRFSGRSGTDFTLTISLQPEDFATY YCQQYSRYSPTFGQGKVEIKGGGGSEVQLQQSGPELVKPG ASMKISCKASGYSTGYTMNWVKQSHGKNLEWMGLINPYK GVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCA RSGYYGDSDWYFDVWGAGTTVTVSSGGGGSGGGGSGGGGS DIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKPDG TVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQEDIATY FCQQGNTLPWTFAGGTKLEIKGGGGSEVQLVESGGGLVQPG GSLRLSCAASGFNVYASGMHWVRQAPGKGLEWVAKIYPDSD YTTYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCS RDSSFYYVYAMDYWGQGTLVTVSS	733
H20-UCHT1-scDb	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGK APKLLIYSASFLYSGVPSRFSGRSGTDFTLTISLQPEDFATYY CQQSNAYPITFGQGKVEIKGGGGSEVQLQQSGPELVKPGAS MKISCKASGYSTGYTMNWVKQSHGKNLEWMGLINPYKGV STYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCARS GYYGDSDWYFDVWGAGTTVTVSSGGGGSGGGGSGGGGSDI QMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKPDGTV KLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQEDIATYFC QQGNTLPWTFAGGTKLEIKGGGGSEVQLVESGGGLVQPGGS LRLSCAASGFNLNSYYMHWVRQAPGKGLEWVAMIIPGYGYT NYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRS YYMYMDYWGQGTLVTVSS	734
H2-UCHT1- tandem scFvs	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGK APKLLIYSAYFLYSGVPSRFSGRSGTDFTLTISLQPEDFATY YCQQYSRYSPTFGQGKVEIKGGGGSGGGGSGGGGSEVQL VESGGGLVQPGGSLRLSCAASGFNVYASGMHWVRQAPGKG LEWVAKIYPDSDYTTYADSVKGRFTISADTSKNTAYLQMNSL RAEDTAVYYCSRDSFYVYAMDYWGQGTLVTVSSGGGGGS EVQLQQSGPELVKPGASMKISCKASGYSTGYTMNWVKQSH GKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYME LLSLTSEDSAVYYCARSGYYGDSDWYFDVWGAGTTVTVSSG GGGSGGGGSGGGGSDIQMTQTTSSLSASLGDRVTISCRASQDI RNYLNWYQQKPDGTVKLLIYYTSRLHSGVPSKFSGSGSGTDY SLTISNLEQEDIATYFCQQGNTLPWTFAGGTKLEIK	735

H2-UCHT1-scDb-bispecific single-chain Fc	DIQMTQSPSSLSASVGDRVITICRASQDVNTAVAWYQQKPGKAPKLLIYSAYFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATY YCQQYSRYSPTFTGQGTKVEIKGGGGSEVQLQQSGPELVKPG ASMKISCKASGYSTGYTMNWVKQSHGKNLEWMGLINPYK GVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCA RSGYYGDSDWYFDVWGAGTTVTVSSGGGGSGGGGSGGGGS DIQMTQTTSSLSASLGDRVITICRASQDIRNYLNWYQQKPDG TVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQEDIATY FCQQGNTLPWTFAGGTKLEIKGGGGSEVQLVESGGGLVQPG GSLRLSCAASGFNVYASGMHWVRQAPGKGLEWVAKIYPDSD YTTYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCS RDSSFYYVYAMDYWGQGLTVTVSSGGGGDKTHTCPPCPAPE LLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPCEEQYGSTYRCVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSD GSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS LSPGKGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSDKTHC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPCEEQYGSTYRCVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP SREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK	736

H2-UCHT1- tandem scFvs single-chain Fc	DIQMTQSPSSLSASVGDRVITTCRASQDVNTAVAWYQQKPGK APKLLIYSAYFLYSGVPSRFSGSRSGTDFLTITSSLPEDFATY YCQQYSRYSPVTFGQGTKVEIKGGGGSGGGGSGGGGSEVQL VESGGGLVQPGGSLRLSCAASGPNVYASGMHWVRQAPGKG LEWVAKIYPDSYTYADSVKGRFTISADTSKNTAYLQMNSL RAEDTAVYYCSRDSFYVYAMDYWGQGTLLTVSSGGGGGS EVQLQQSGPELVKPGASMKISCKASGYSTGYTMNWVKQSH GKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYME LLSLTSEDSAVYYCARSGYYGDSDWYFDVWGAGTTVTVSSG GGGSGGGGSGGGGSDIQMTQTTSSLSASLGDRVITISCRASQDI RNYLNWYQQKPDGTVKLLIYYTSRLHSGVPSKFSGSGSGTDY SLTISNLEQEDIATYFCQQGNTLPWTFAGGKLEIKGGGGDKT HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS HEDPEVKFNWYVDGVEVHNAKTKPCEEQYGSTYRCVSVLTV LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYT LPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGKGGGGSGGGGSGGGGSGGGGSGGGGSGGGG GSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPCEEQYGSTYRC VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPGK	737

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not
5 limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A molecule comprising an antigen-binding domain that can bind to a peptide-HLA complex, wherein said peptide is derived from a modified p53 polypeptide.
2. The molecule of claim 1, wherein said modified p53 polypeptide comprises from 7 amino acids to 25 amino acids.
3. The molecule of claim 2, wherein said modified p53 polypeptide comprises 9 amino acids.
4. The molecule of any one of claim 1 to claim 3, wherein said modified p53 polypeptide comprises an amino acid sequence set forth in SEQ ID NO:1.
5. The molecule of claim 4, wherein said antigen binding domain comprises an amino acid sequence set forth in SEQ ID NO:137, SEQ ID NO:138, SEQ ID NO:139, SEQ ID NO:140, or SEQ ID NO:141.
6. The molecule of any one of claim 1 to claim 5, wherein said molecule is selected from the group consisting of an antibody, an antibody fragment, a single chain variable fragment (scFv), a chimeric antigen receptor (CAR), a T cell receptor (TCR), a TCR mimic, a tandem scFv, a bispecific T cell engager, a diabody, a single-chain diabody (scDb), an scFv-Fc, a bispecific antibody, and a dual-affinity re-targeting antibody (DART).
7. The molecule of any one of claim 1 to claim 6, wherein said molecule further comprises an antigen-binding domain that can bind to an effector cell receptor selected from the group consisting of CD3, CD28, CD4, CD8, CD16a, NKG2D, PD-1, CTLA-4, 4-1BB, OX40, ICOS, and CD27.

8. The molecule of claim 7, wherein said antigen-binding domain that can bind to an effector cell can bind to CD3, wherein said antigen-binding domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO:170, SEQ ID NO:171, SEQ ID NO:172, SEQ ID NO:173, SEQ ID NO:174, SEQ ID NO:175, SEQ ID NO:176, SEQ ID NO:177, SEQ ID NO:178, SEQ ID NO:179, SEQ ID NO:180, SEQ ID NO:181, SEQ ID NO:182, and SEQ ID NO:183.
9. A molecule comprising an antigen-binding domain that can bind to a peptide-HLA complex, wherein said peptide is derived from a modified RAS polypeptide.
10. The molecule of claim 9, wherein said modified RAS peptide comprises from 7 amino acids to 25 amino acids.
11. The molecule of claim 10, wherein said modified RAS peptide comprises 10 amino acids.
12. The molecule of any one of claim 9 to claim 11, wherein said modified RAS peptide comprises an amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4.
13. The molecule of claim 12, wherein said modified RAS peptide comprises SEQ ID NO:2, and wherein said antigen binding domain comprises an amino acid sequence set forth in SEQ ID NO:142, SEQ ID NO:143, SEQ ID NO:144, SEQ ID NO:145, SEQ ID NO:146, SEQ ID NO:147, SEQ ID NO:148, or SEQ ID NO:149.
14. The molecule of claim 12, wherein said modified RAS peptide comprises SEQ ID NO:3, and wherein said antigen binding domain comprises an amino acid sequence set forth in SEQ ID NO:150, SEQ ID NO:151, SEQ ID NO:152, SEQ ID NO:153, SEQ ID NO:154, SEQ ID NO:155, SEQ ID NO:156, SEQ ID NO:157, SEQ ID NO:158, SEQ ID NO:159, or SEQ ID NO:160.

15. The molecule of claim 12, wherein said modified RAS peptide comprises SEQ ID NO:4, and wherein said antigen binding domain comprises an amino acid sequence set forth in SEQ ID NO:161, SEQ ID NO:162, SEQ ID NO:163, SEQ ID NO:164, SEQ ID NO:165, SEQ ID NO:166, SEQ ID NO:167, SEQ ID NO:168, or SEQ ID NO:169.

16. The molecule of any one of claim 9 to claim 15, wherein said molecule is selected from the group consisting of an antibody, an antibody fragment, a single chain variable fragment (scFv), a chimeric antigen receptor (CAR), a T cell receptor (TCR), a TCR mimic, a tandem scFv, a bispecific T cell engager, a diabody, a single-chain diabody (scDb), an scFv-Fc, a bispecific antibody, and a dual-affinity re-targeting antibody (DART).

17. The molecule of any one of claim 9 to claim 16, wherein said molecule further comprises an antigen-binding domain that can bind to an effector cell receptor selected from the group consisting of CD3, CD28, CD4, CD8, CD16a, NKG2D, PD-1, CTLA-4, 4-1BB, OX40, ICOS, and CD27.

18. The molecule of claim 17, wherein said antigen-binding domain that can bind to an effector cell can bind to CD3, wherein said antigen-binding domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO:170, SEQ ID NO:171, SEQ ID NO:172, SEQ ID NO:173, SEQ ID NO:174, SEQ ID NO:175, SEQ ID NO:176, SEQ ID NO:177, SEQ ID NO:178, SEQ ID NO:179, SEQ ID NO:180, SEQ ID NO:181, SEQ ID NO:182, and SEQ ID NO:183.

19. A method for treating a mammal having a cancer, said method comprising:
administering to said mammal a molecule of any one of claim 1 to claim 20, wherein said cancer comprises cancer cells expressing said modified peptide.

20. The method of claim 19, wherein said mammal is a human.

21. The method of any one of claim 19 to claim 20, wherein said cancer is selected from the group consisting of Hodgkin's lymphoma, non-Hodgkin's lymphoma, acute myeloid leukemia, acute lymphoblastic leukemia, multiple myeloma, a myelodysplastic syndrome (MDS), a myeloproliferative disease, lung cancer, pancreatic cancer, gastric cancer, colorectal cancer, ovarian cancer, endometrial cancer, biliary tract cancer, liver cancer, breast cancer, prostate cancer, esophageal cancer, stomach cancer, kidney cancer, bone cancer, soft tissue cancer, head and neck cancer, glioblastoma multiforme, astrocytoma, thyroid cancer, germ cell tumor, and melanoma.

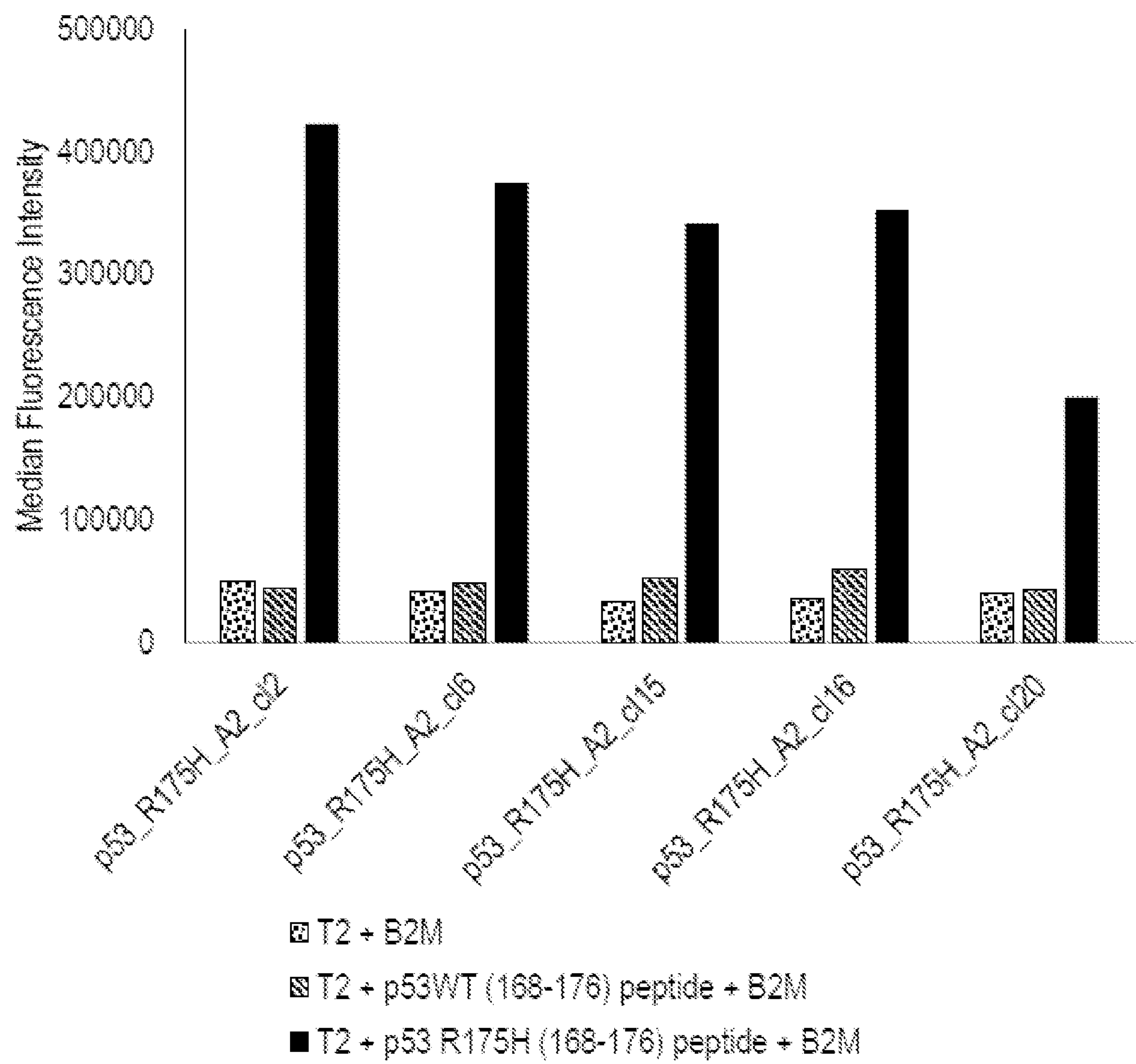


FIG. 1

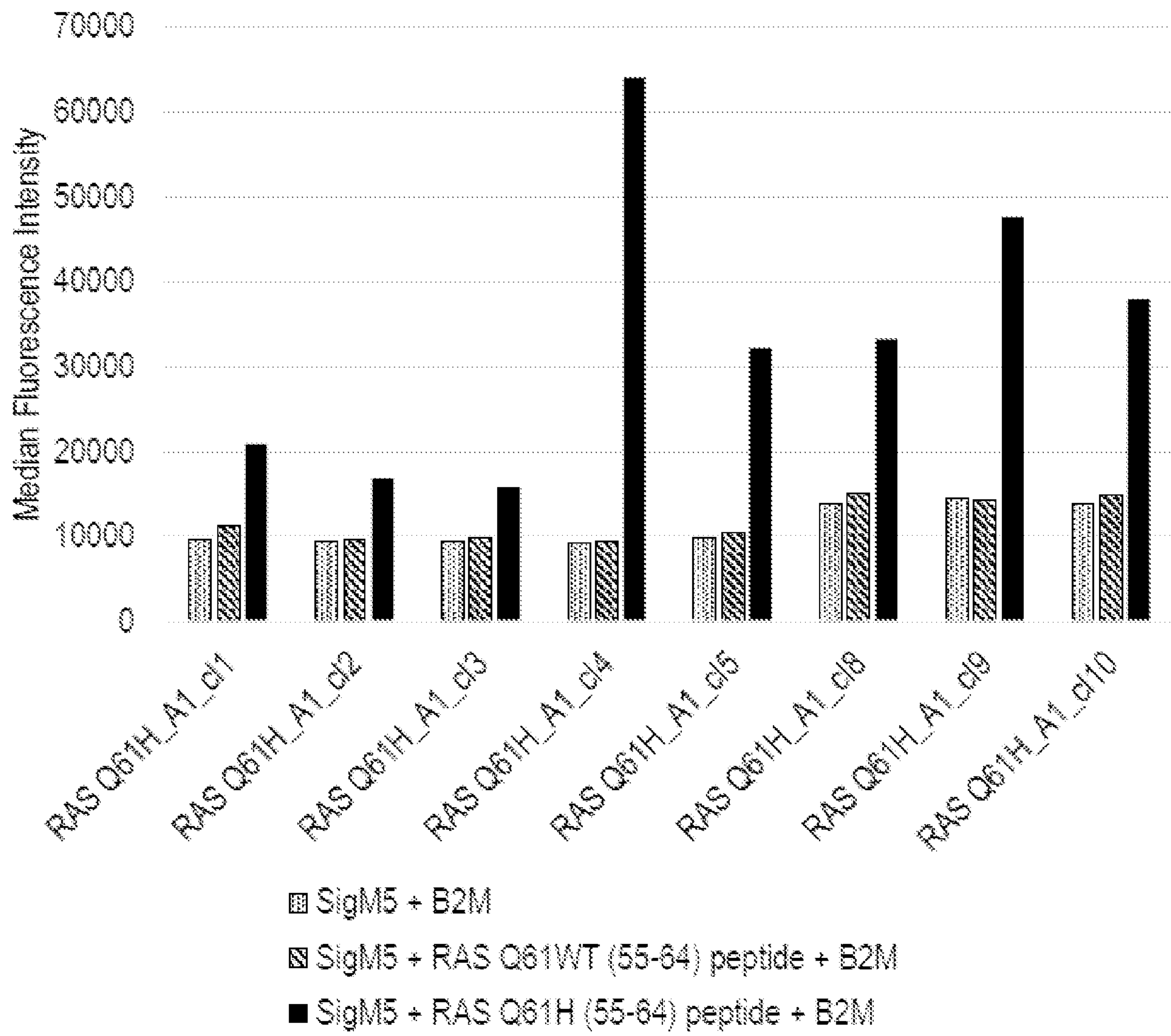


FIG. 2

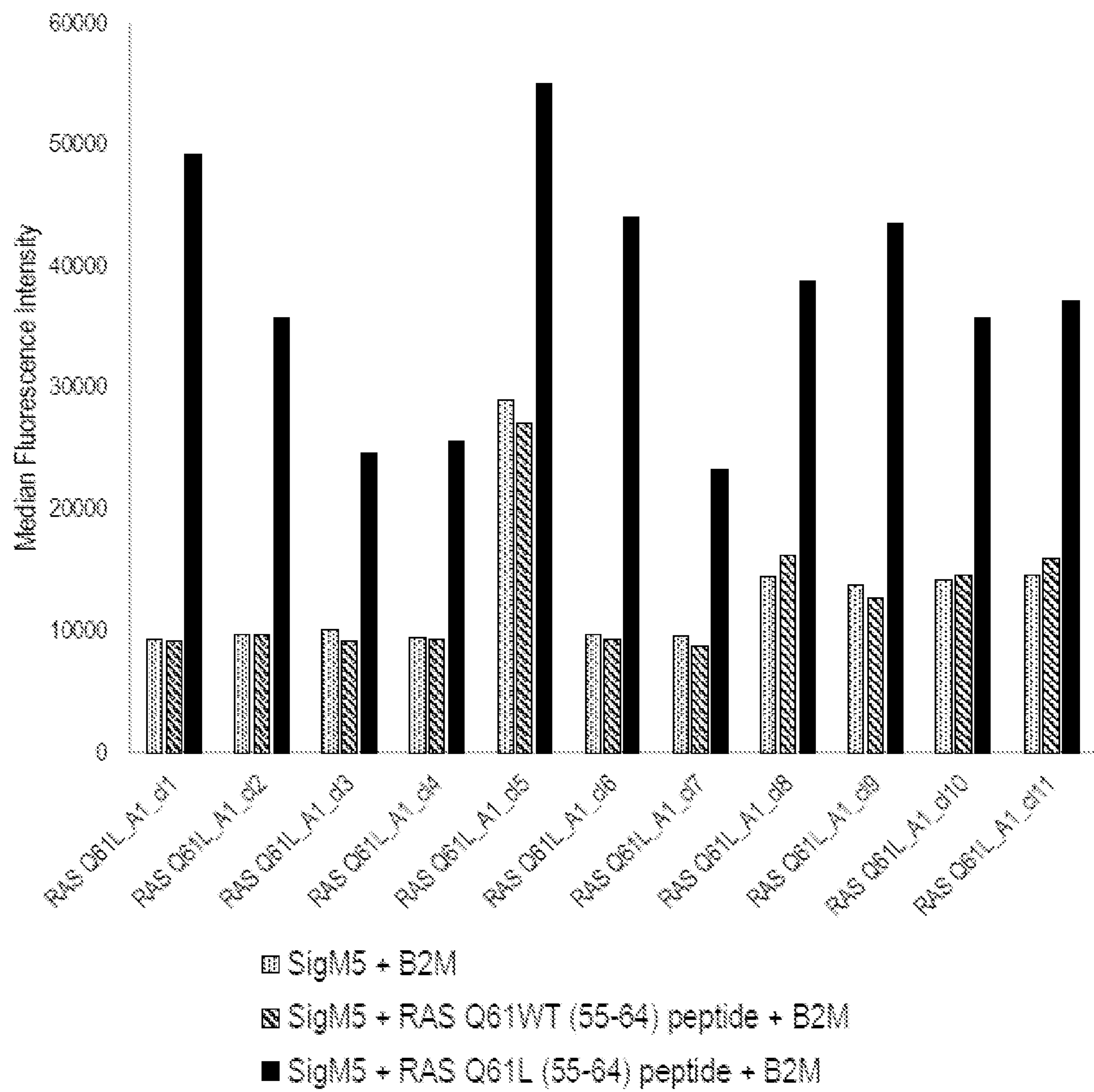


FIG. 3

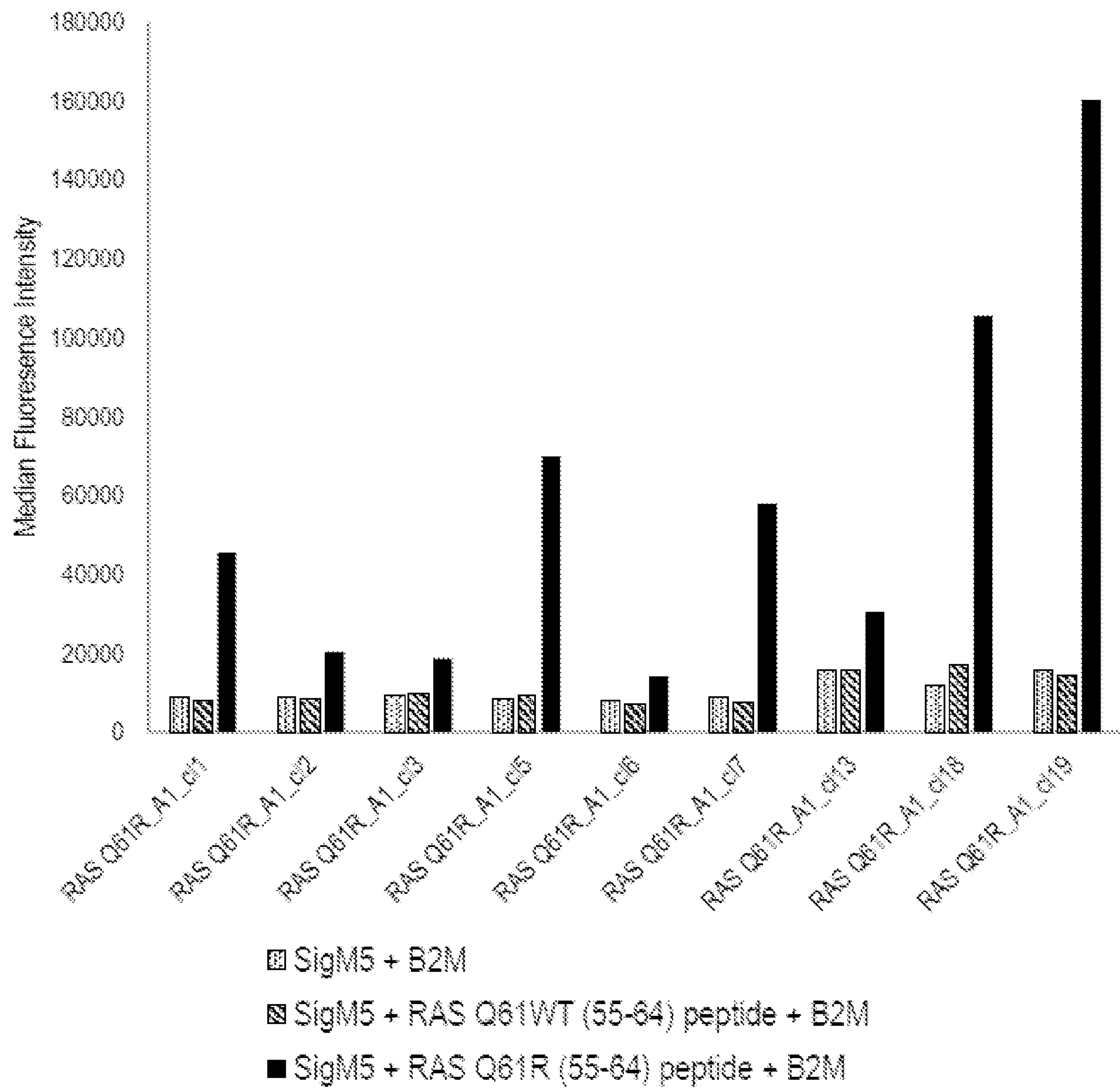


FIG. 4

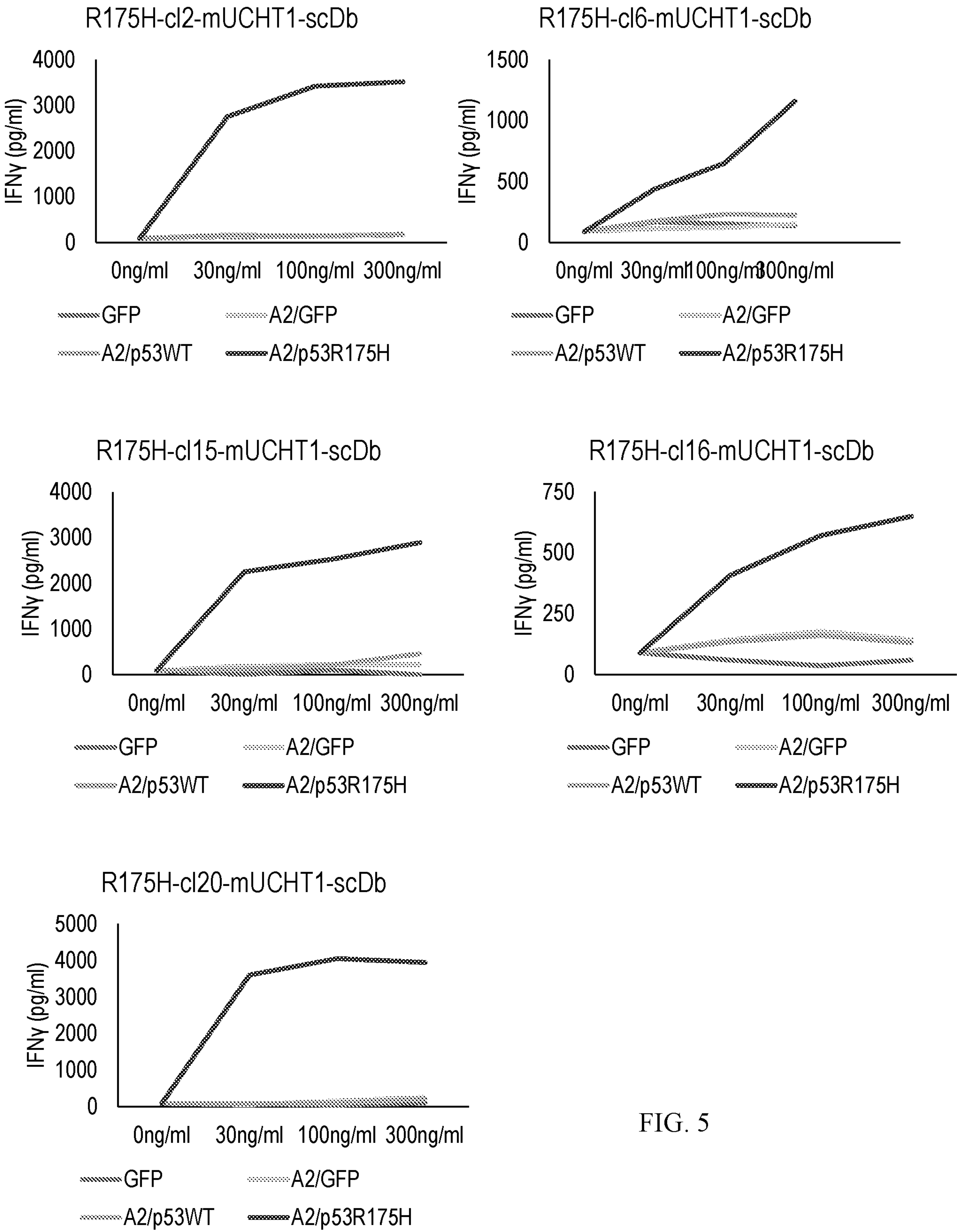


FIG. 5

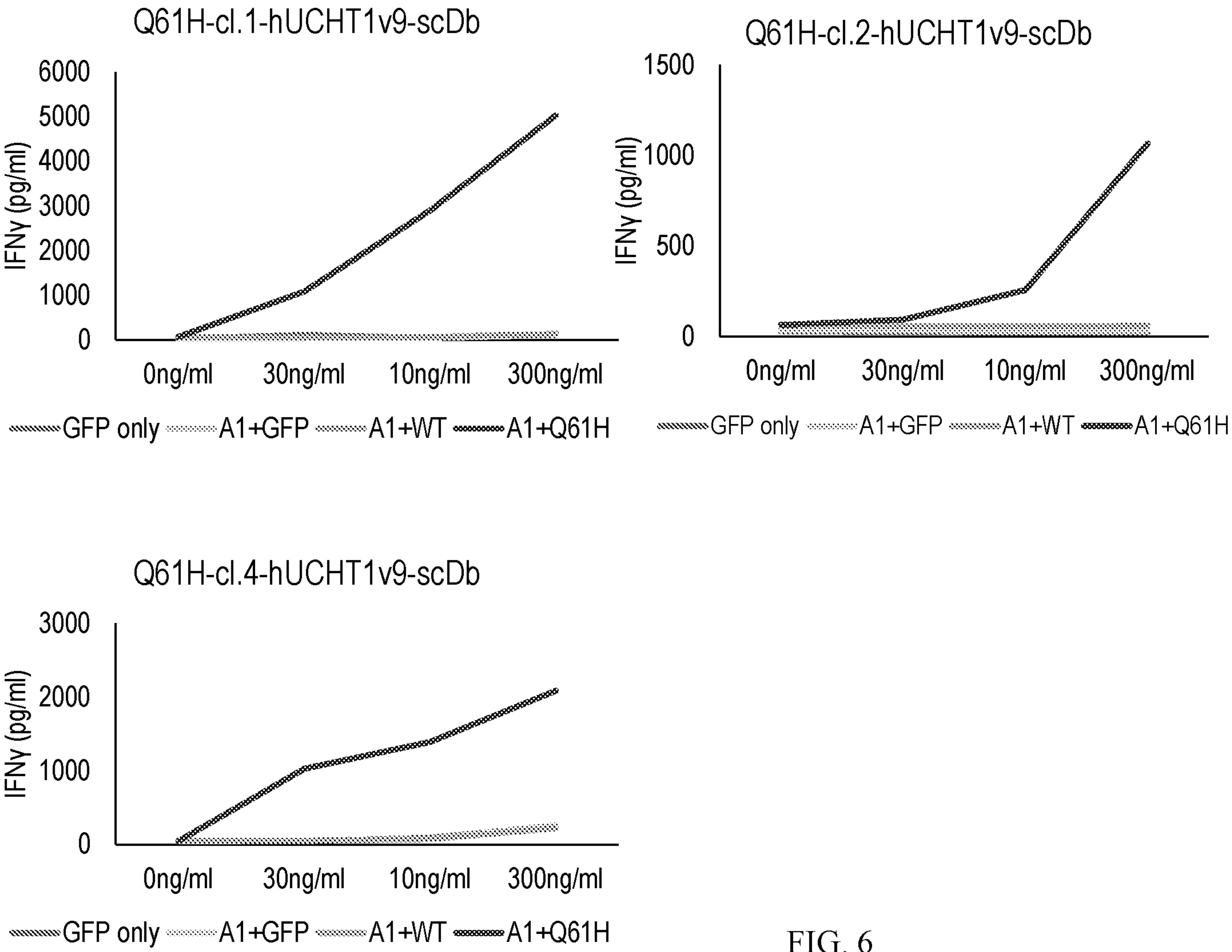


FIG. 6

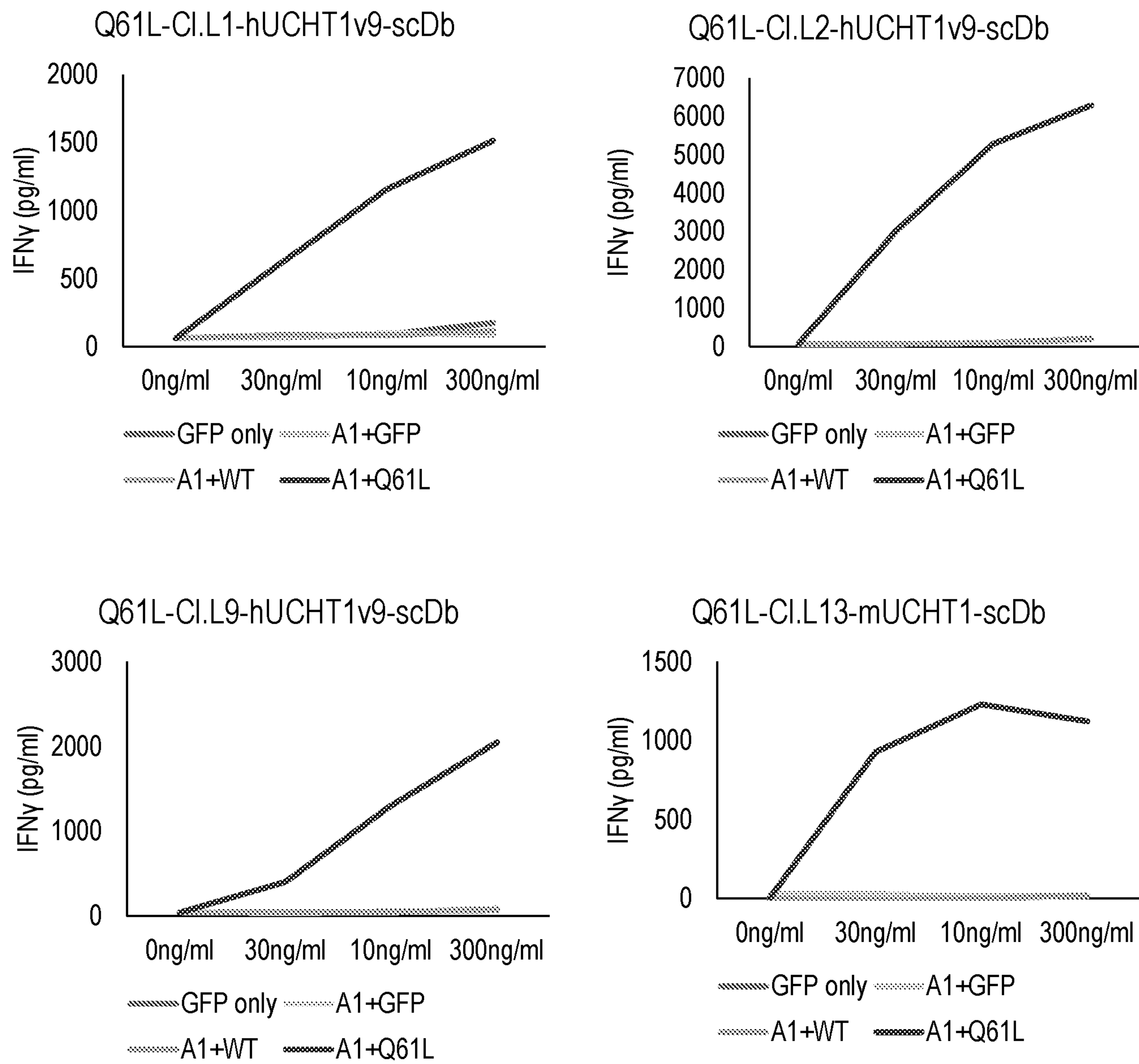


FIG. 7

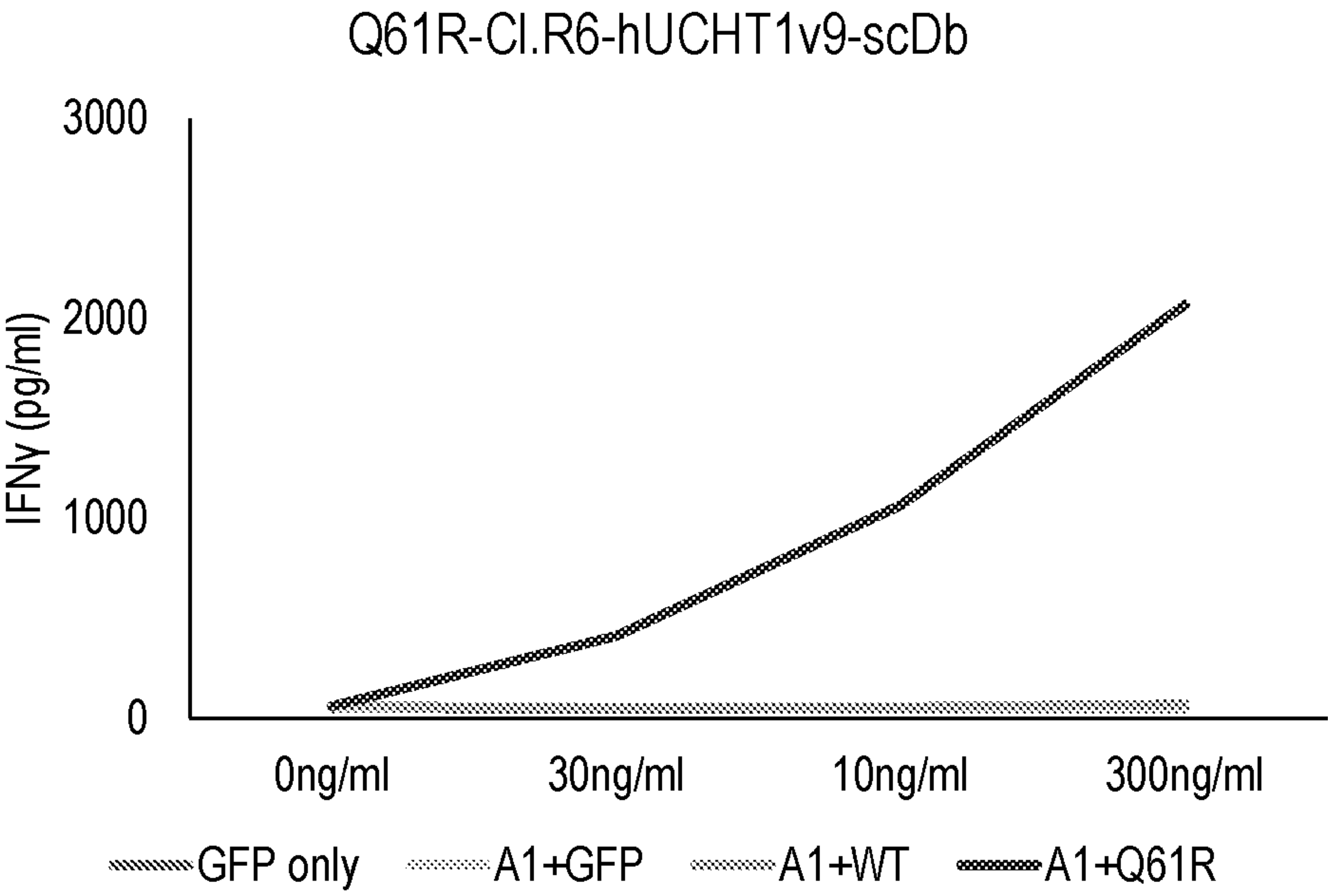


FIG. 8

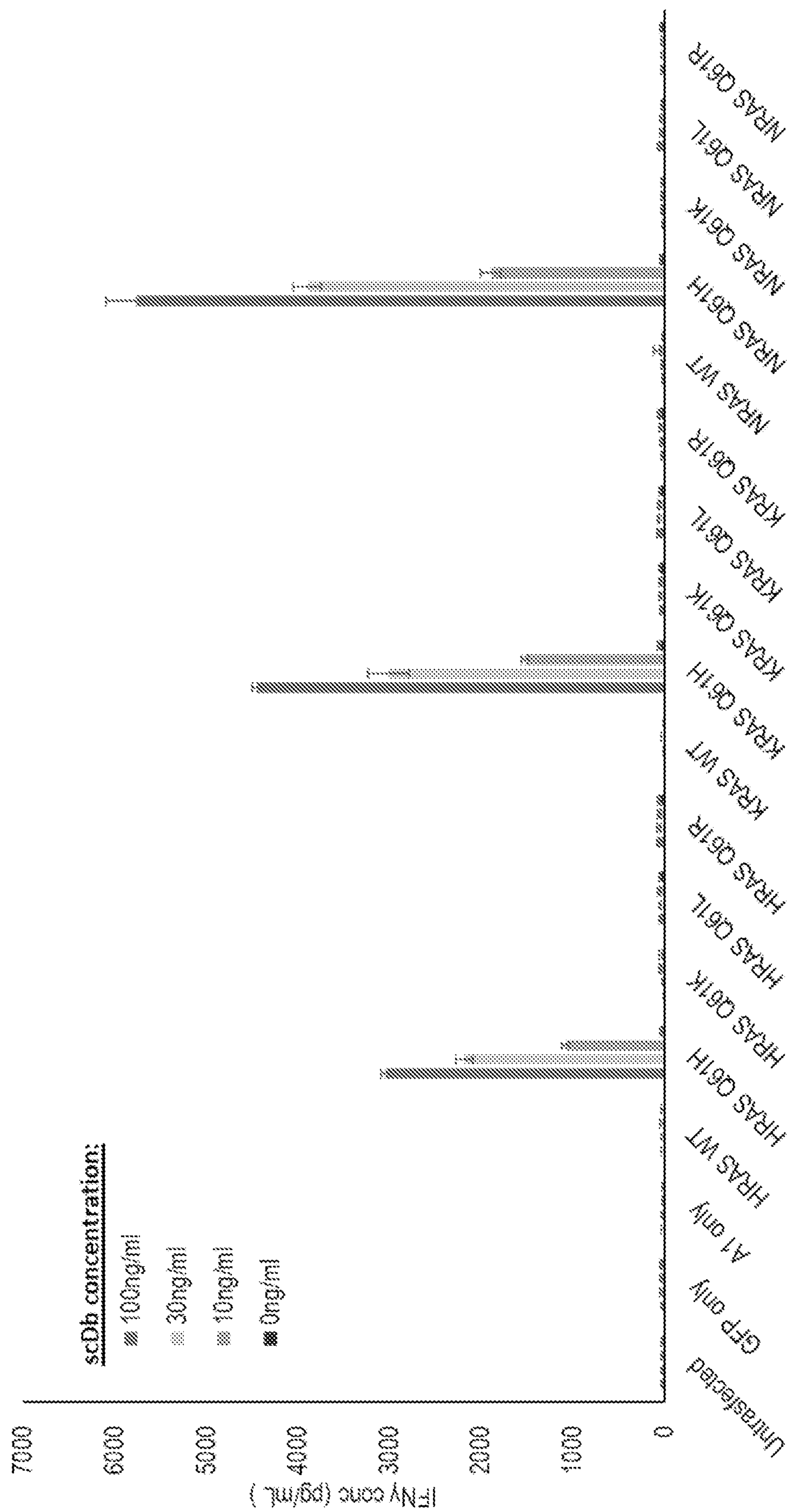


FIG. 9A

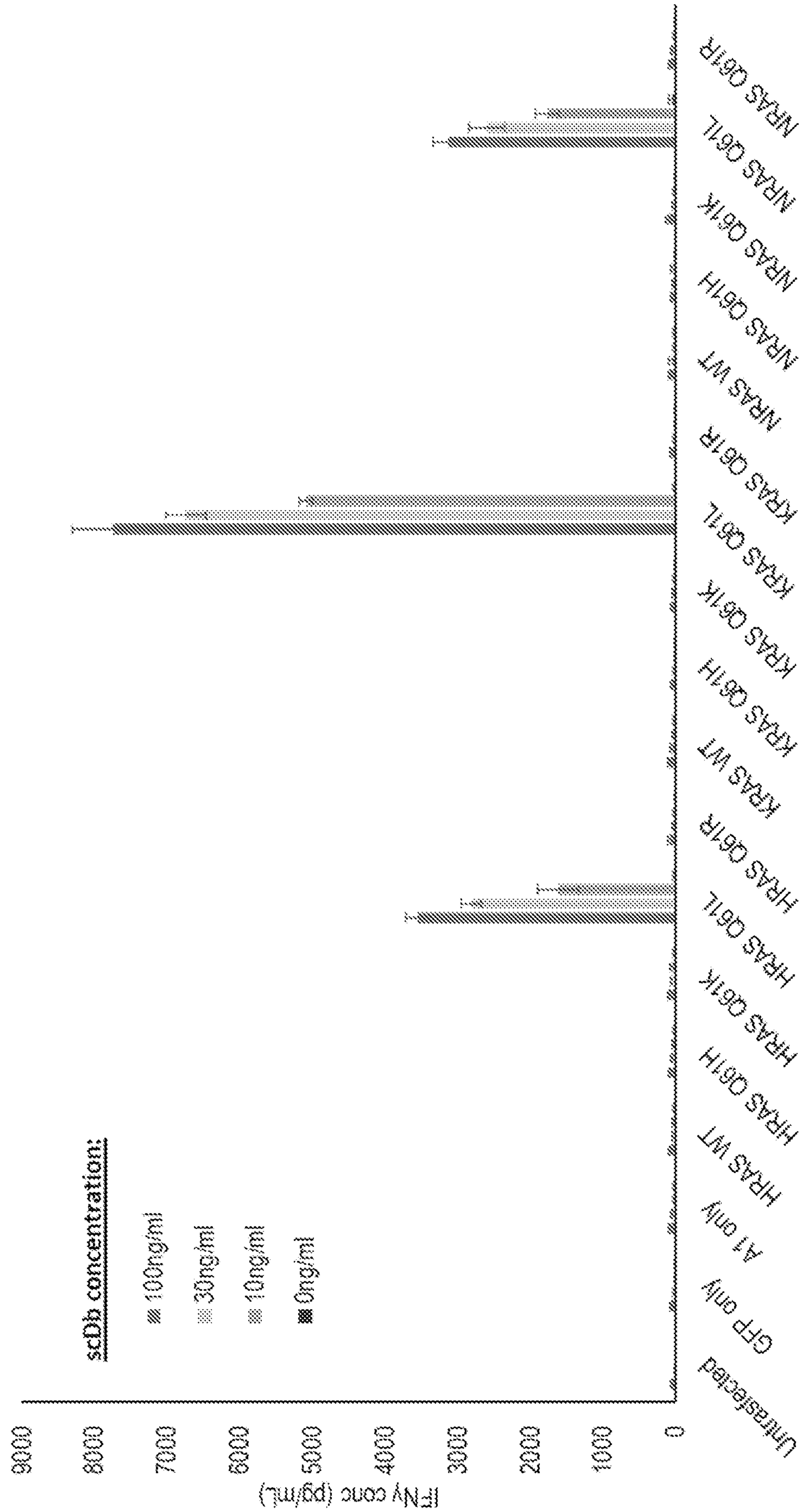


FIG. 9B

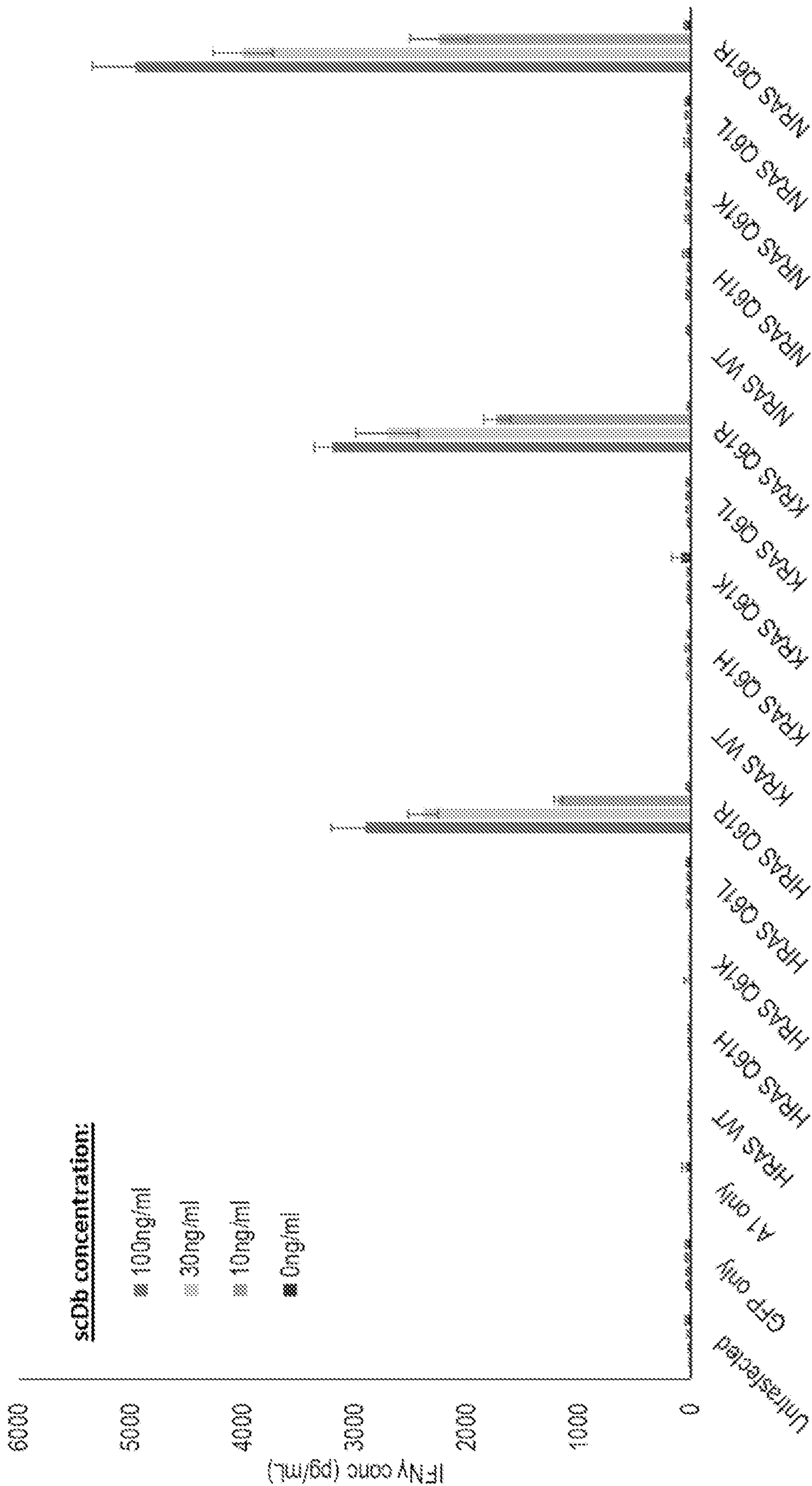


FIG. 9C

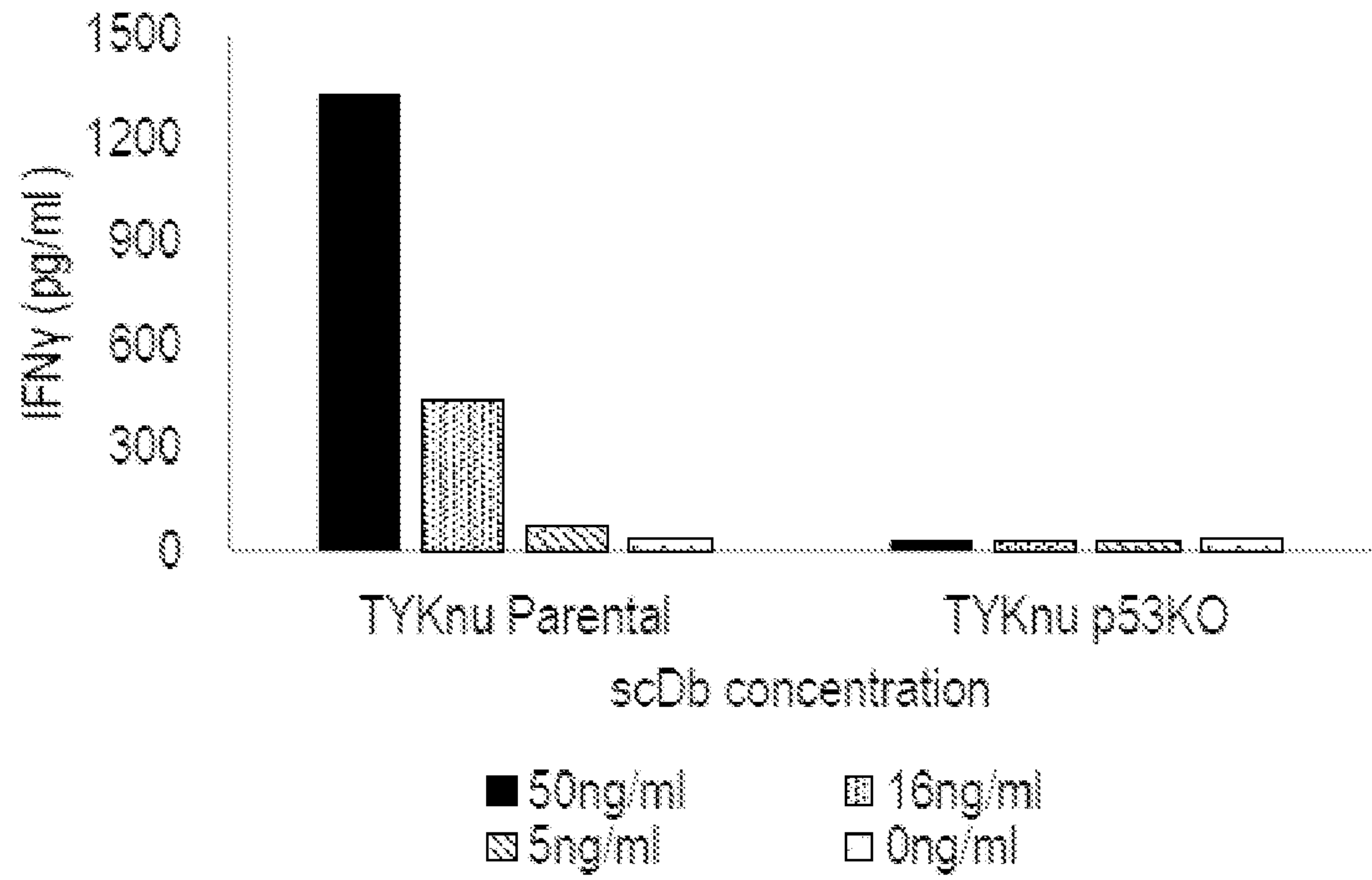


FIG. 10

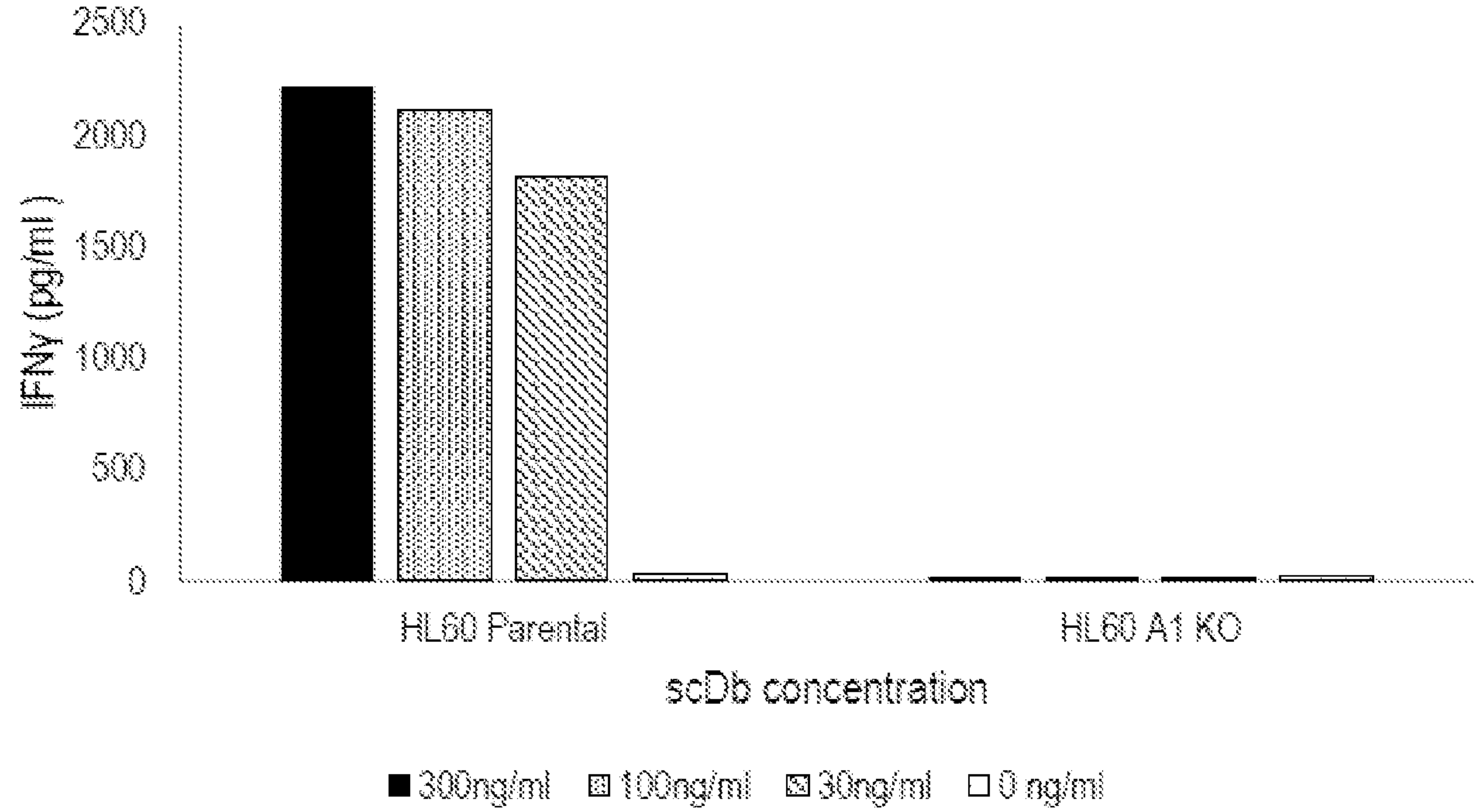


FIG. 11

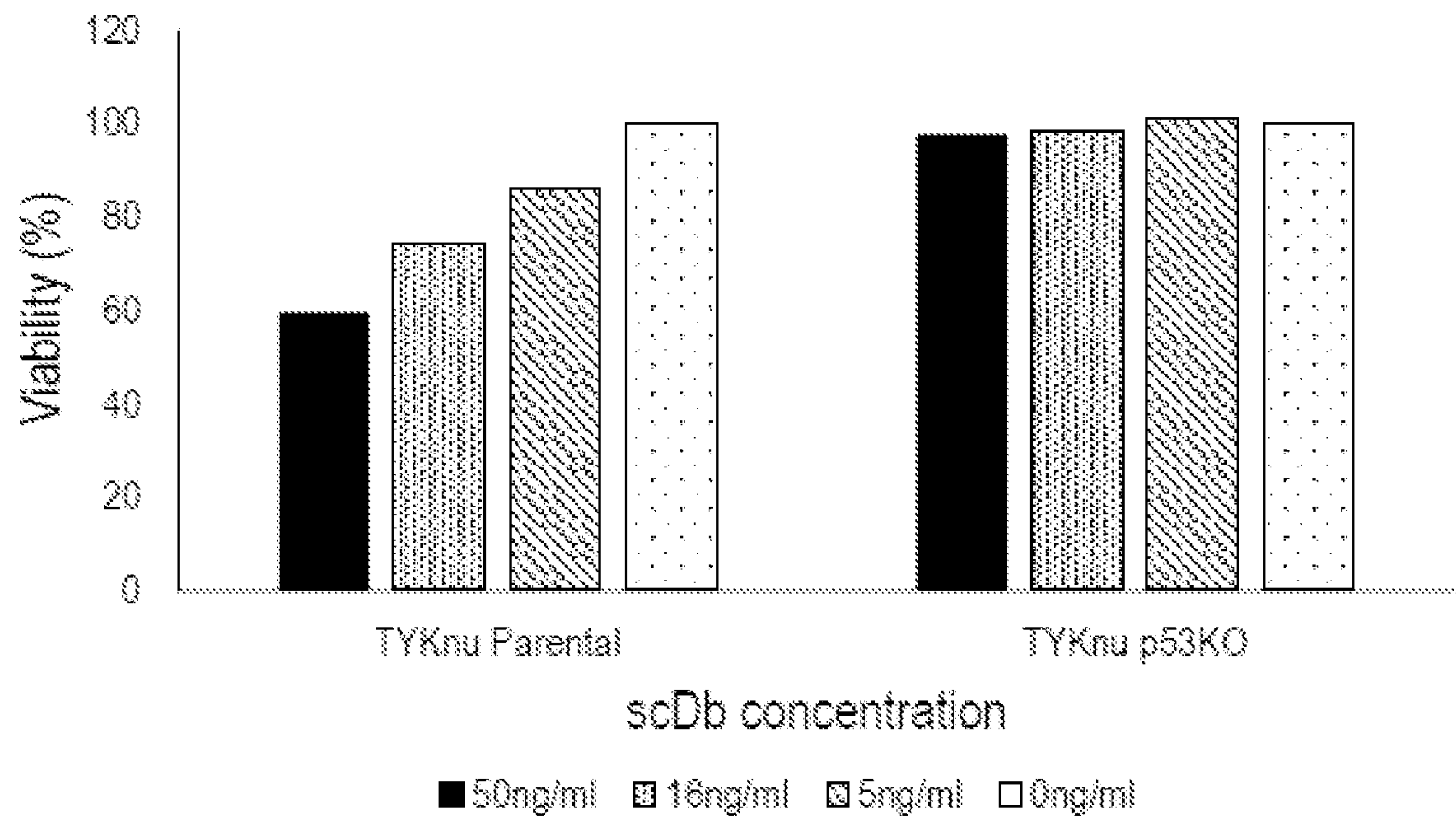


FIG. 12

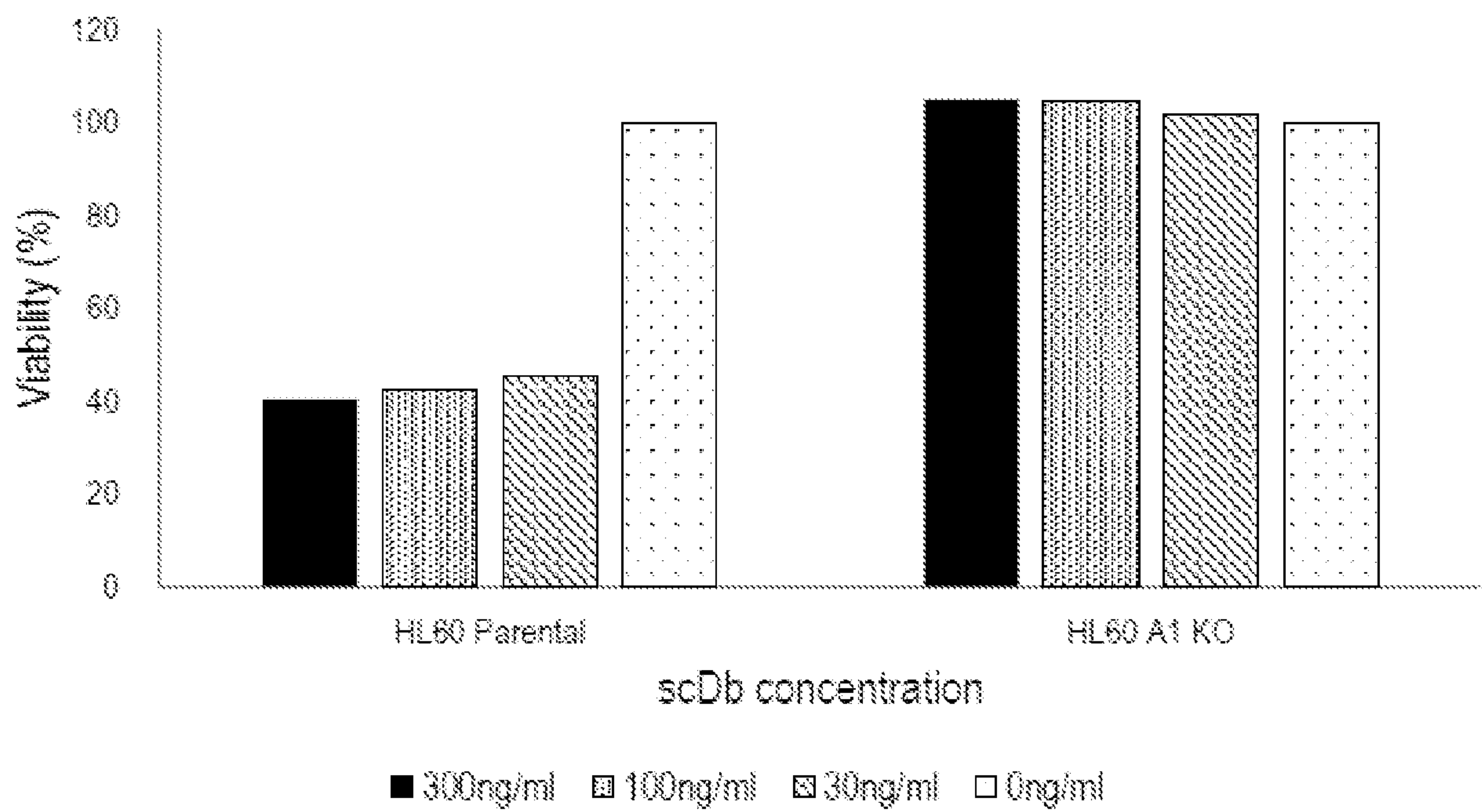
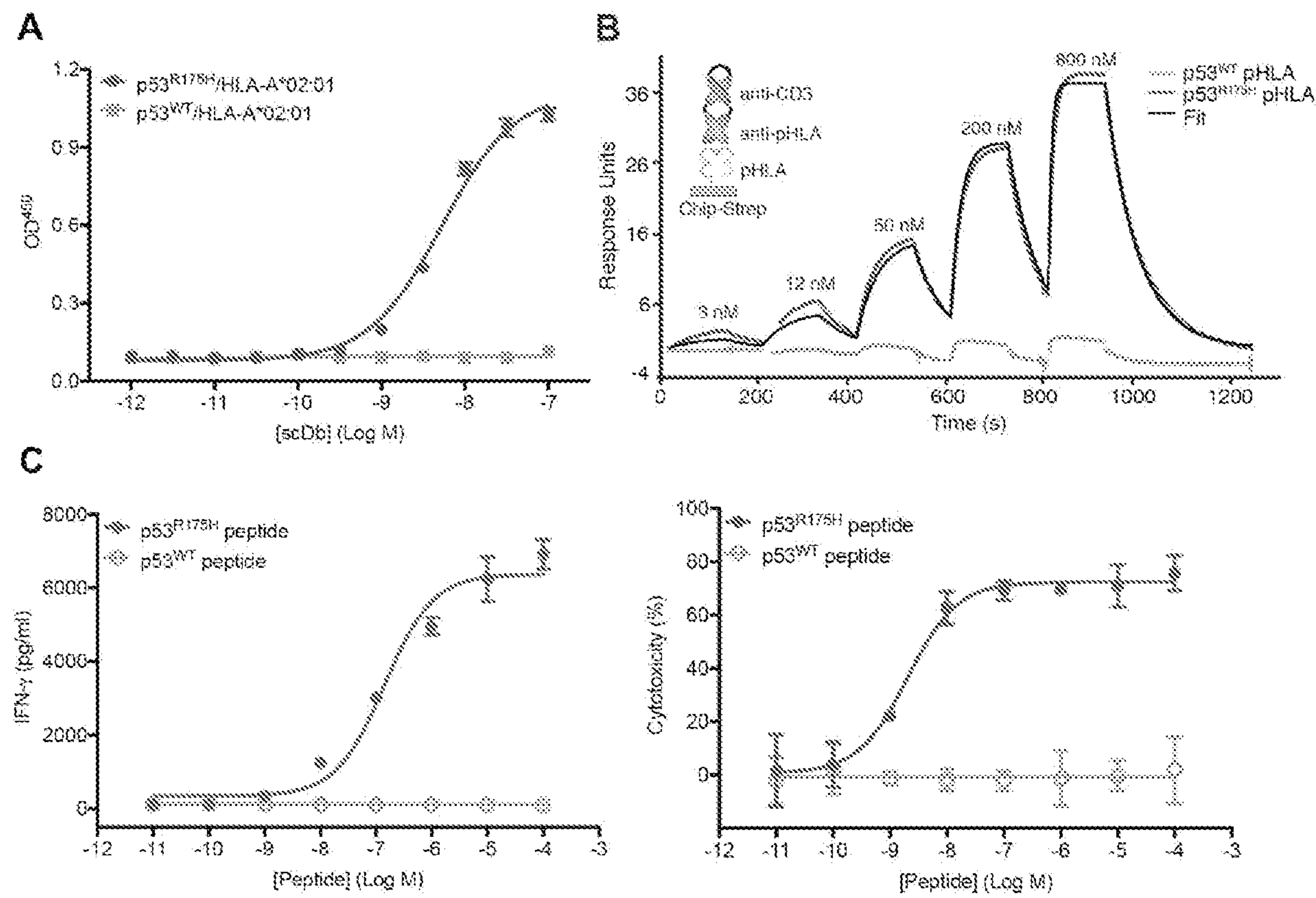


FIG. 13



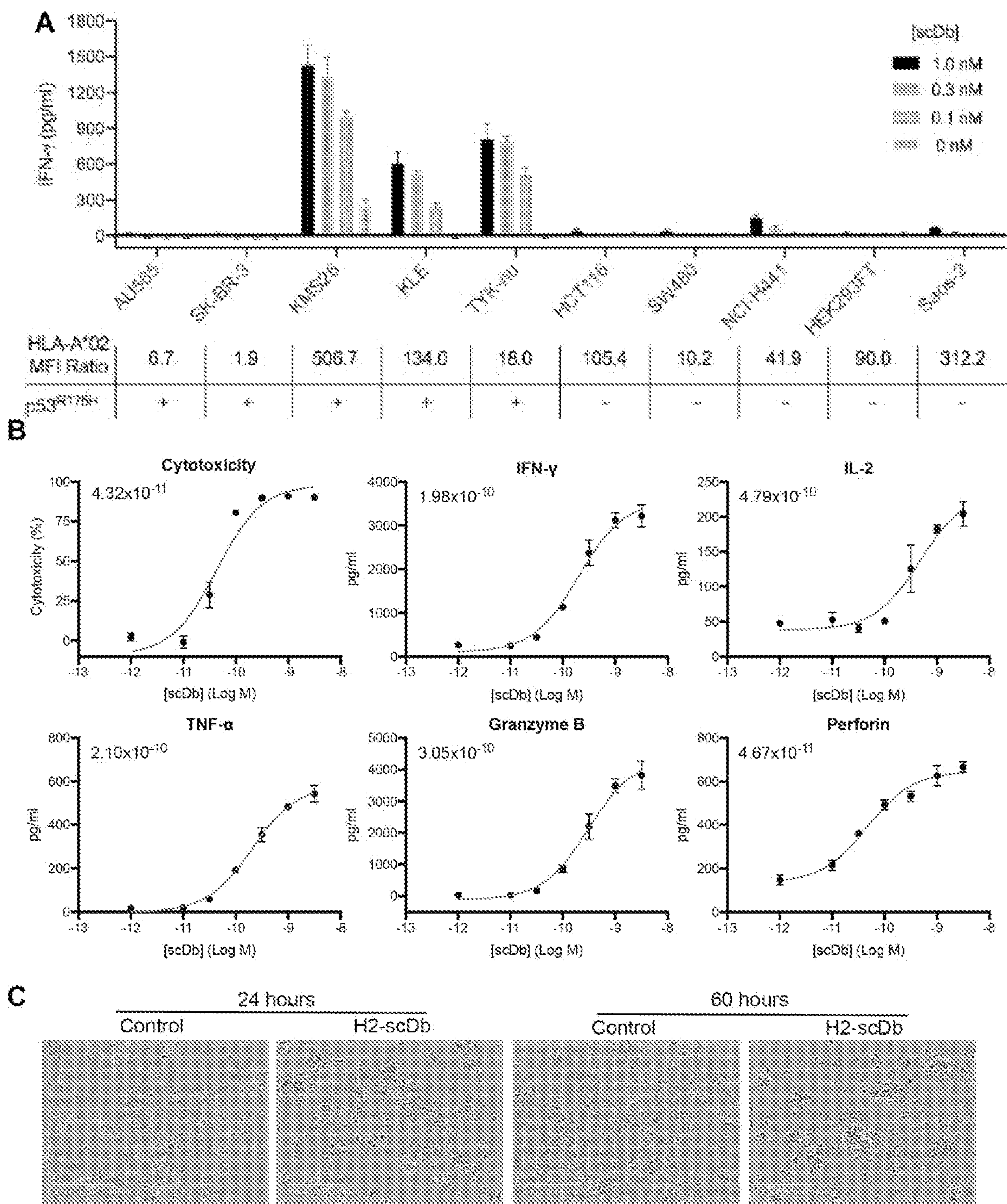


FIG. 15

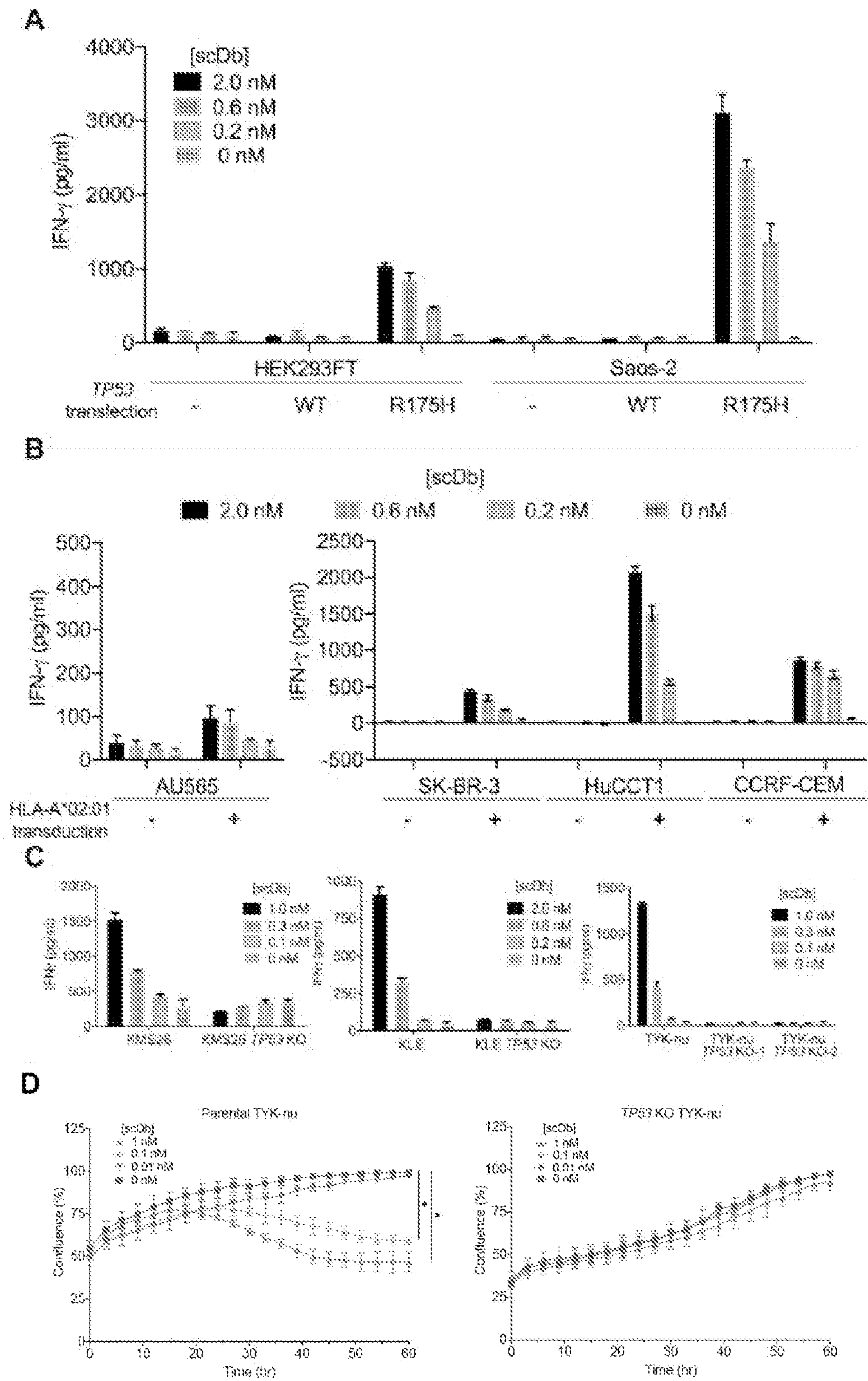


FIG. 16

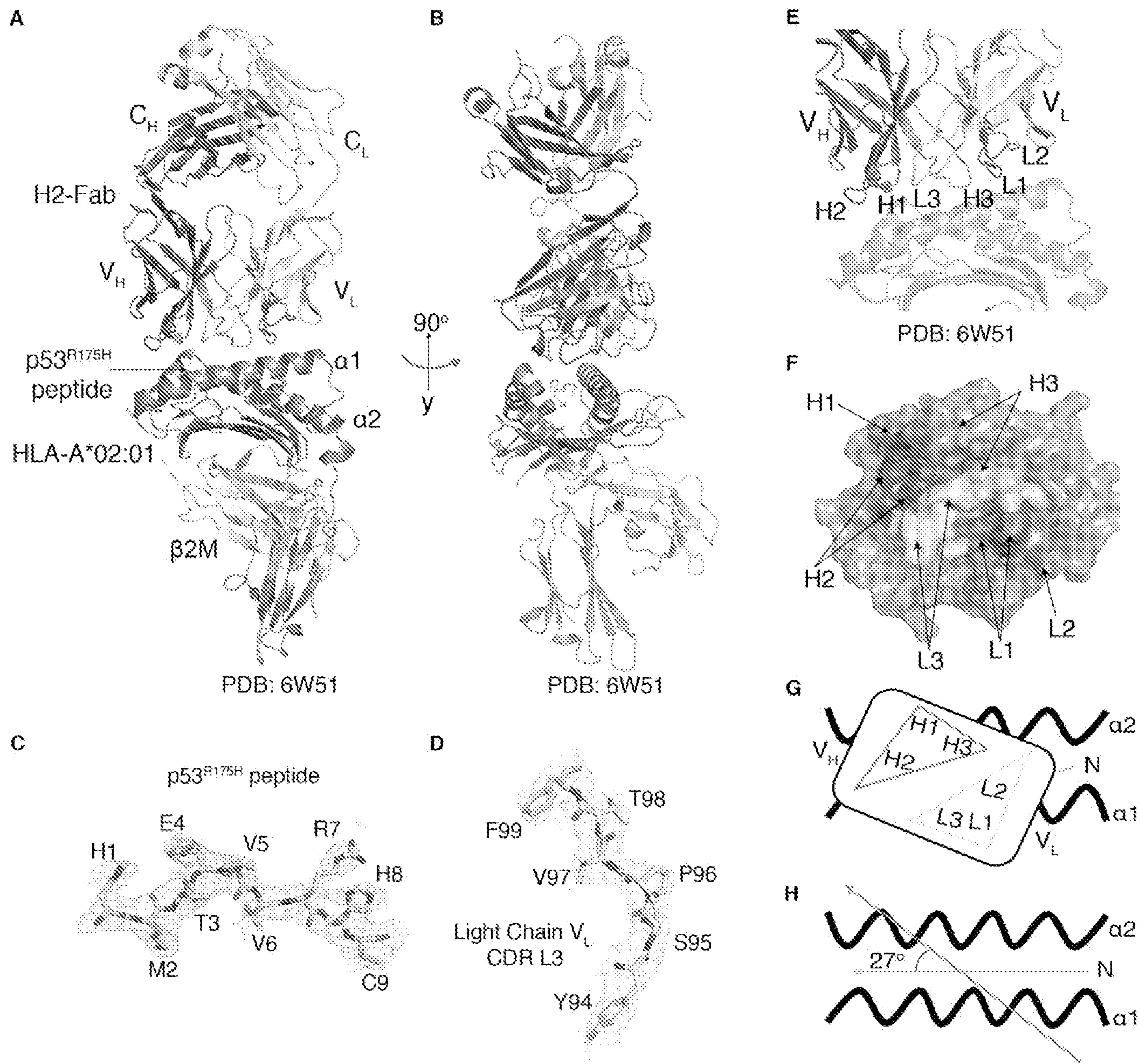


FIG. 17

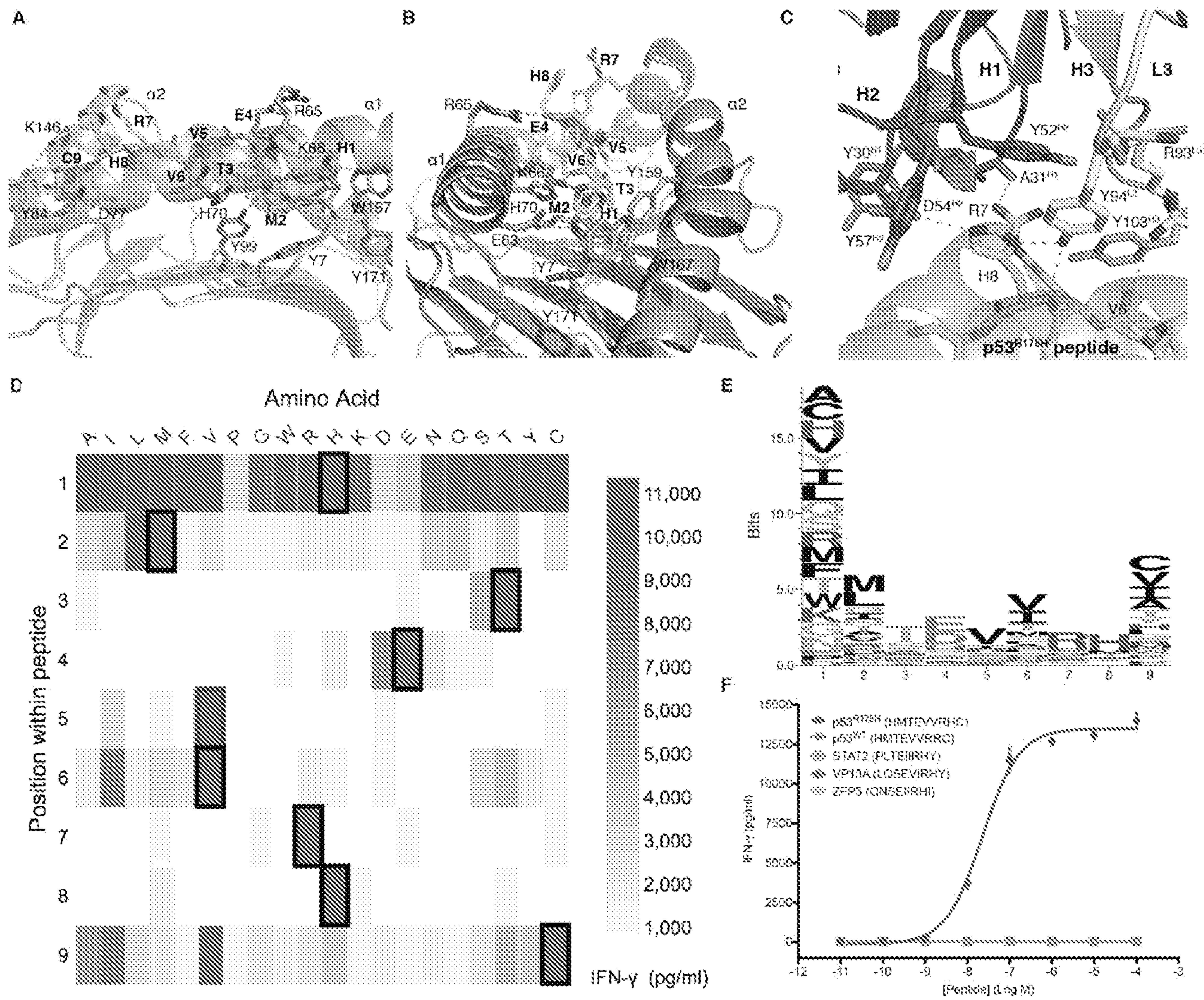
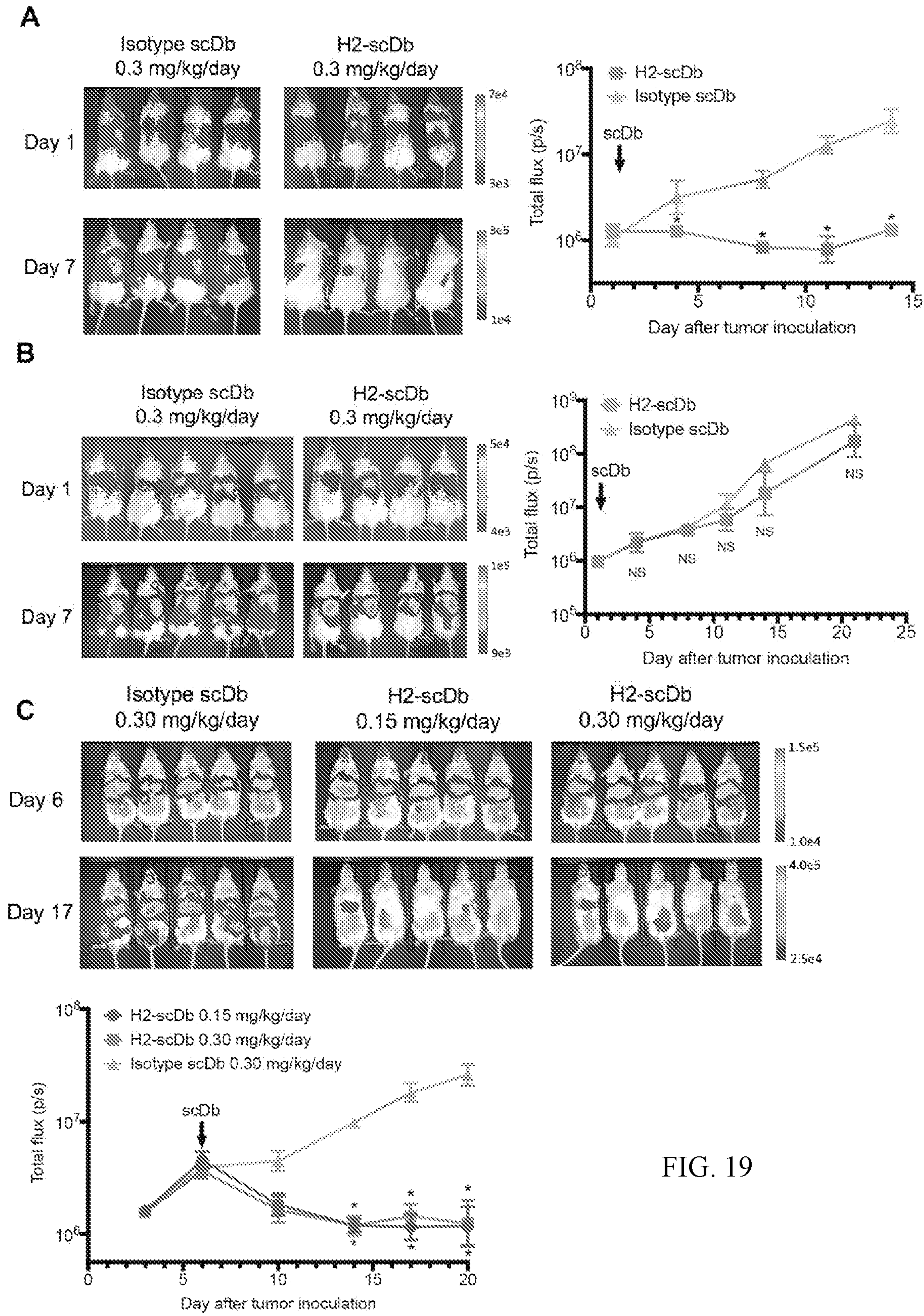


FIG. 18



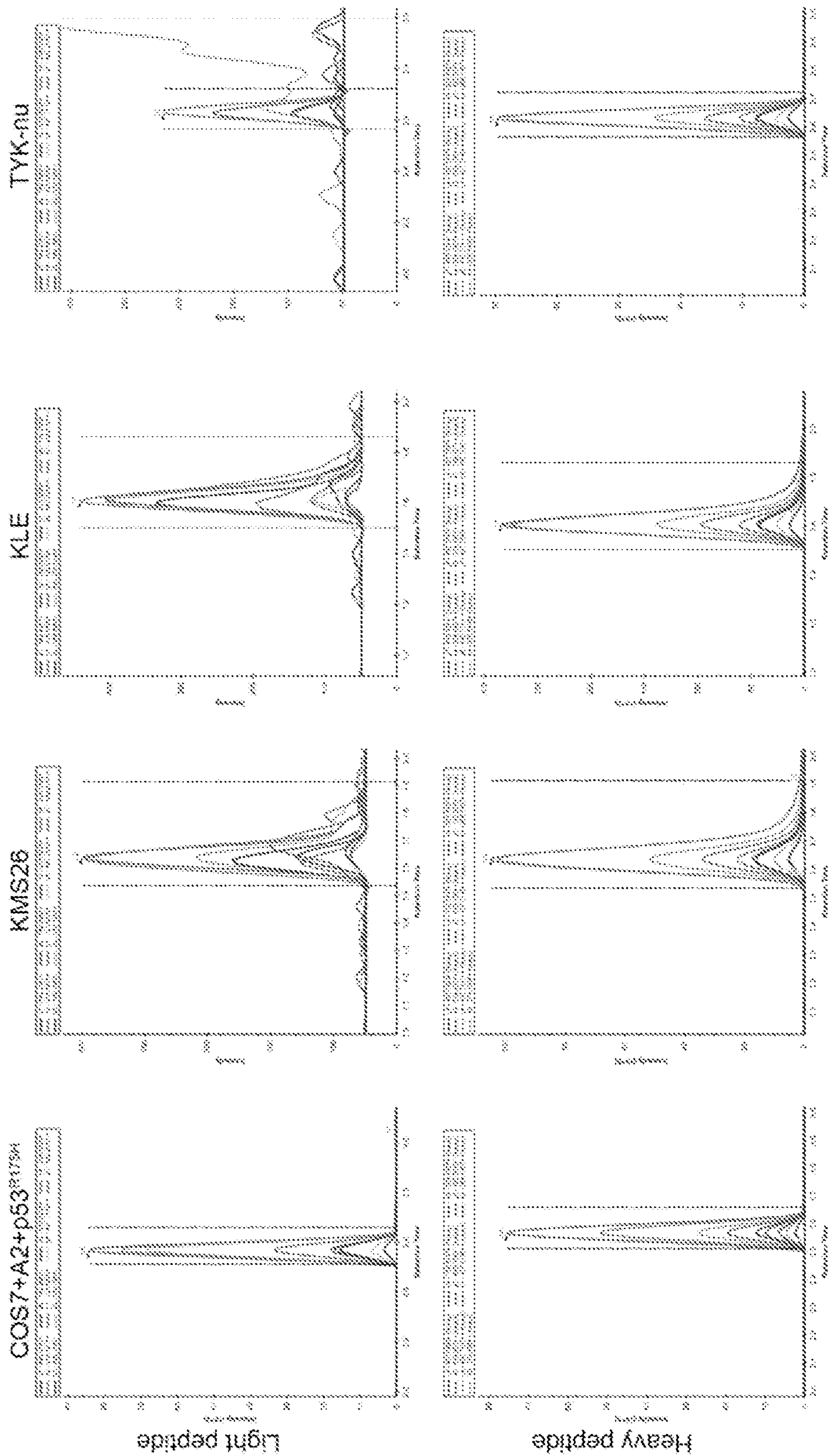


FIG. 20

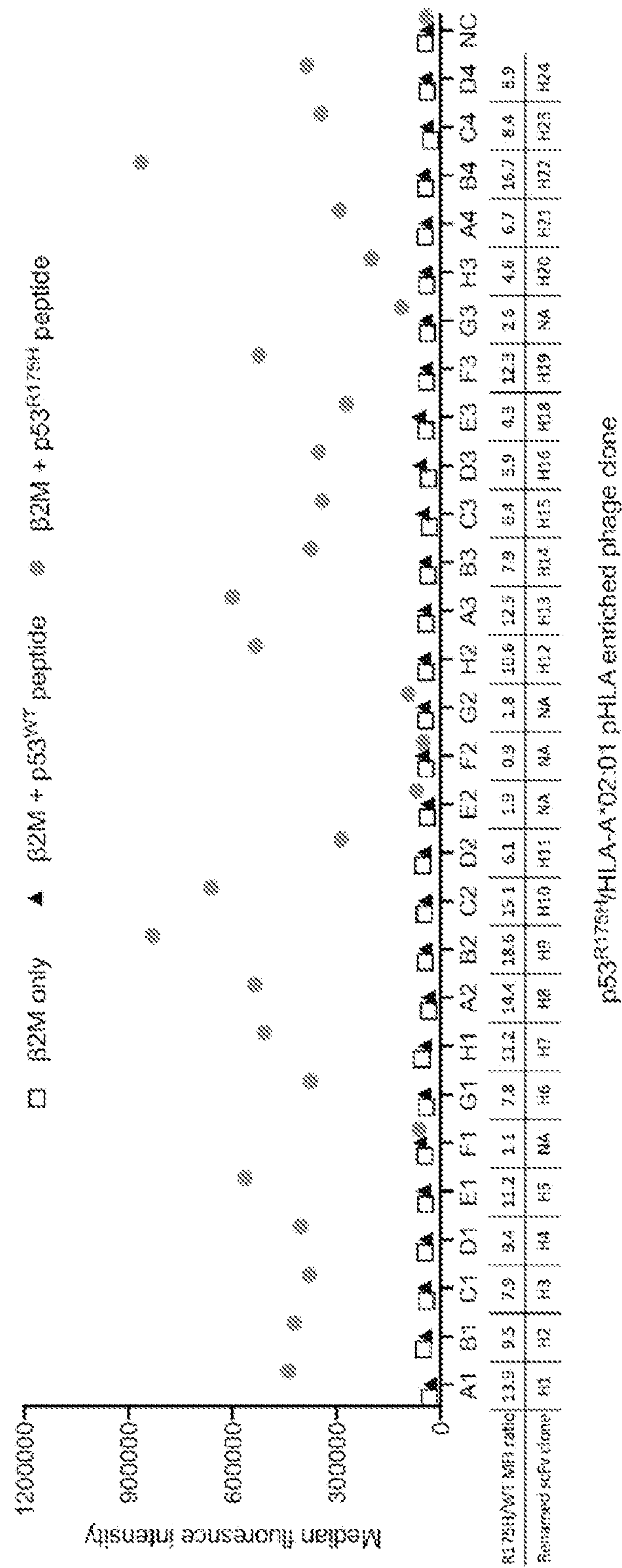


FIG. 21

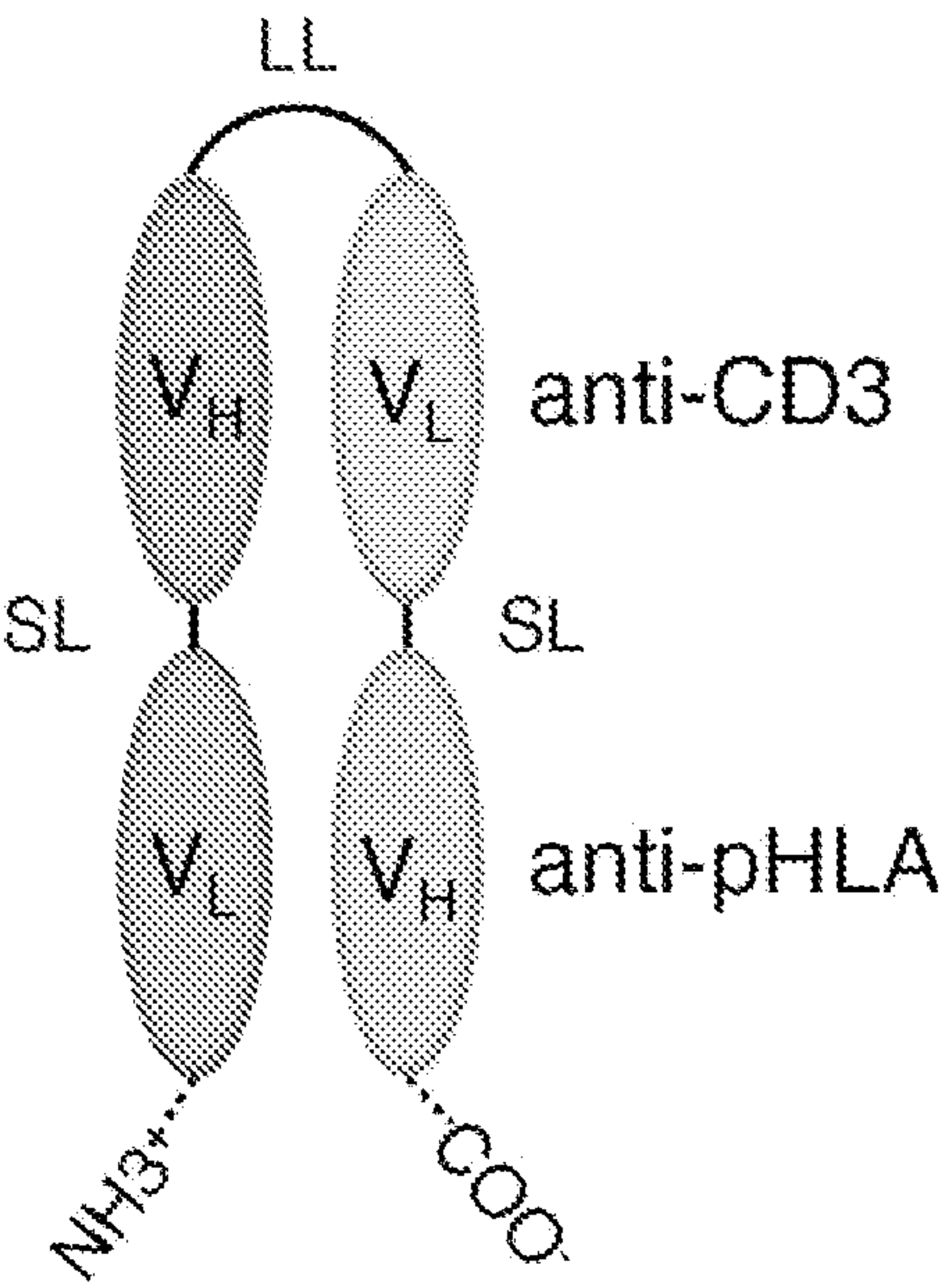


FIG. 22

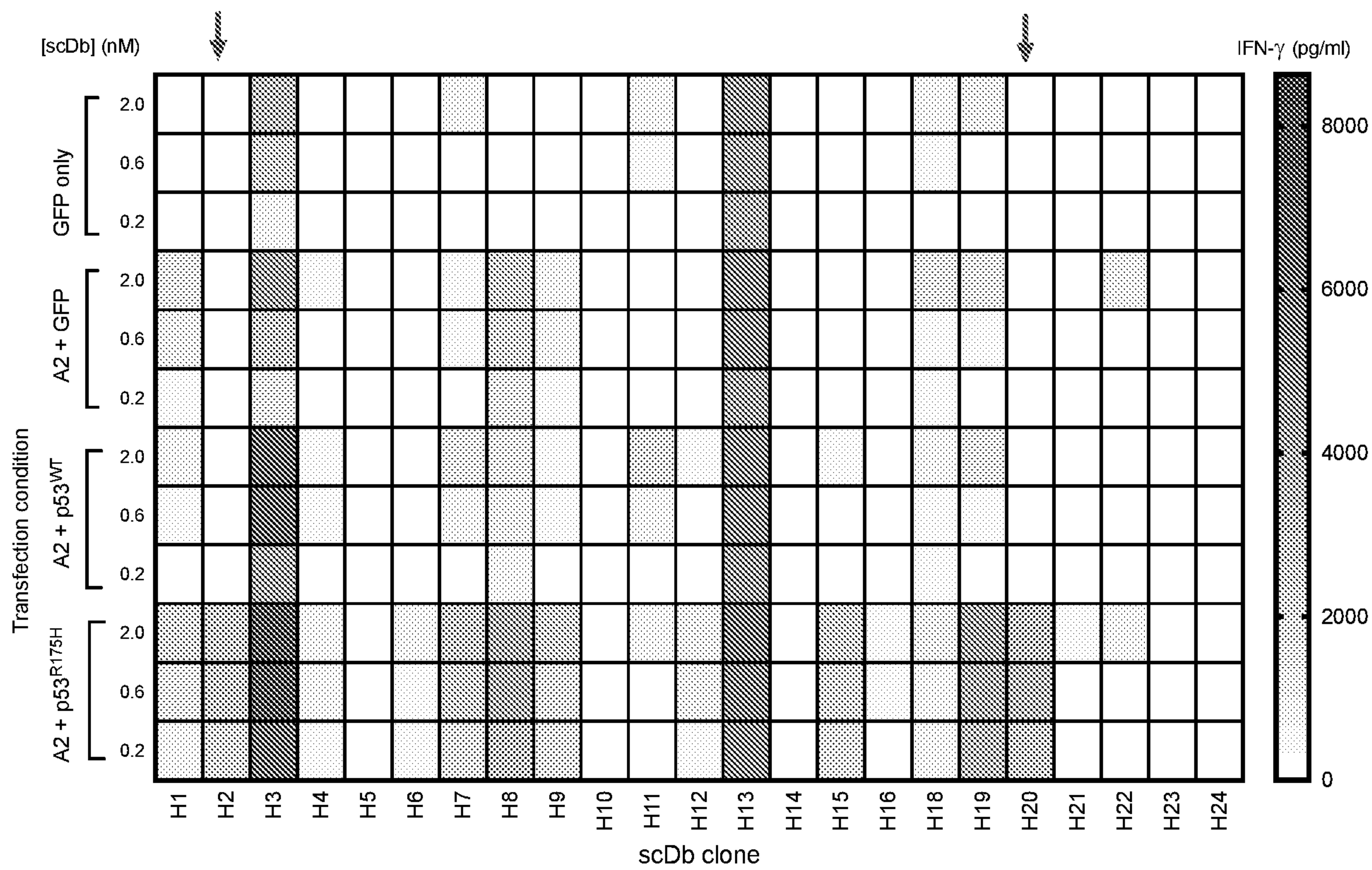


FIG. 23

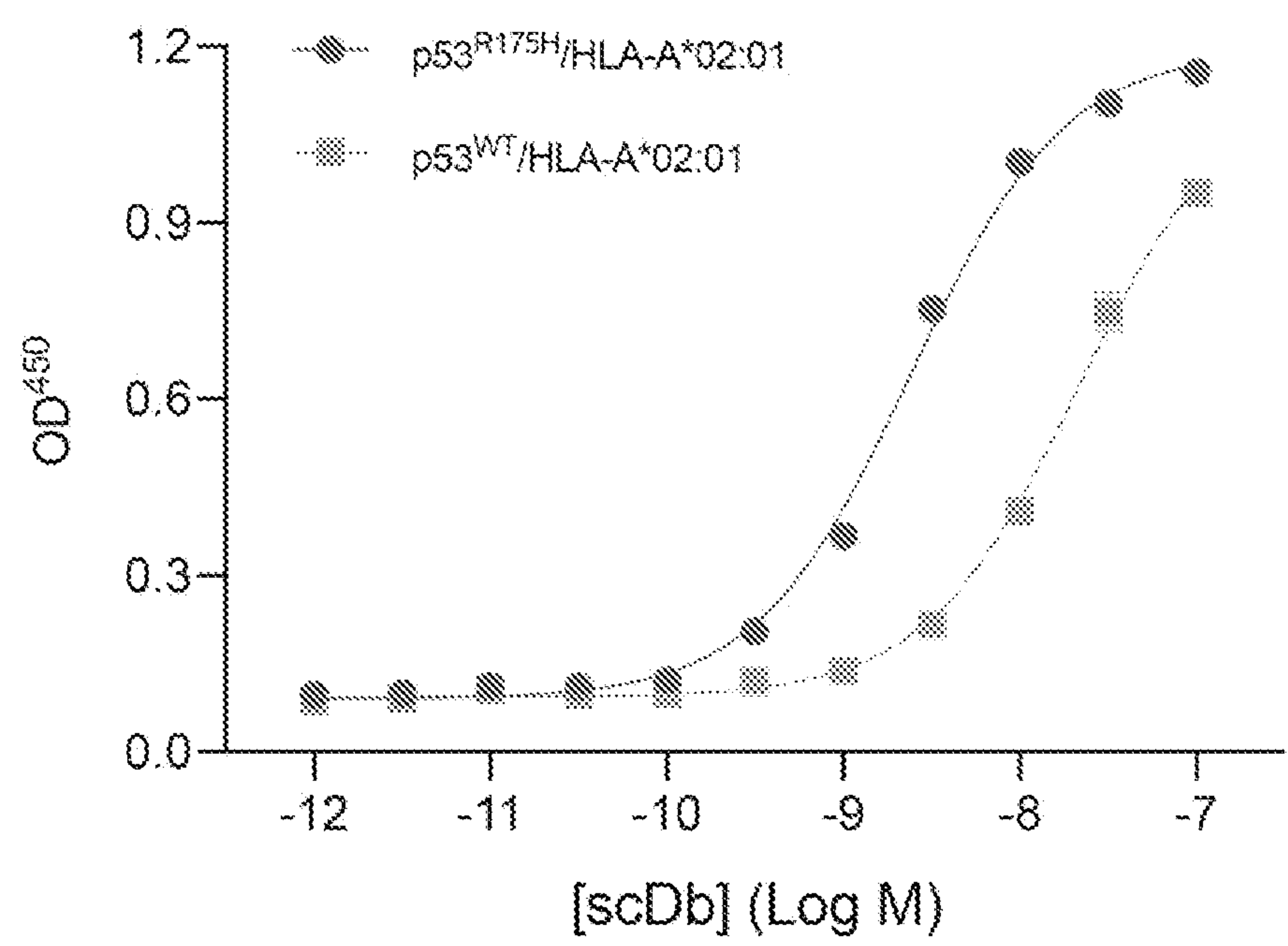


FIG. 24

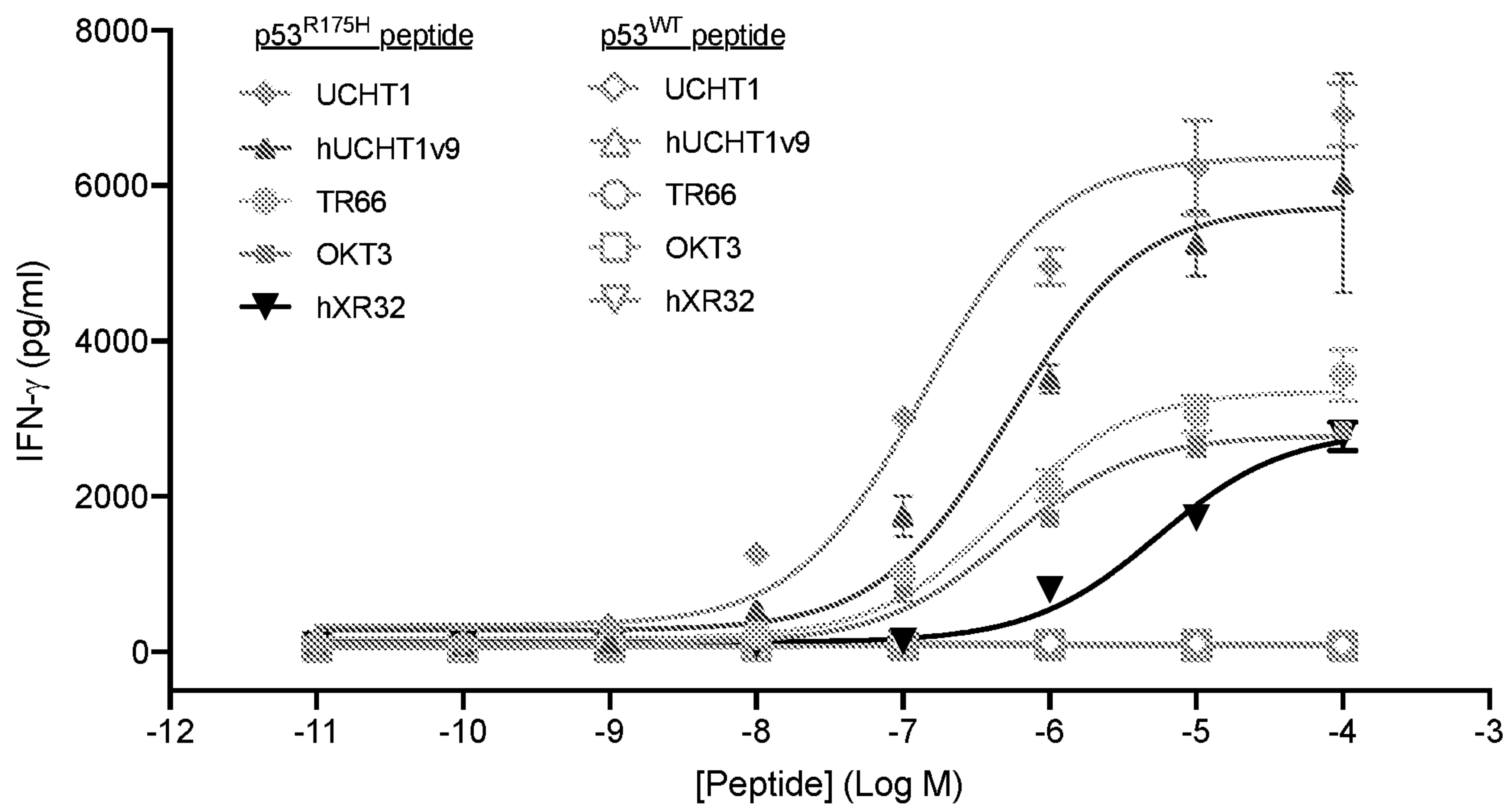


FIG. 25

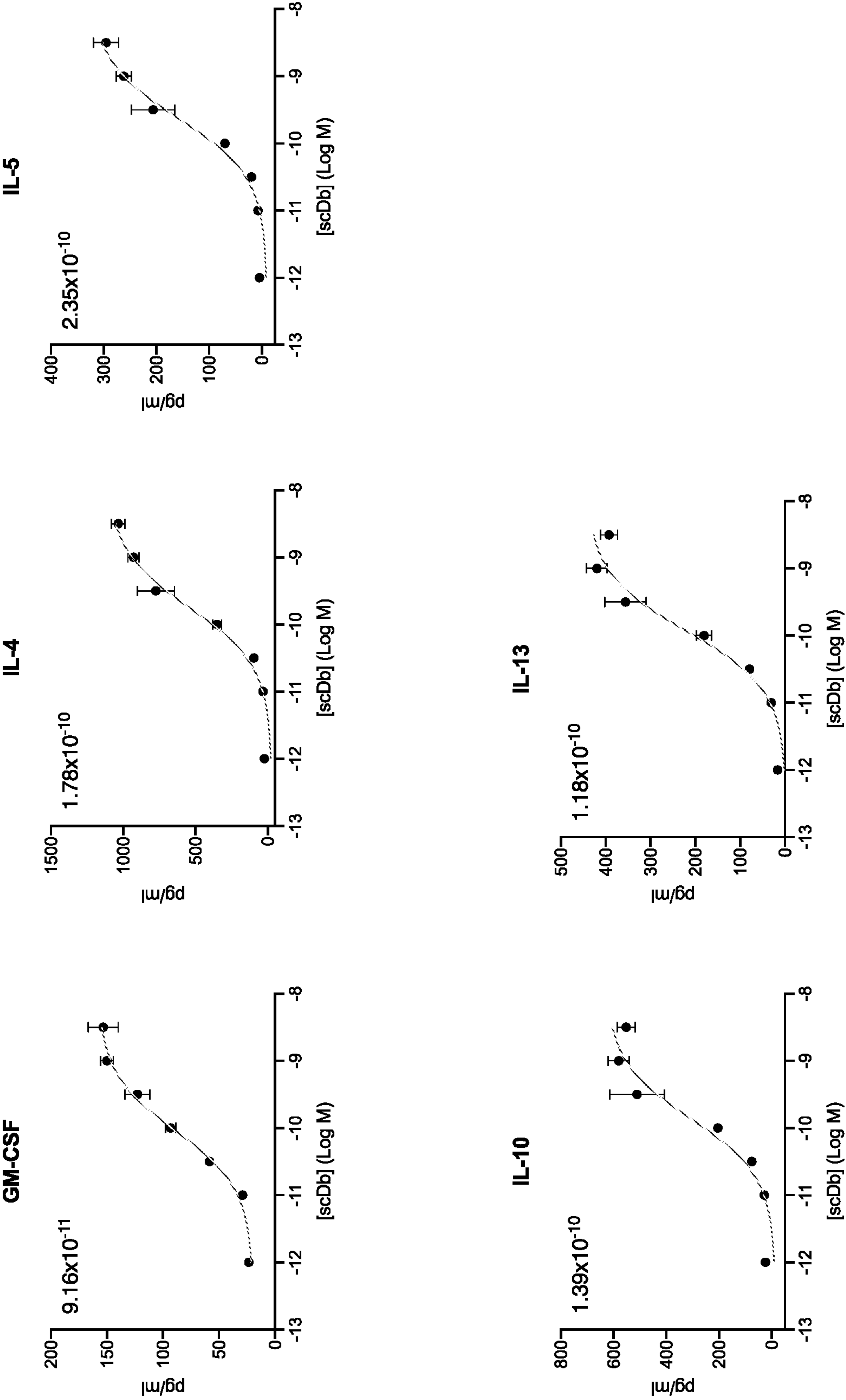


FIG. 26

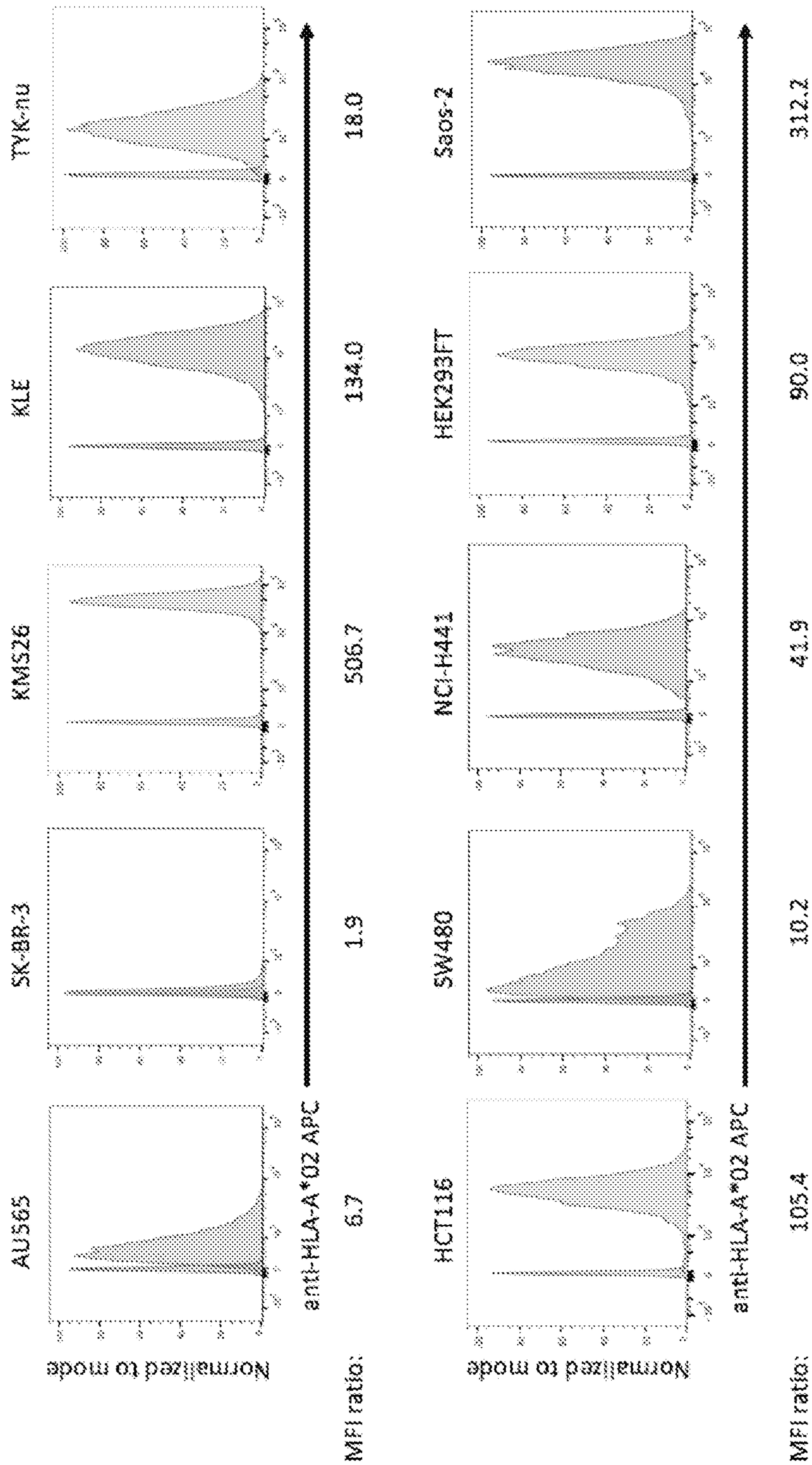


FIG. 27

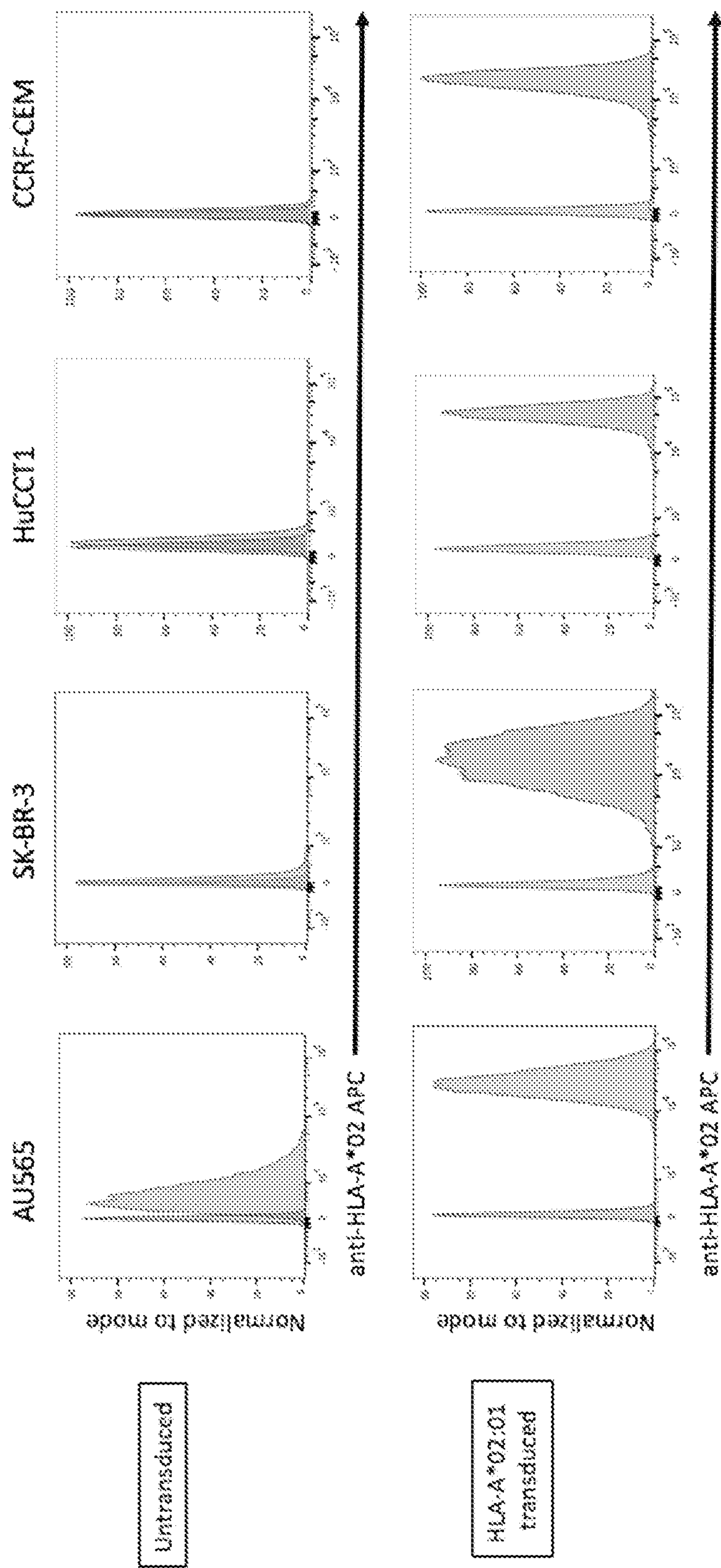


FIG. 28

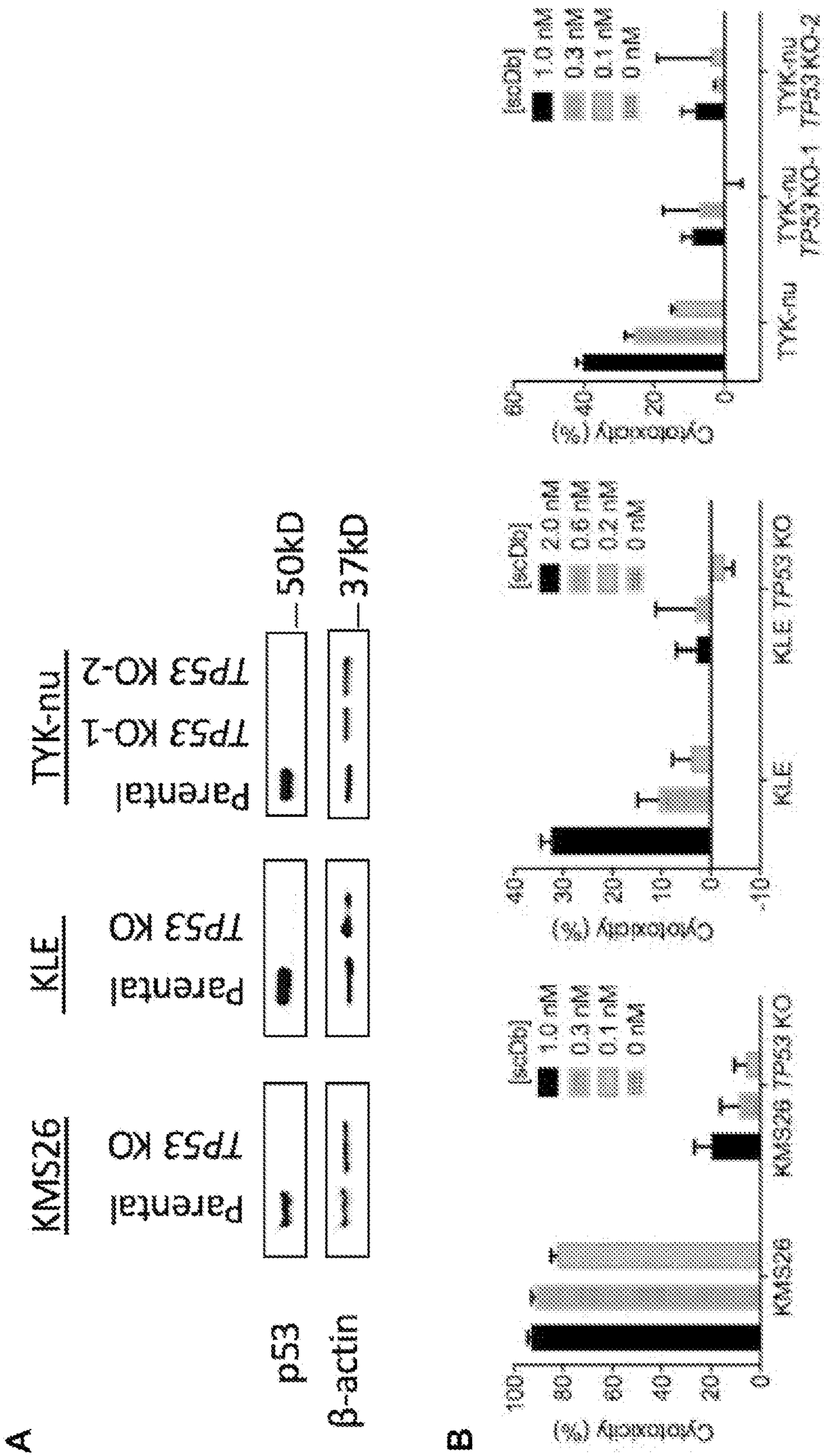
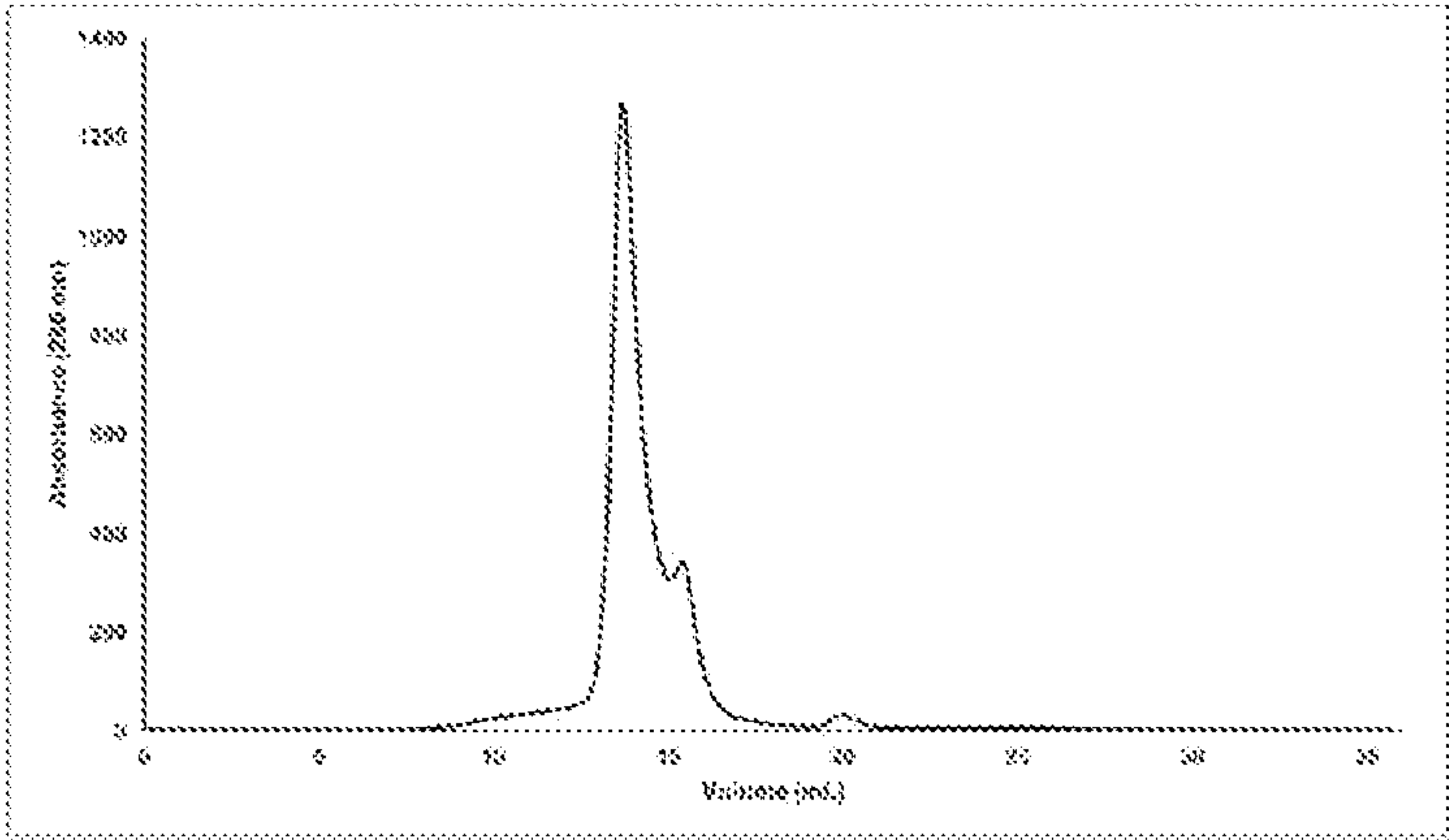


FIG. 29

A



B

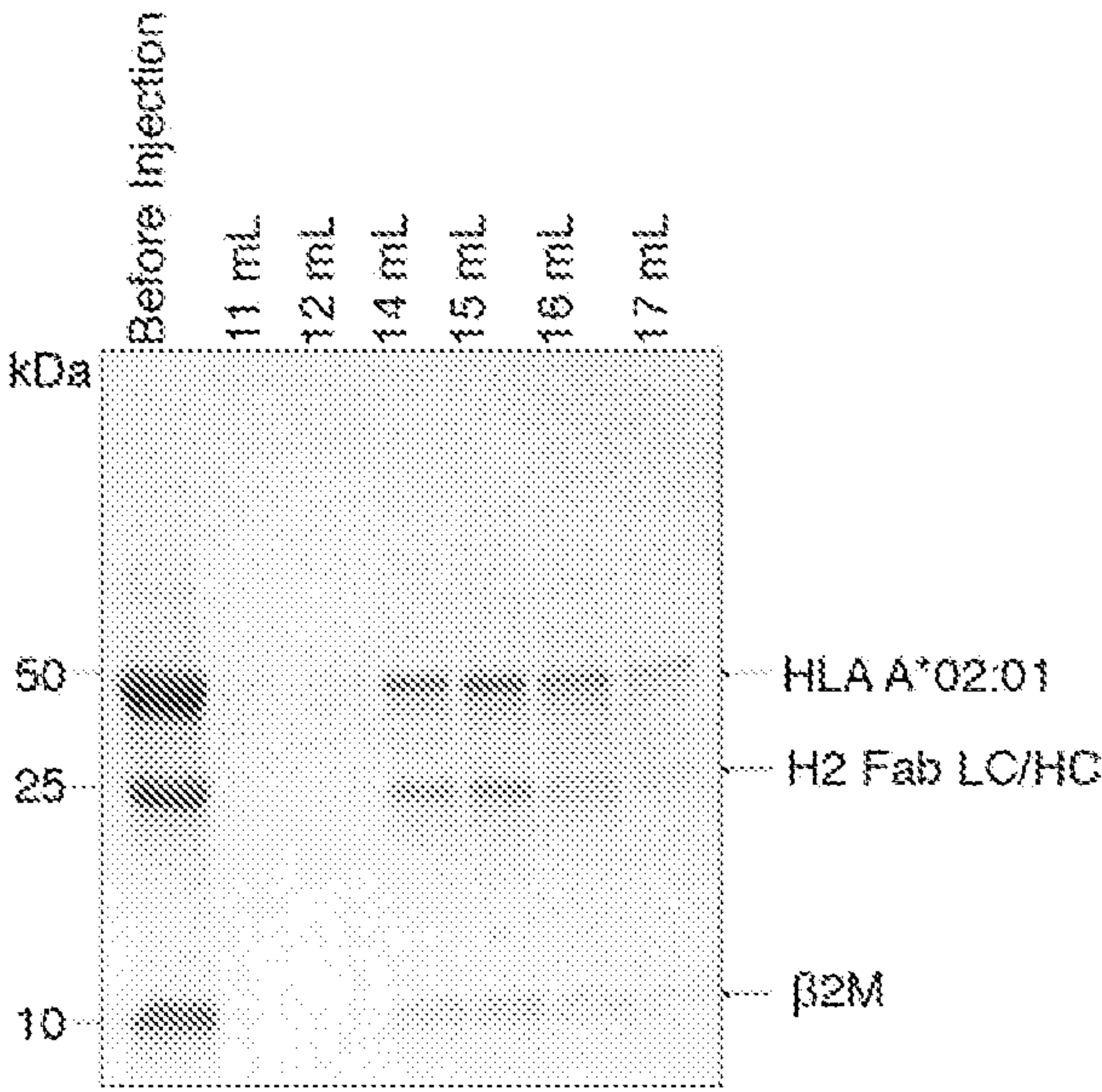


FIG. 30

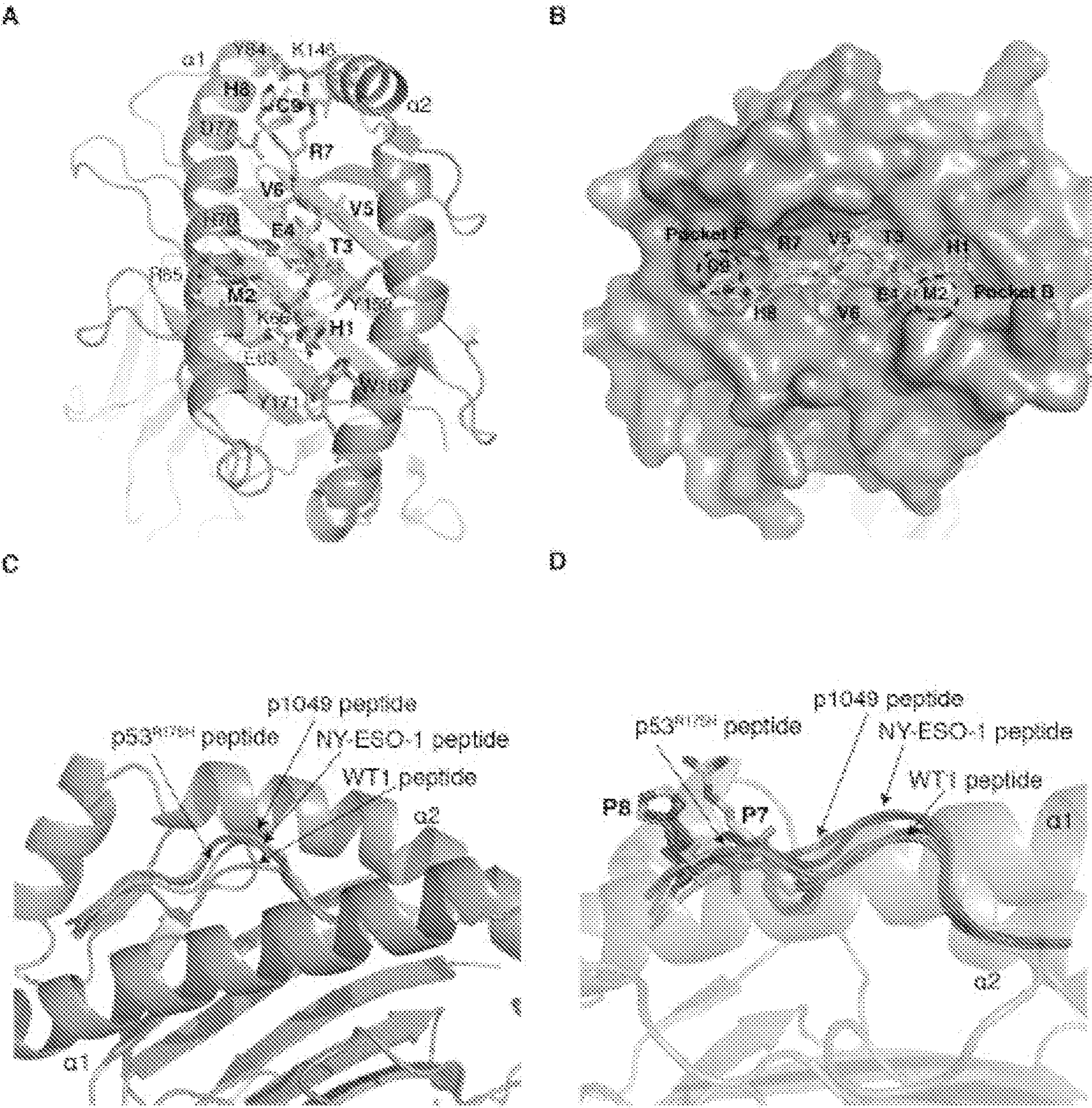


FIG. 31

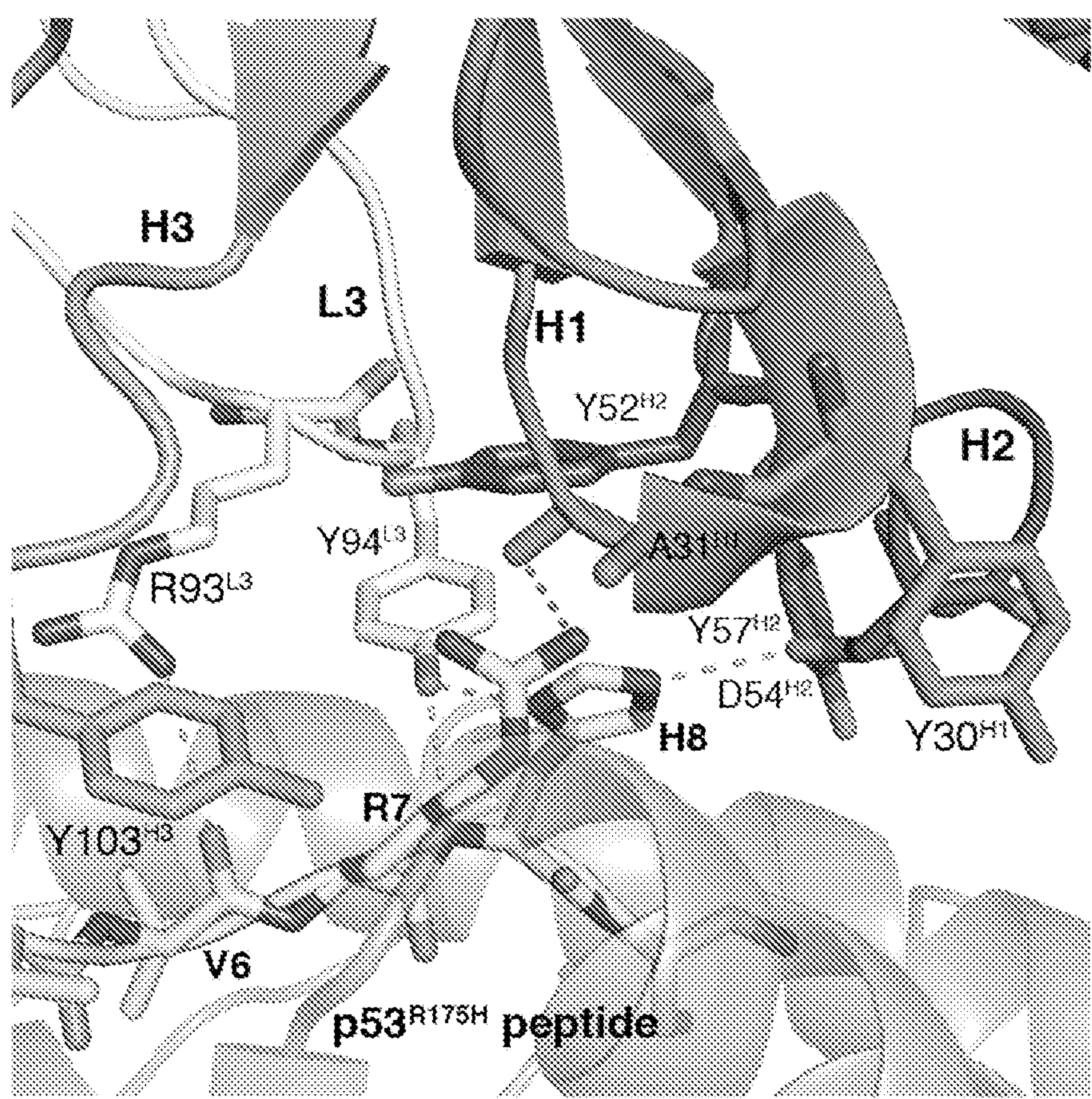


FIG. 32

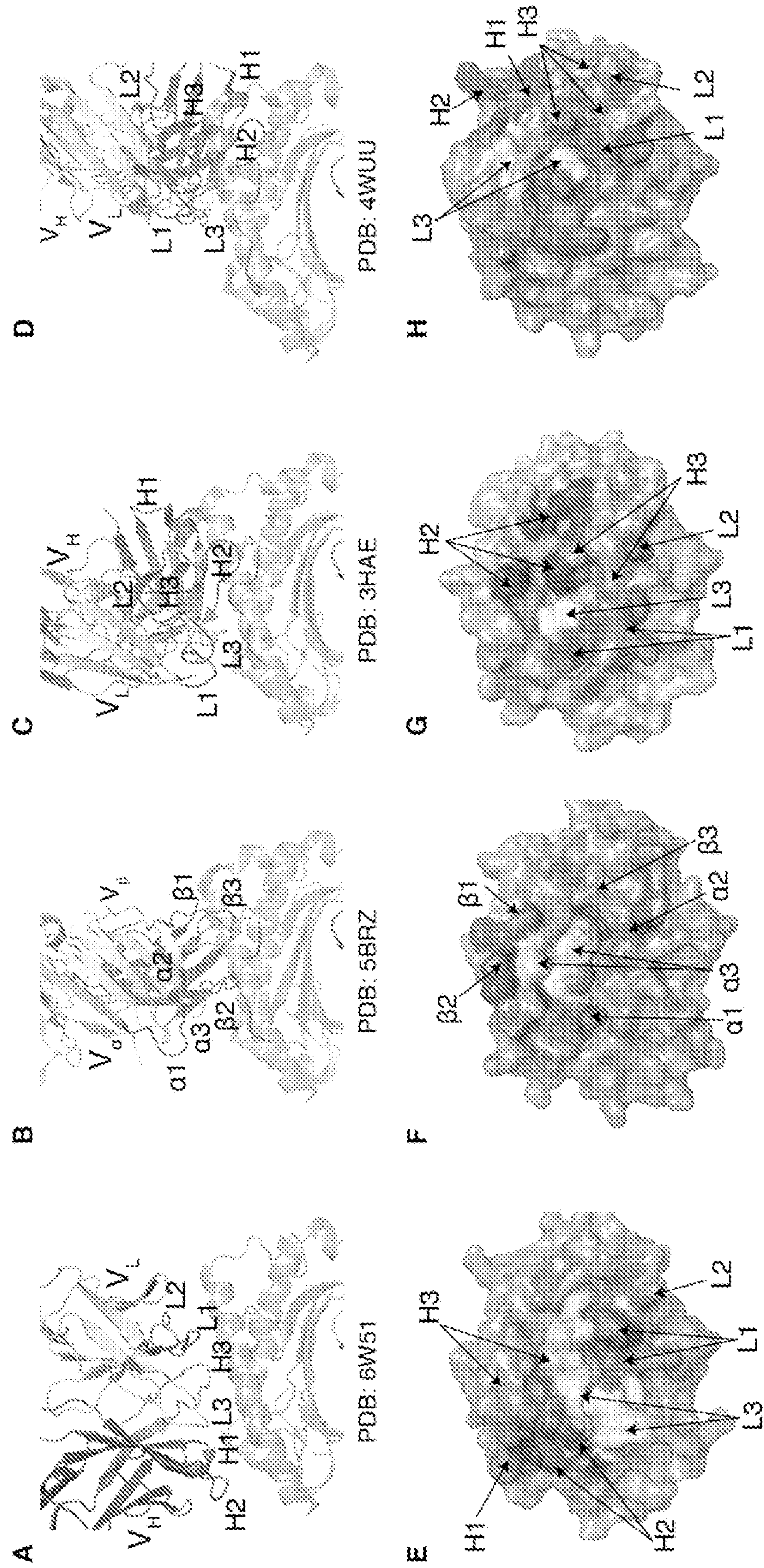


FIG. 33

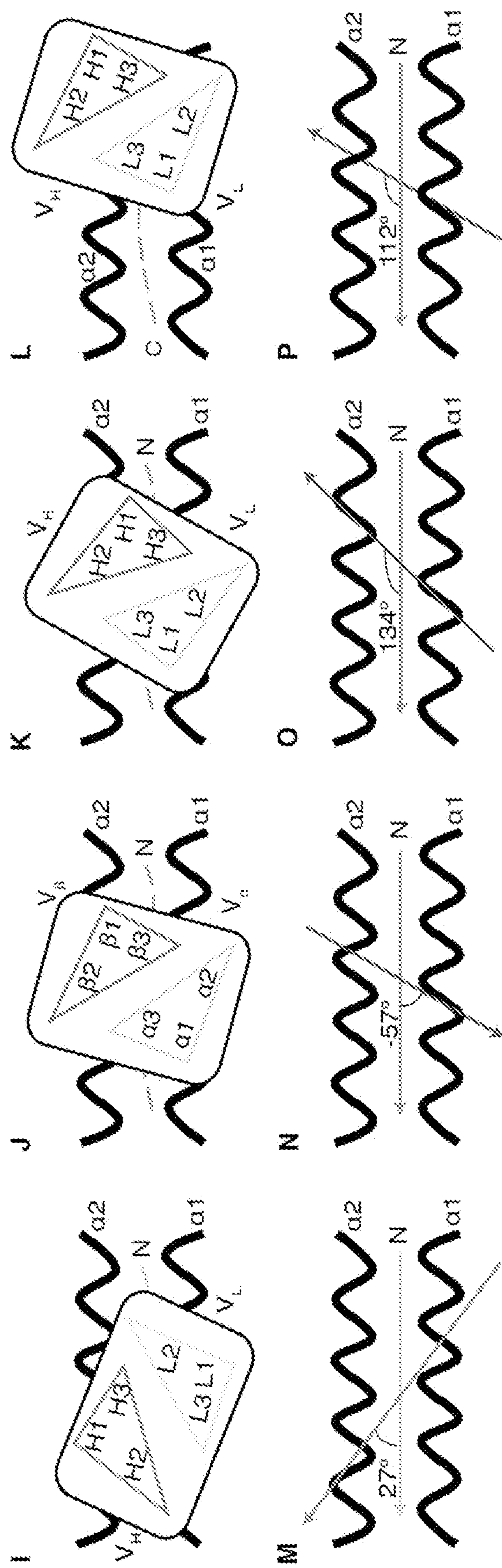


FIG. 33 (continued)

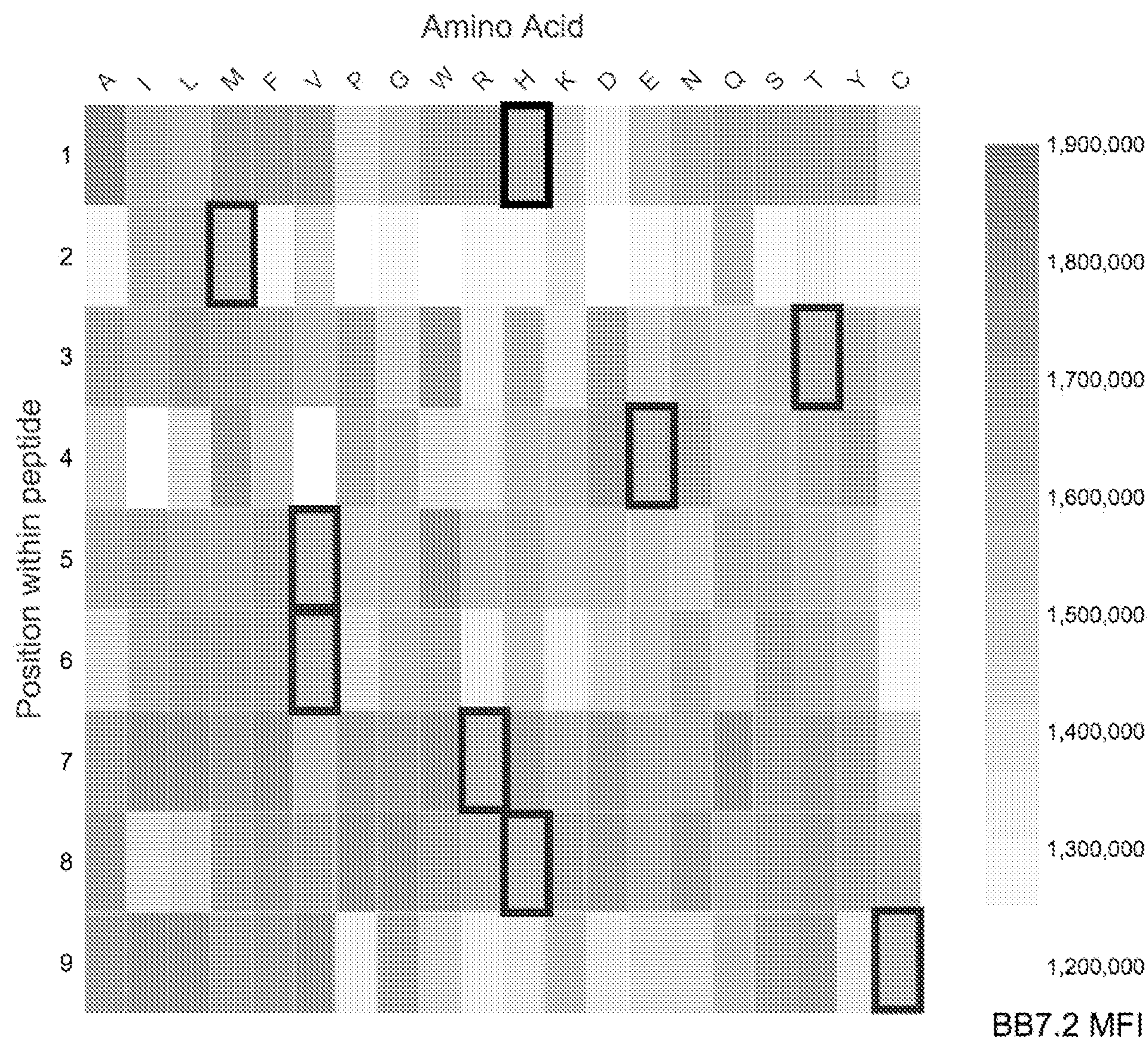


FIG. 34

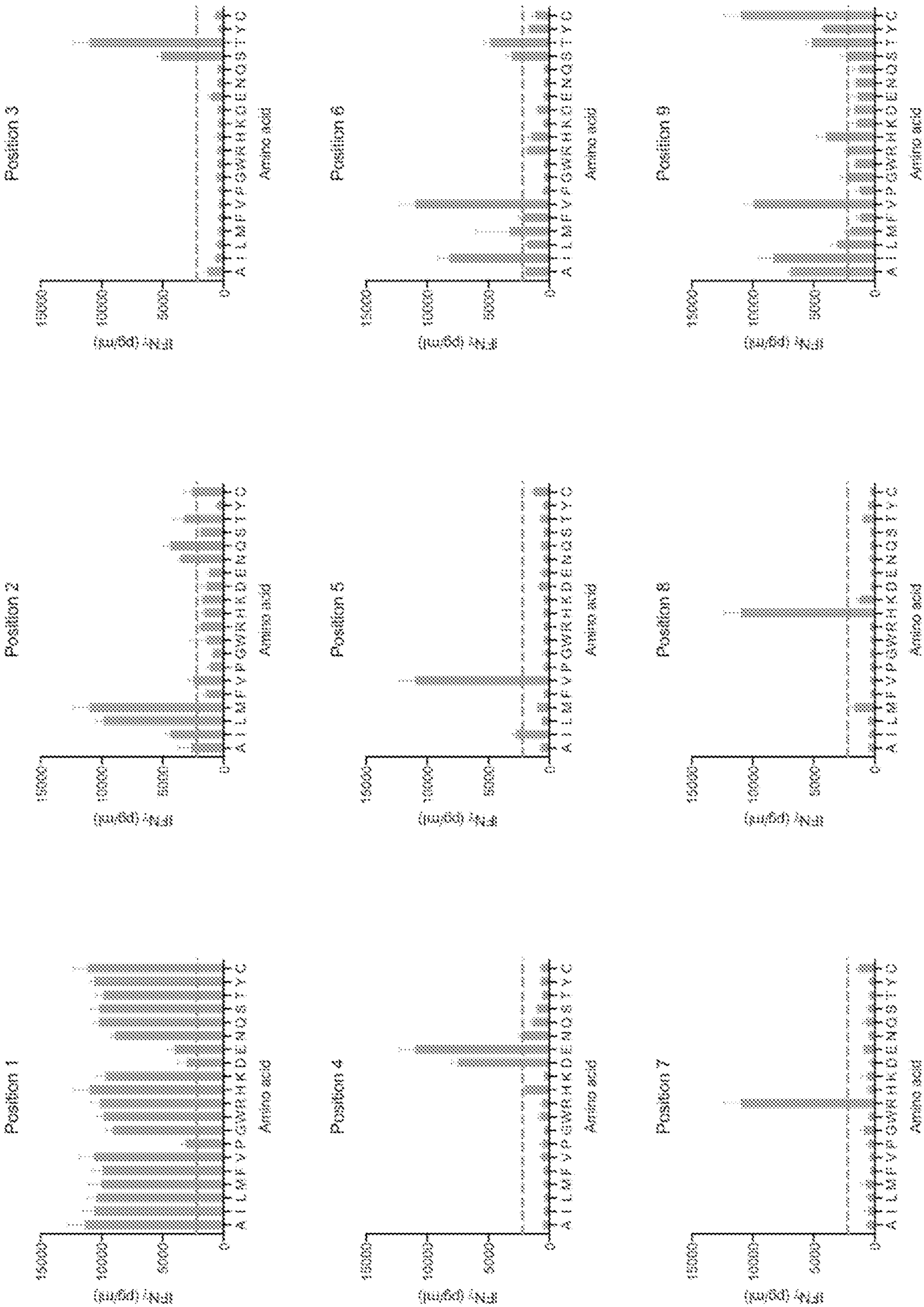


FIG. 35

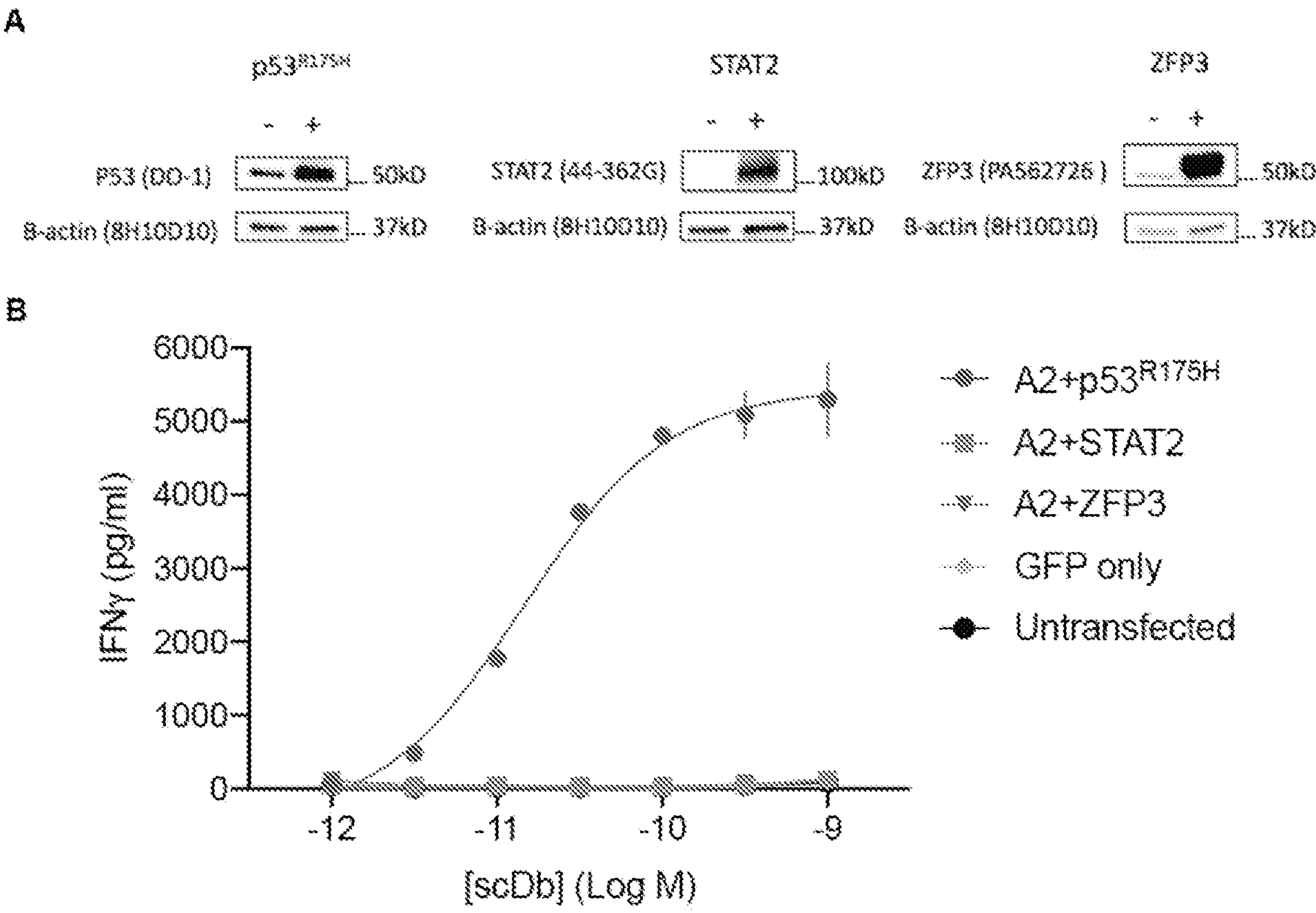


FIG. 36

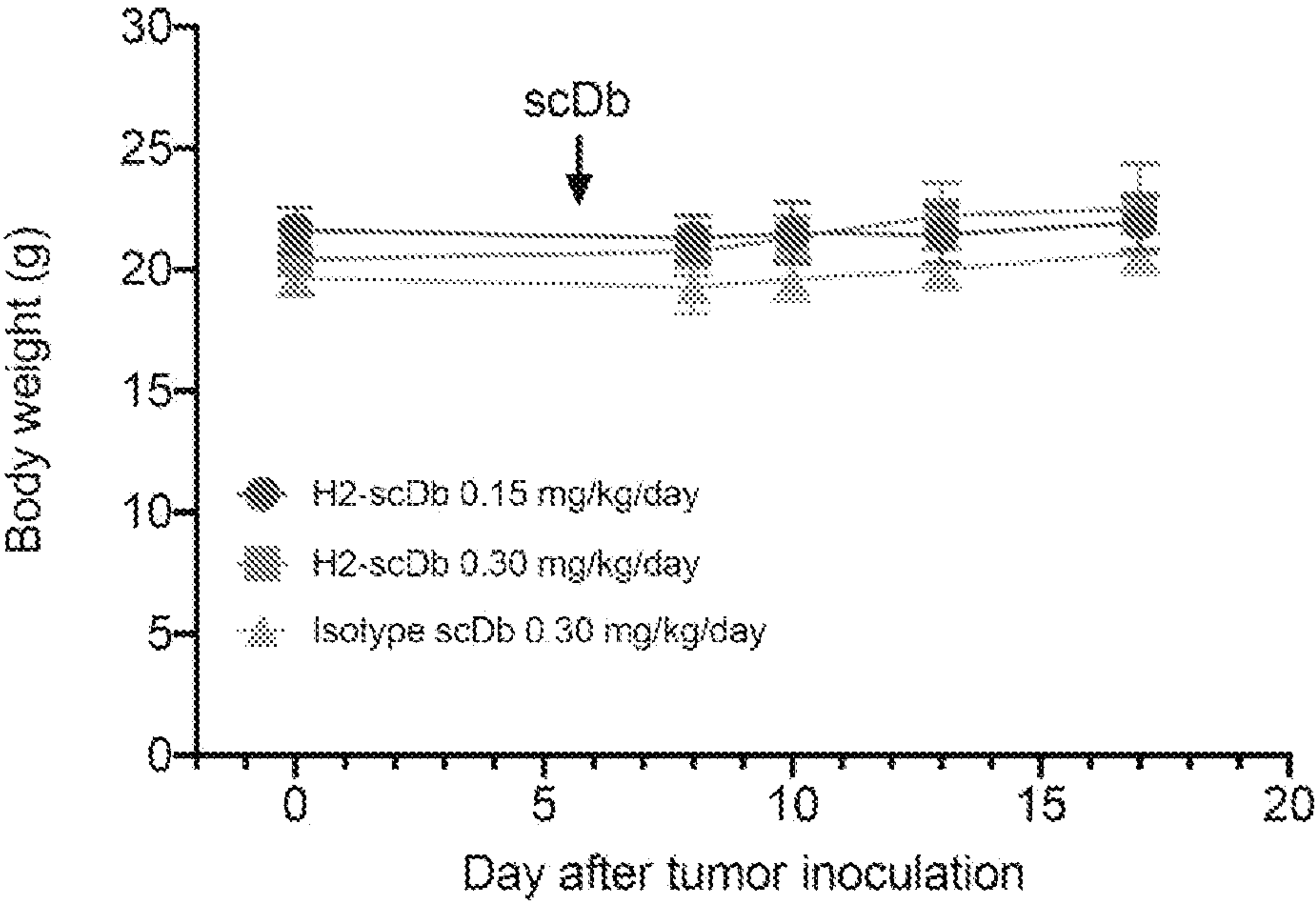


FIG. 37

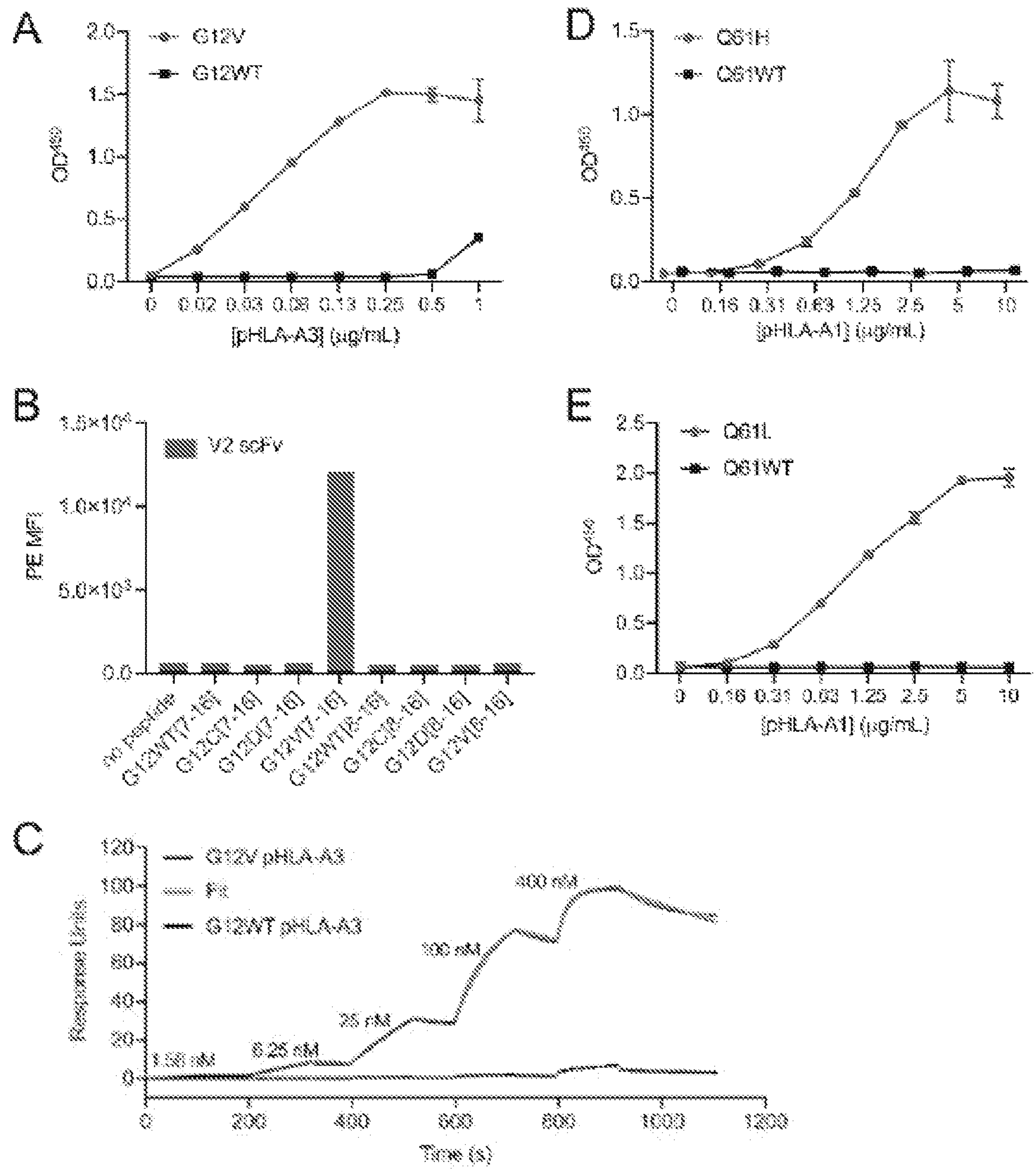


FIG. 38

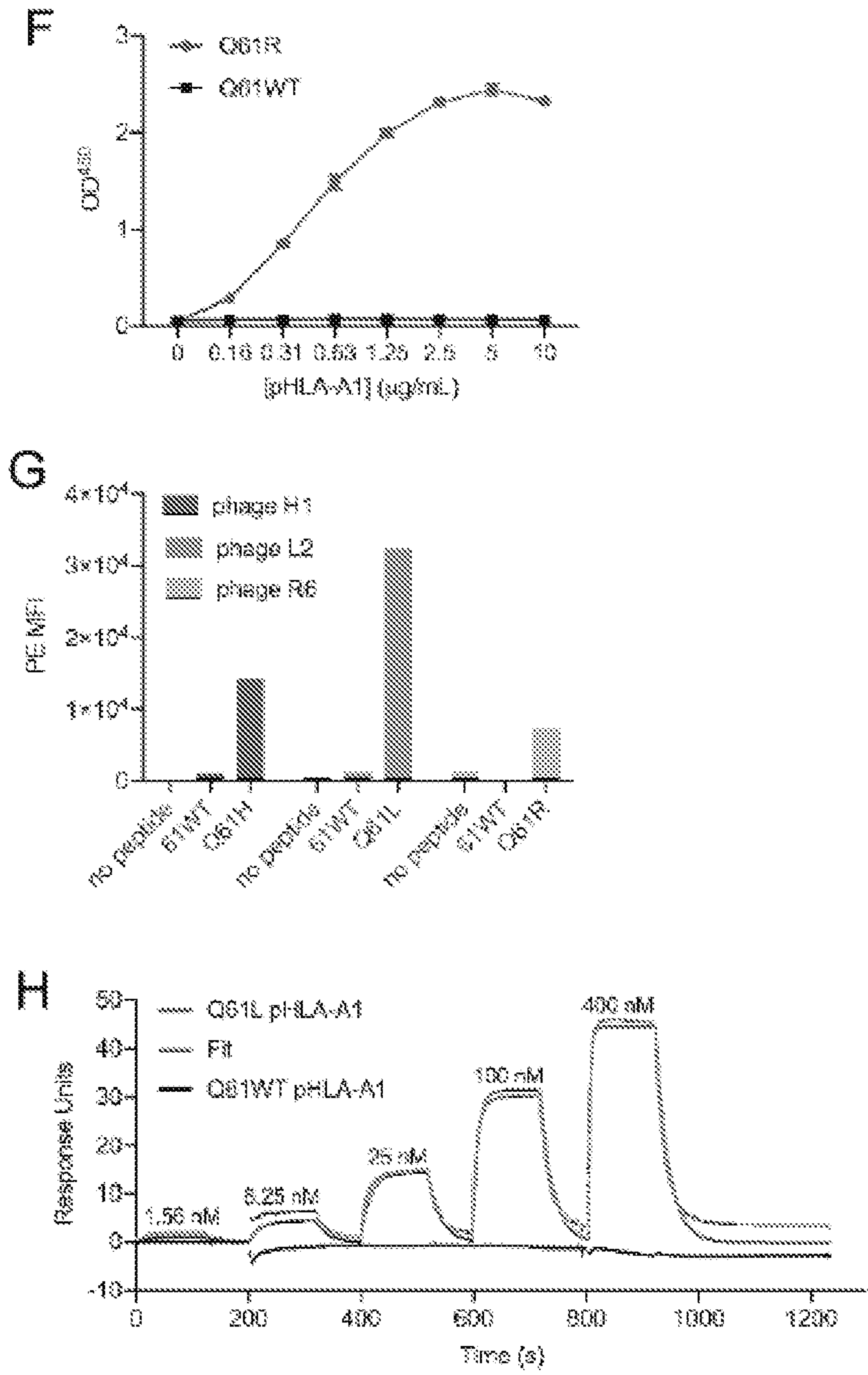


FIG. 38 (continued)

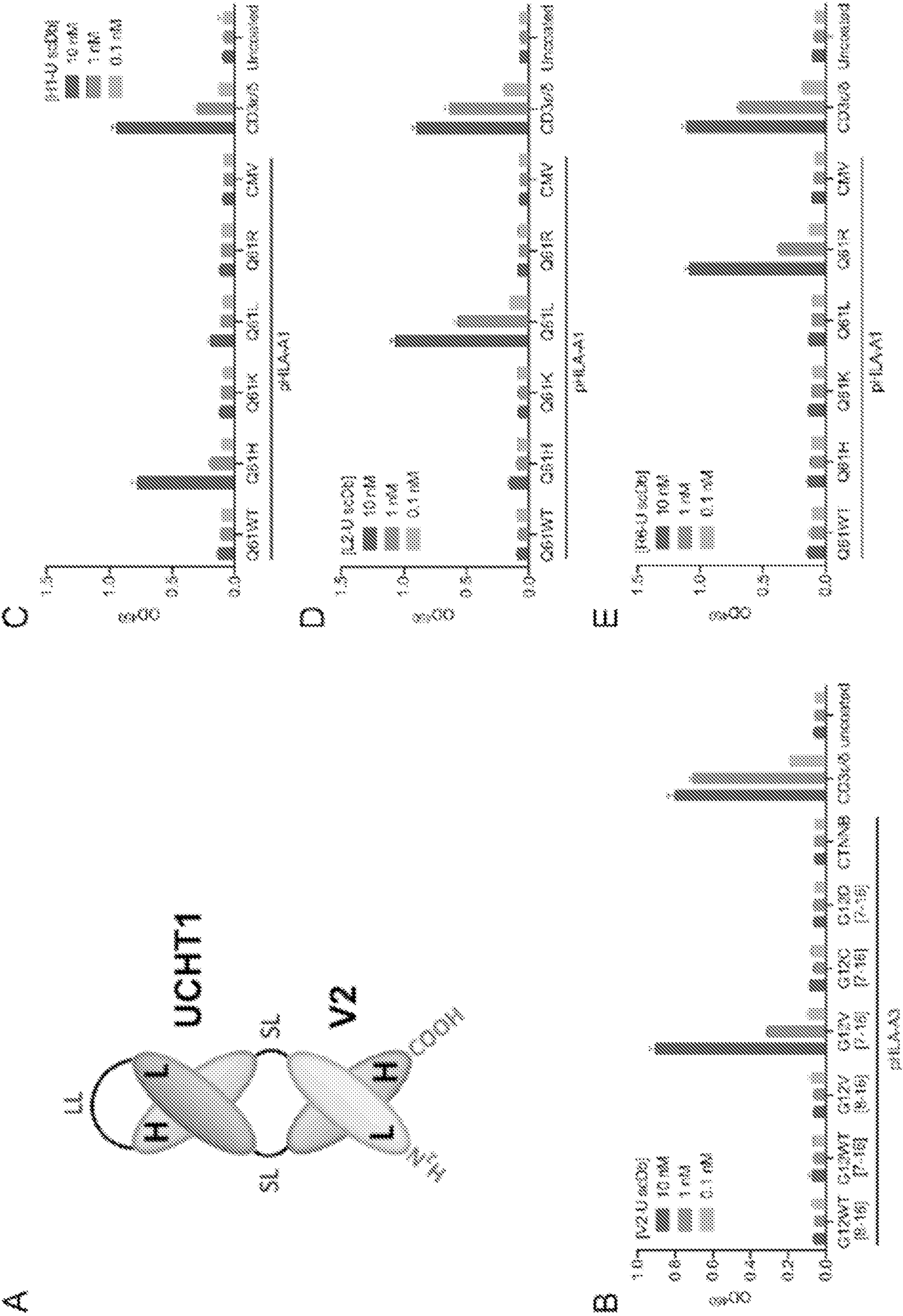


FIG. 39

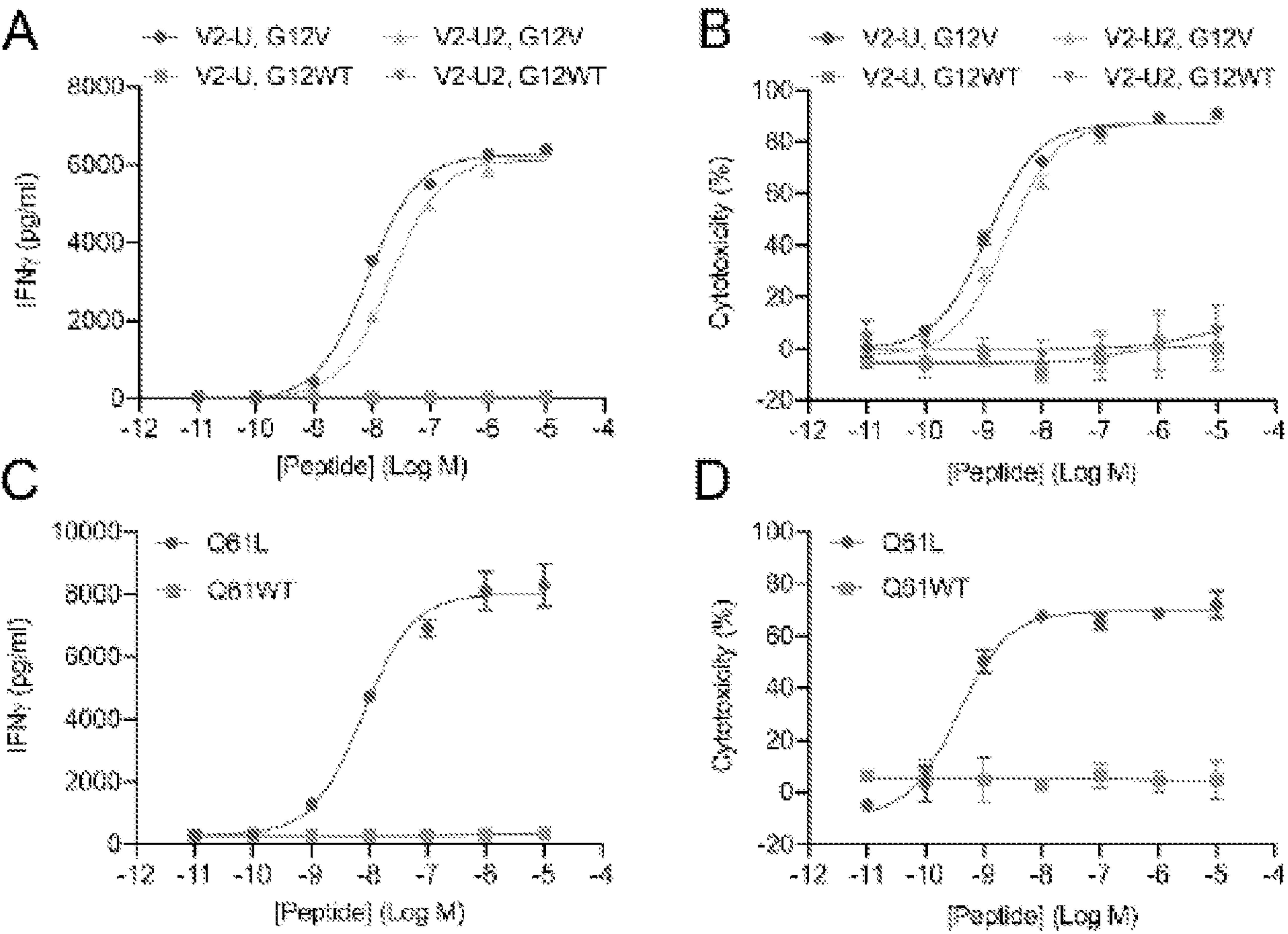


FIG. 40

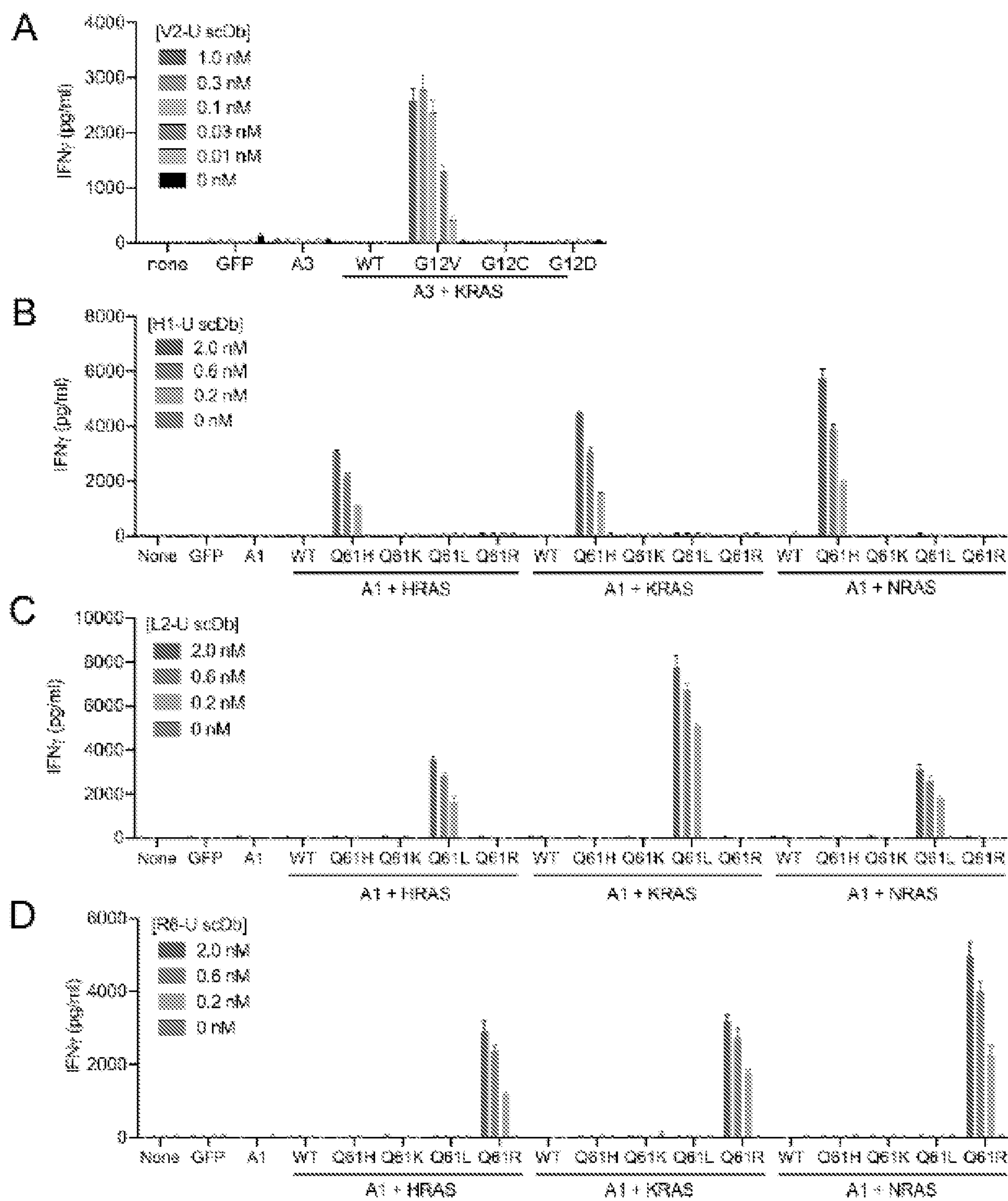


FIG. 41

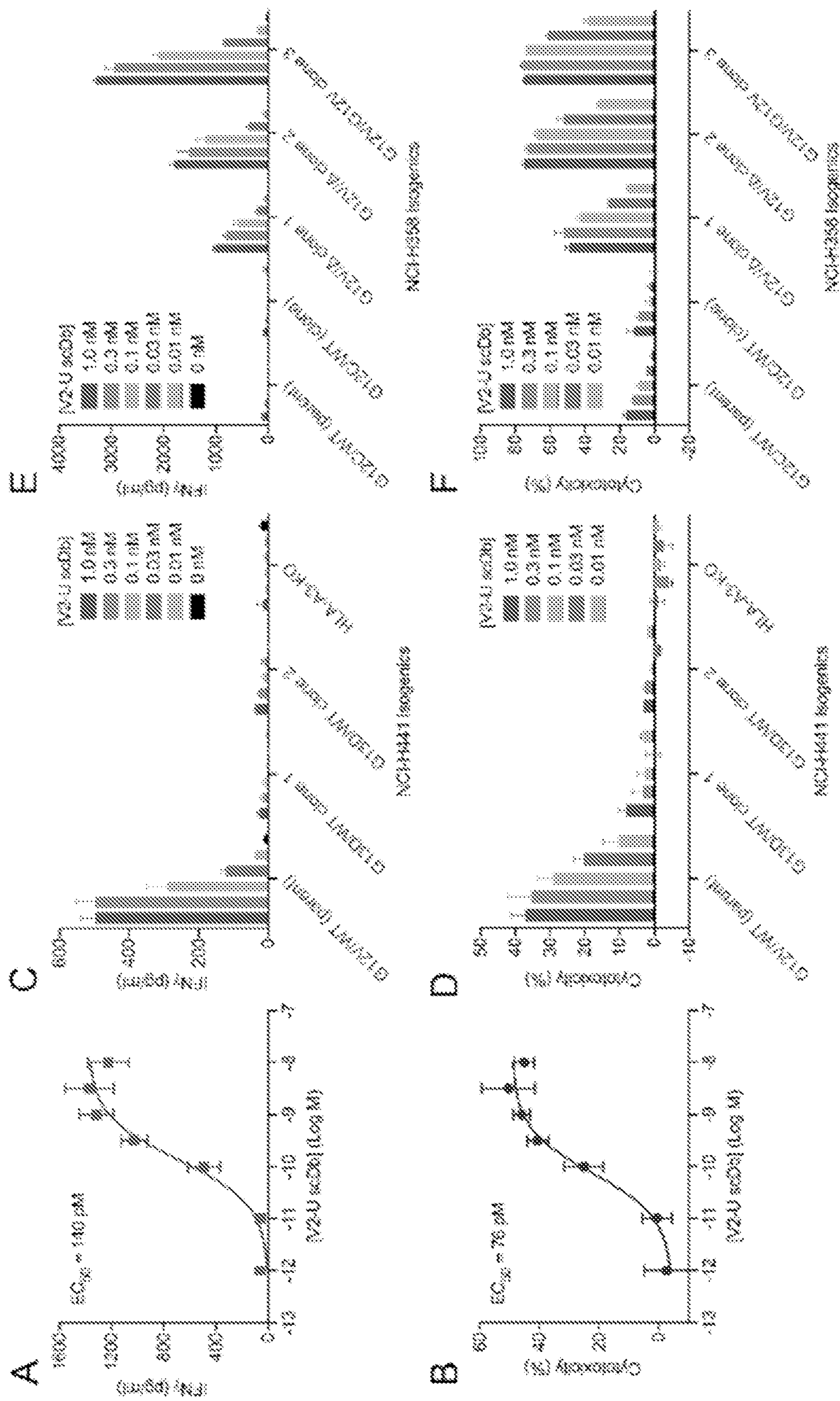


FIG. 42

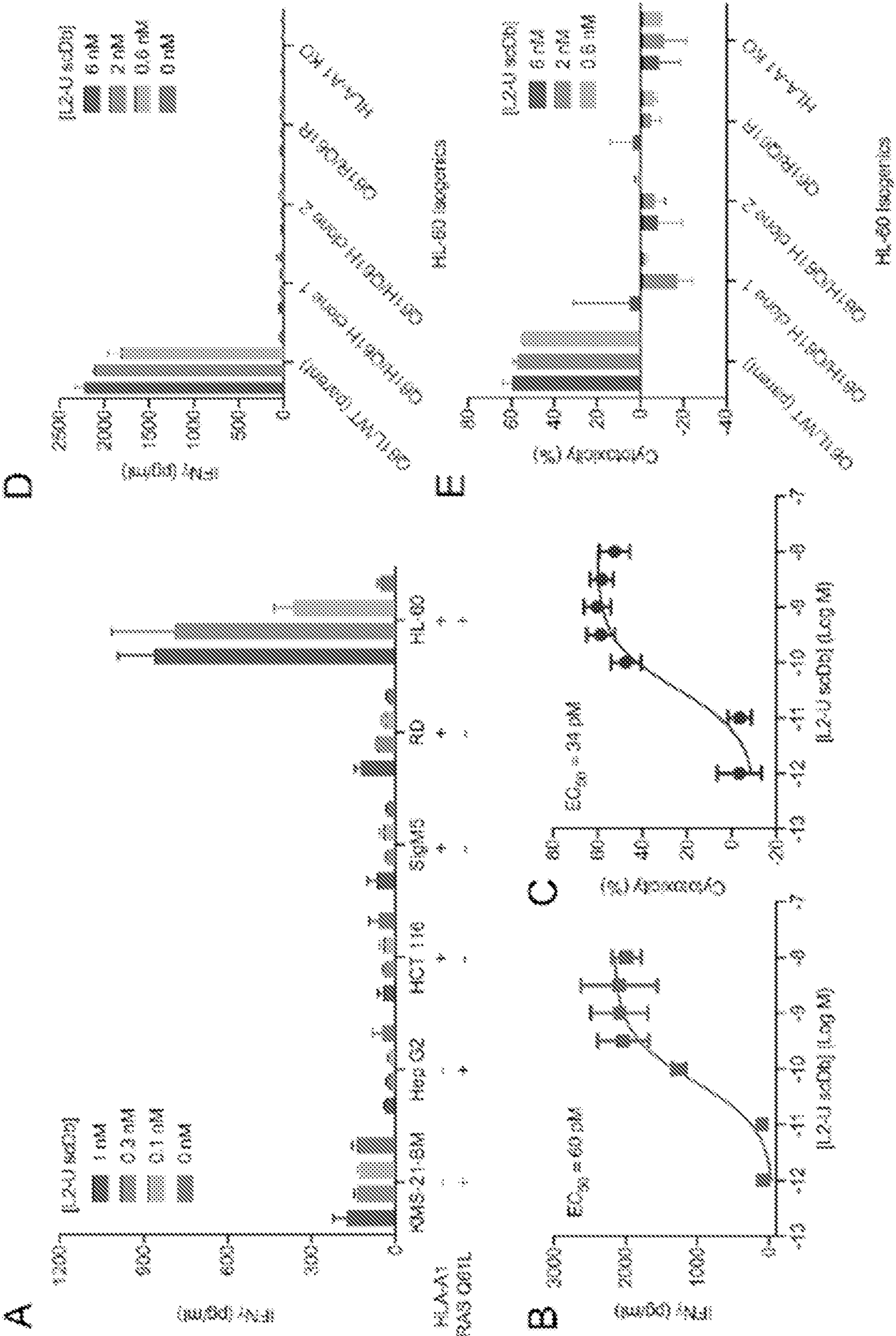


FIG. 43

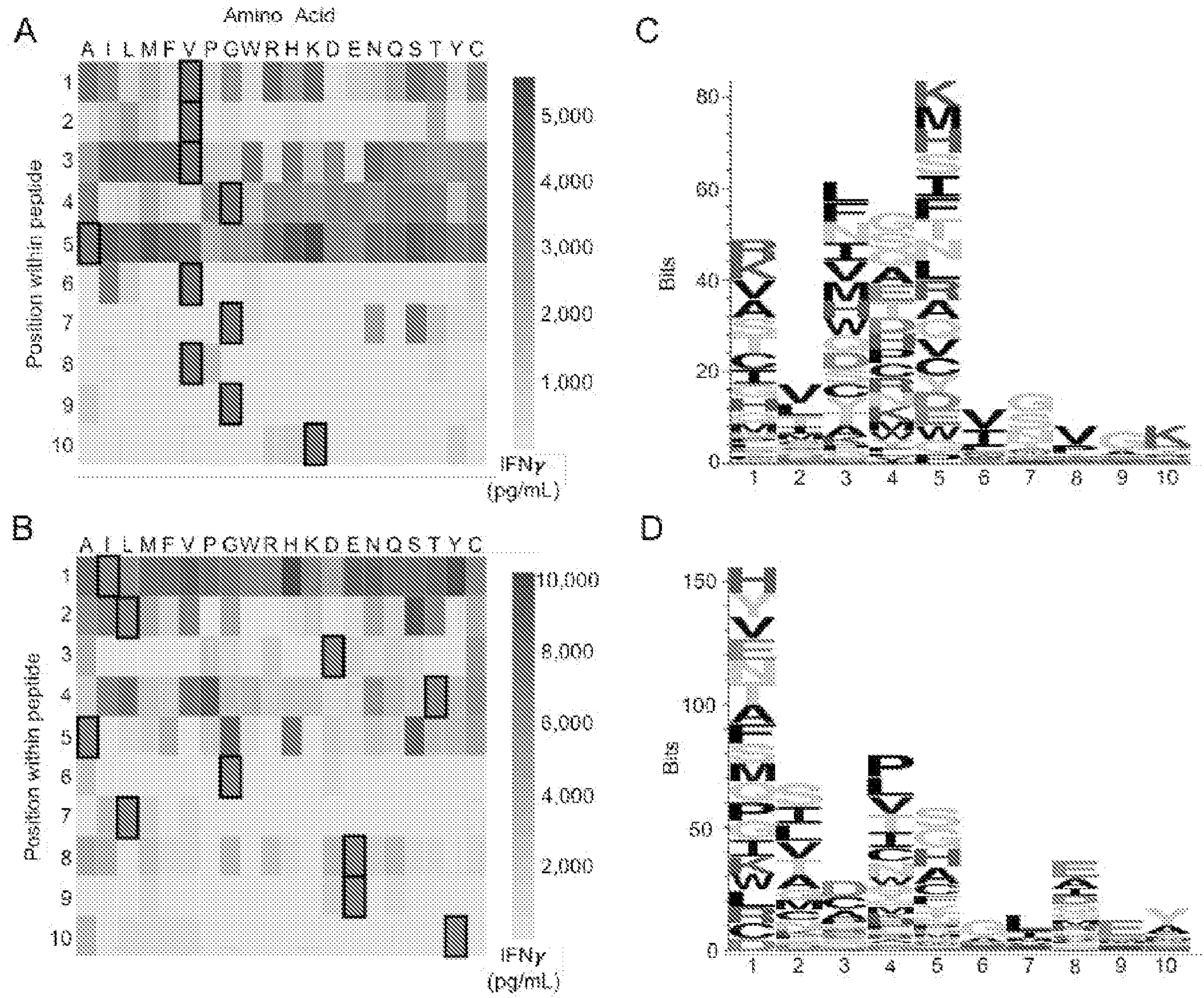


FIG. 44

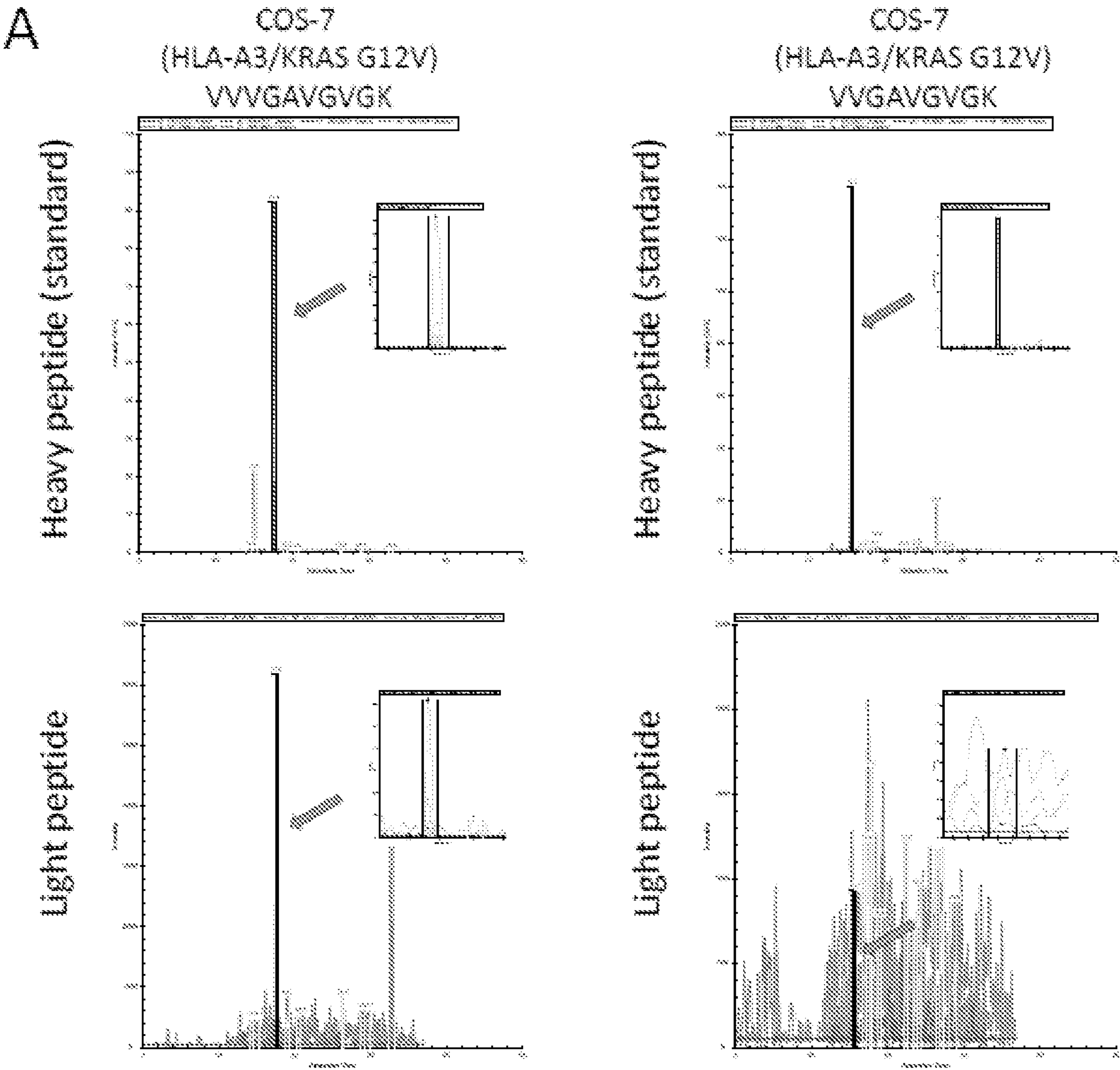


FIG. 45

B

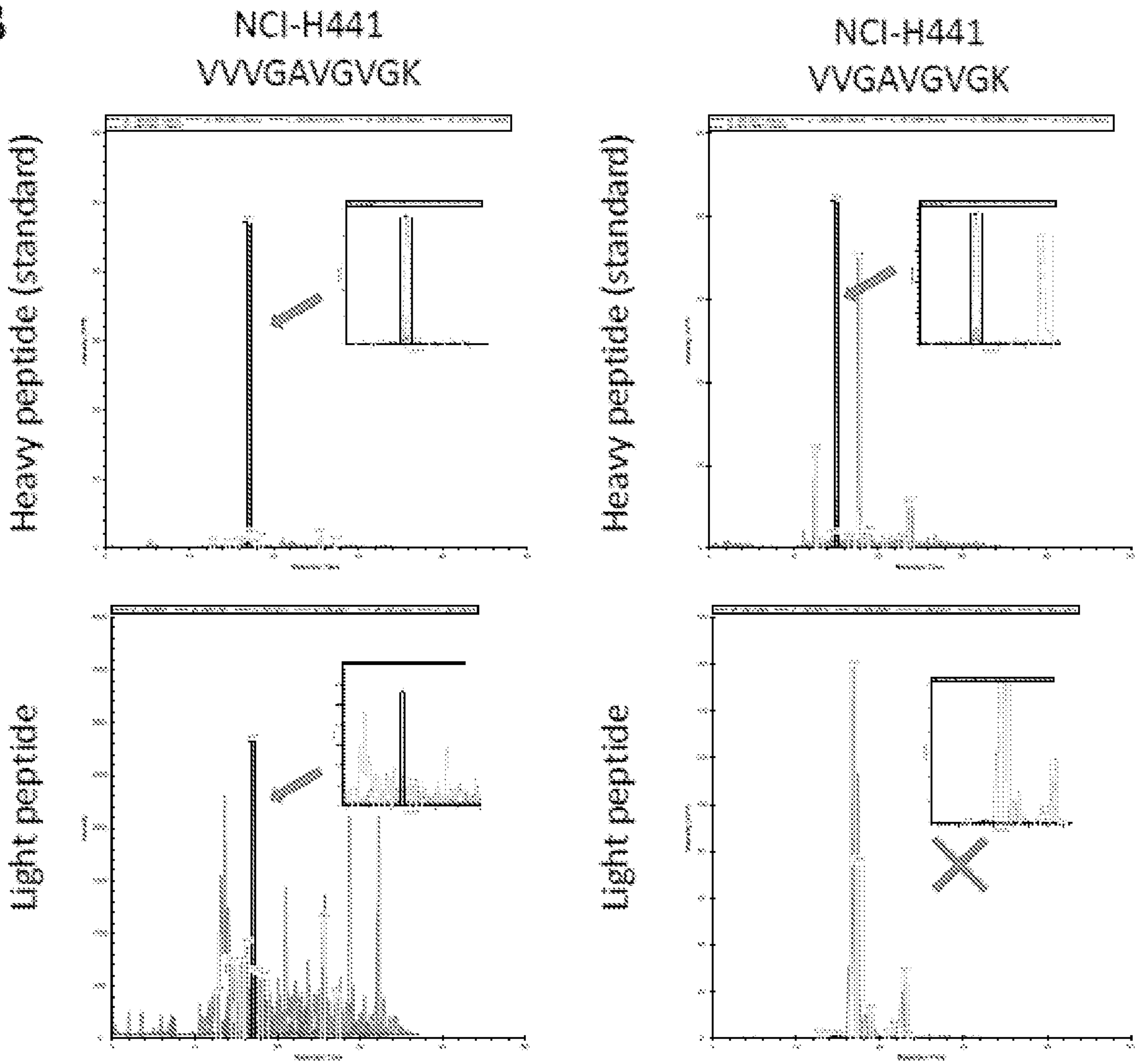


FIG. 45 (continued)

C

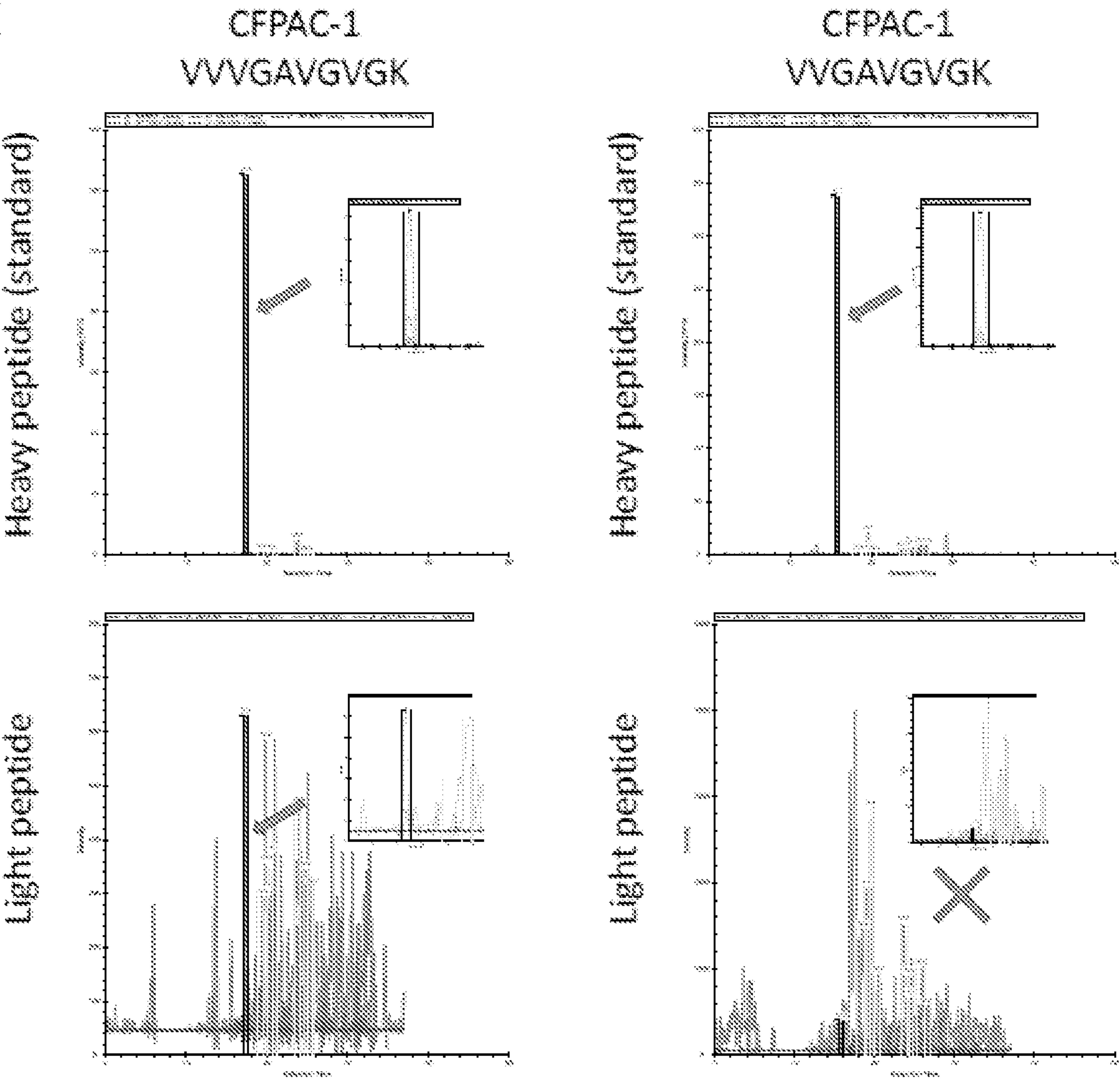


FIG. 45 (continued)

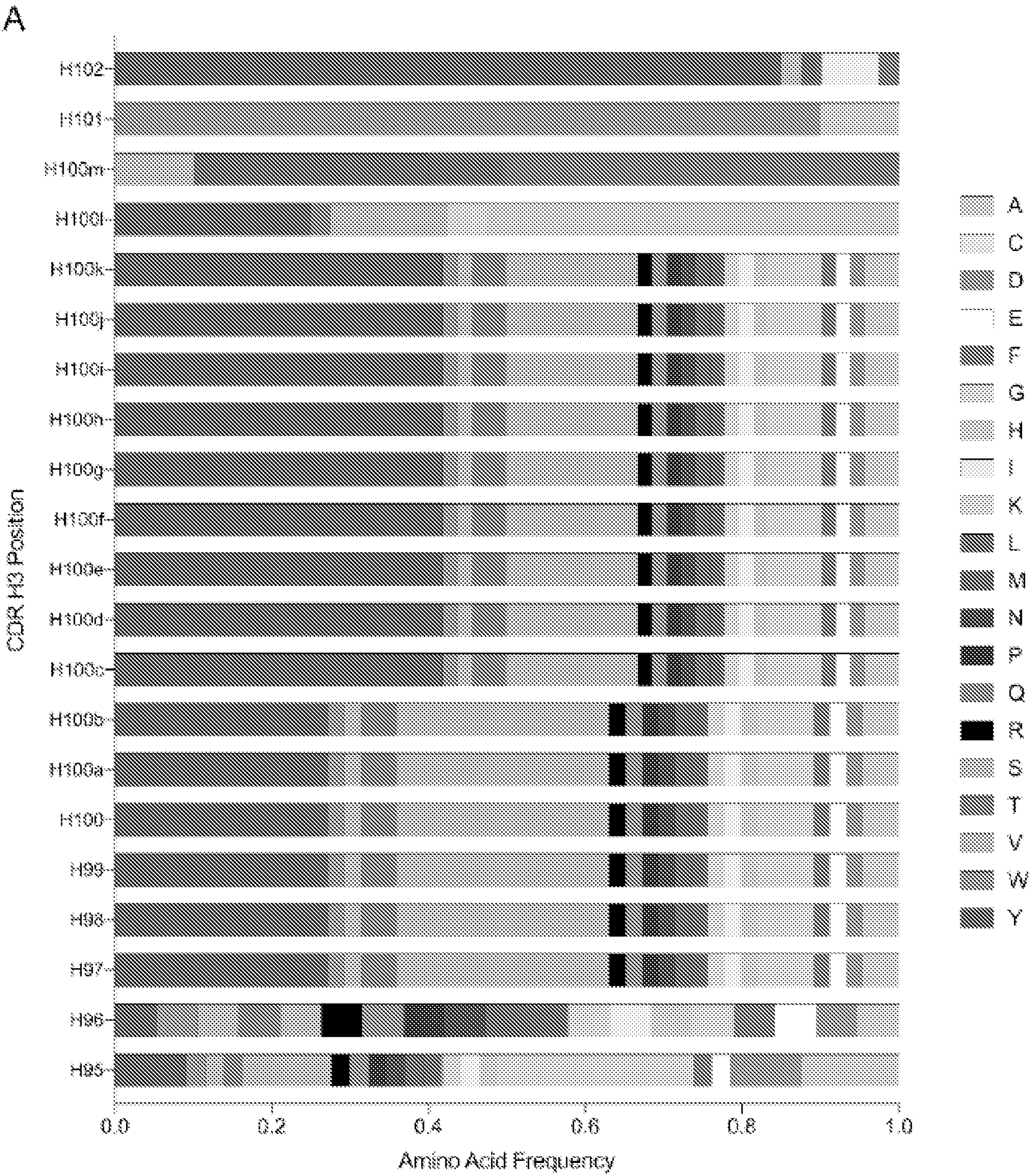


FIG. 46

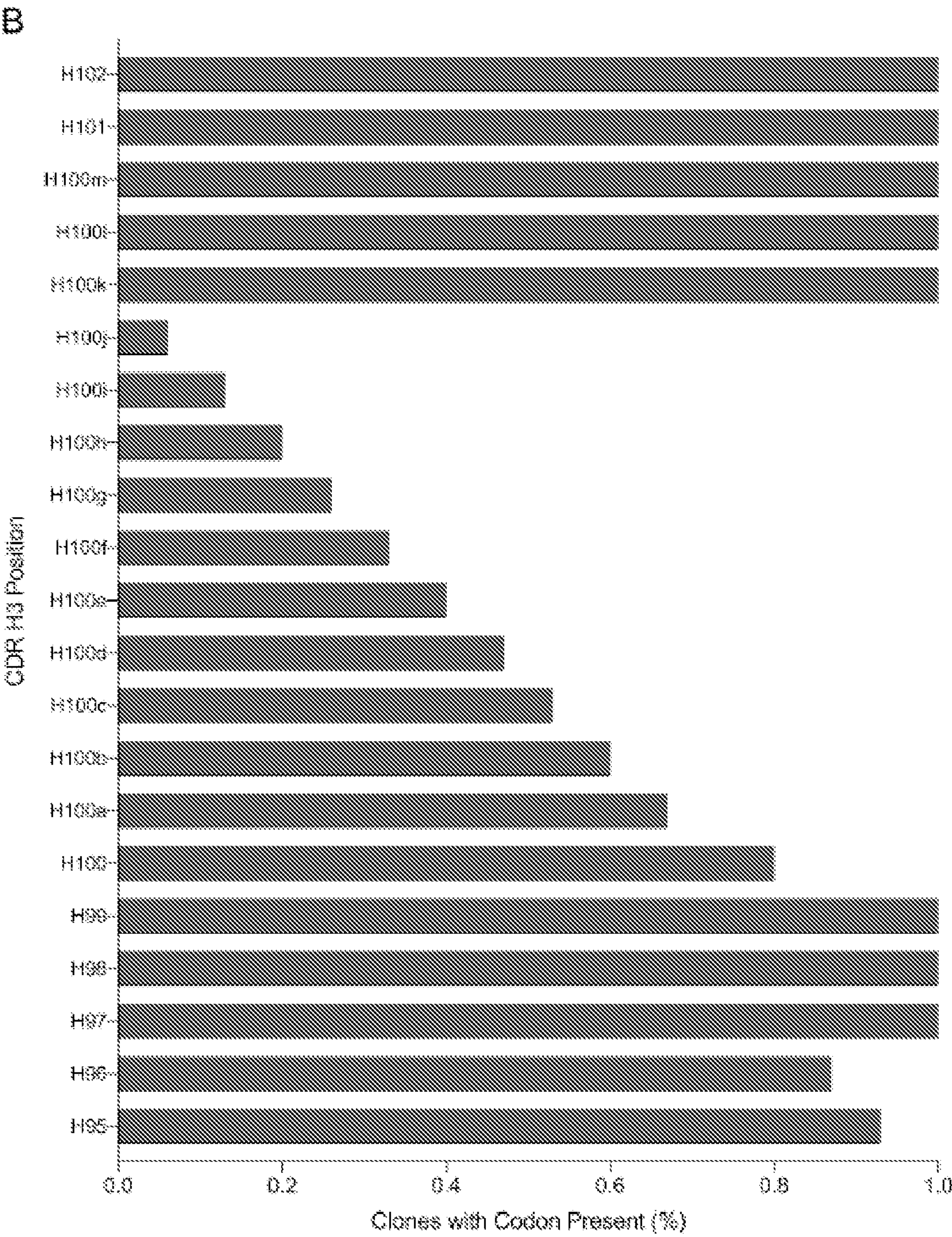


FIG. 46 (continued)

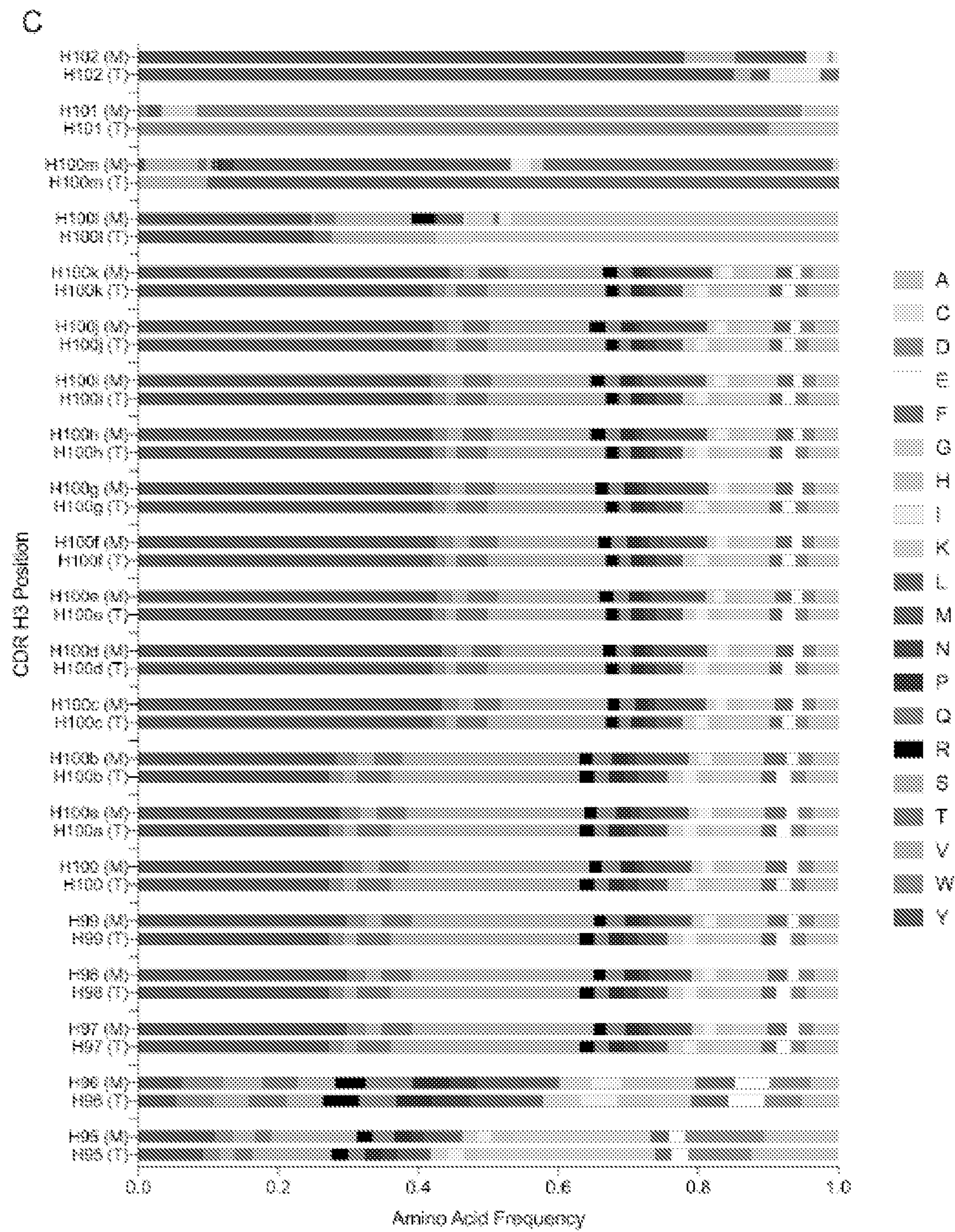


FIG. 46 (continued)

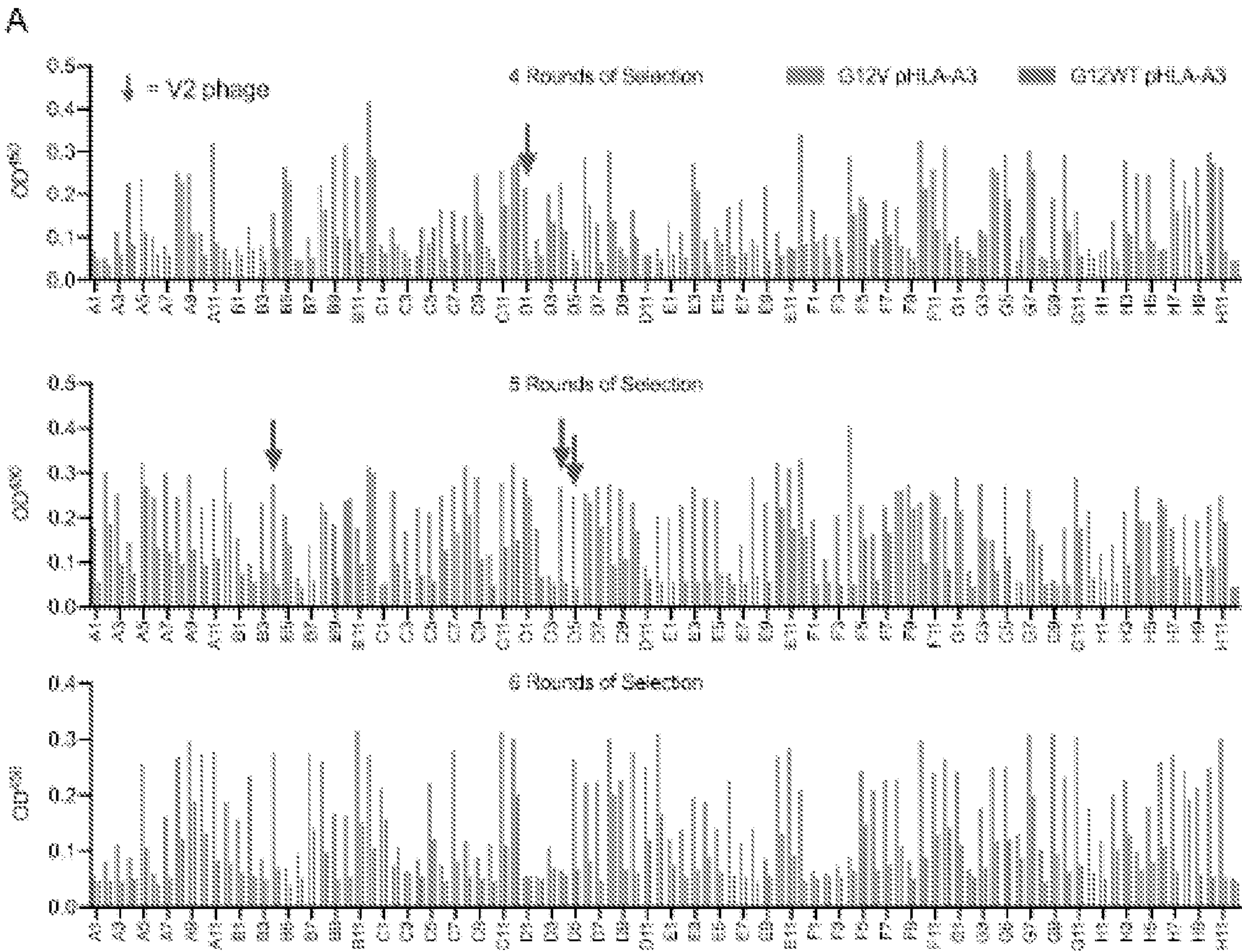


FIG. 47

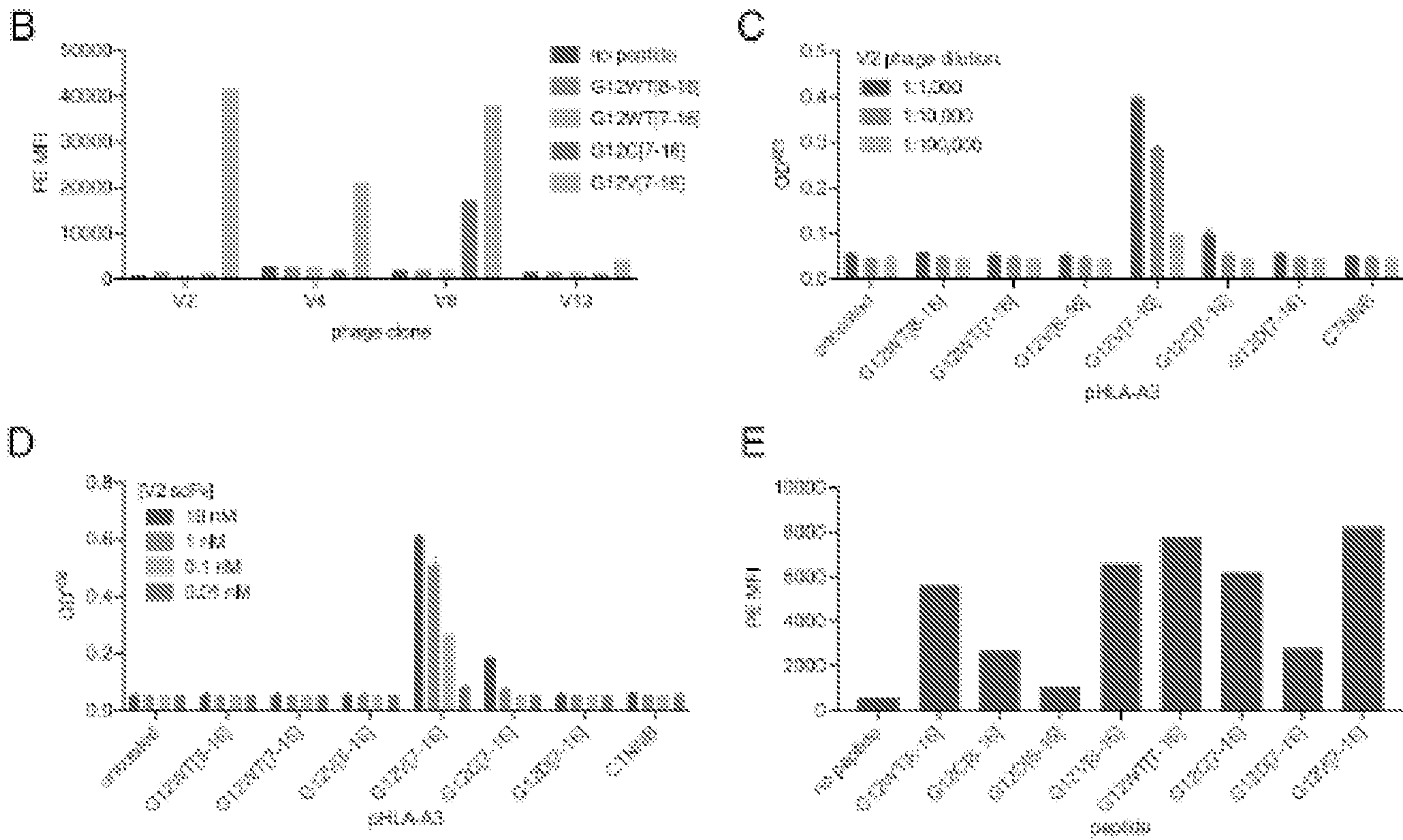


FIG. 47 (continued)

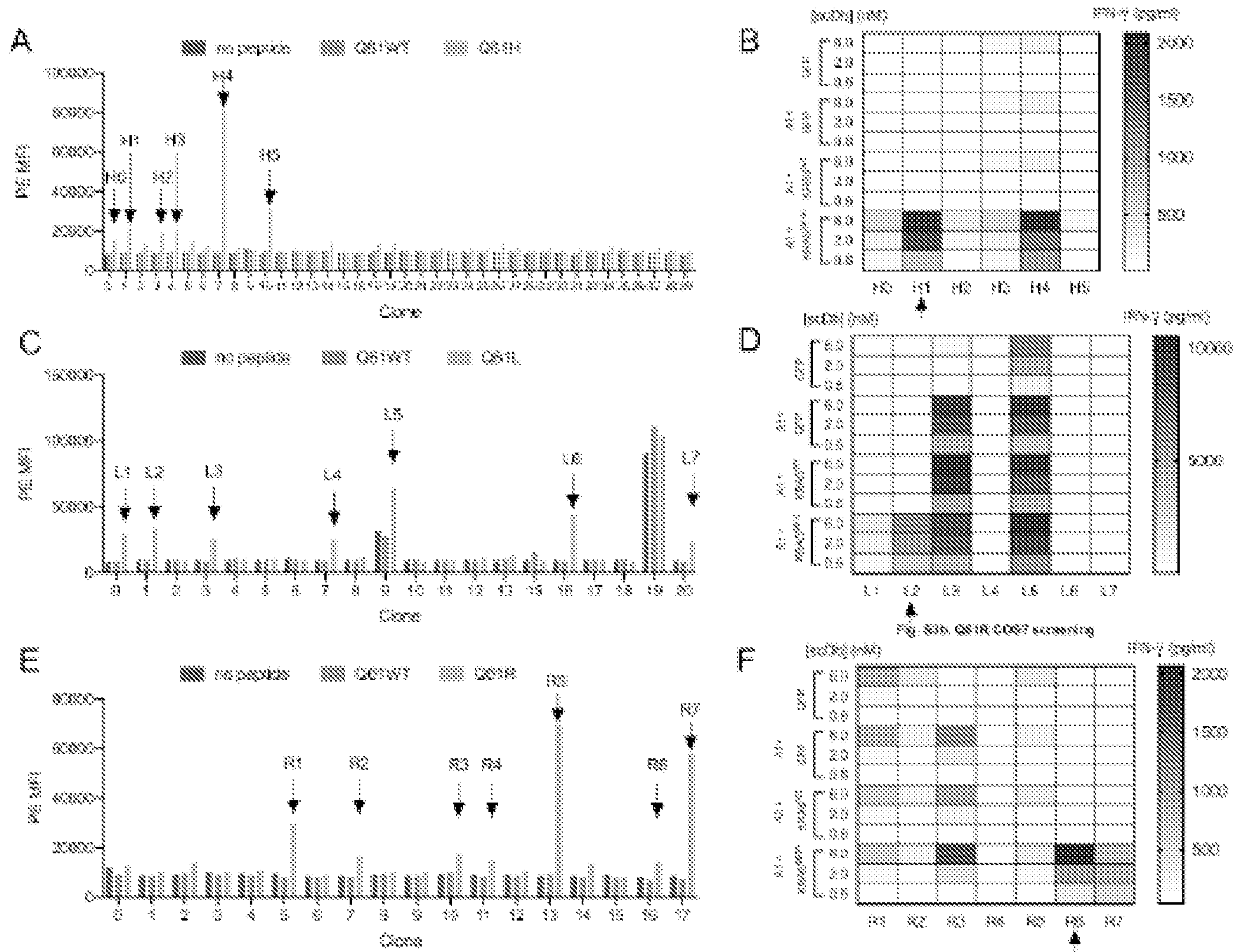


FIG. 48

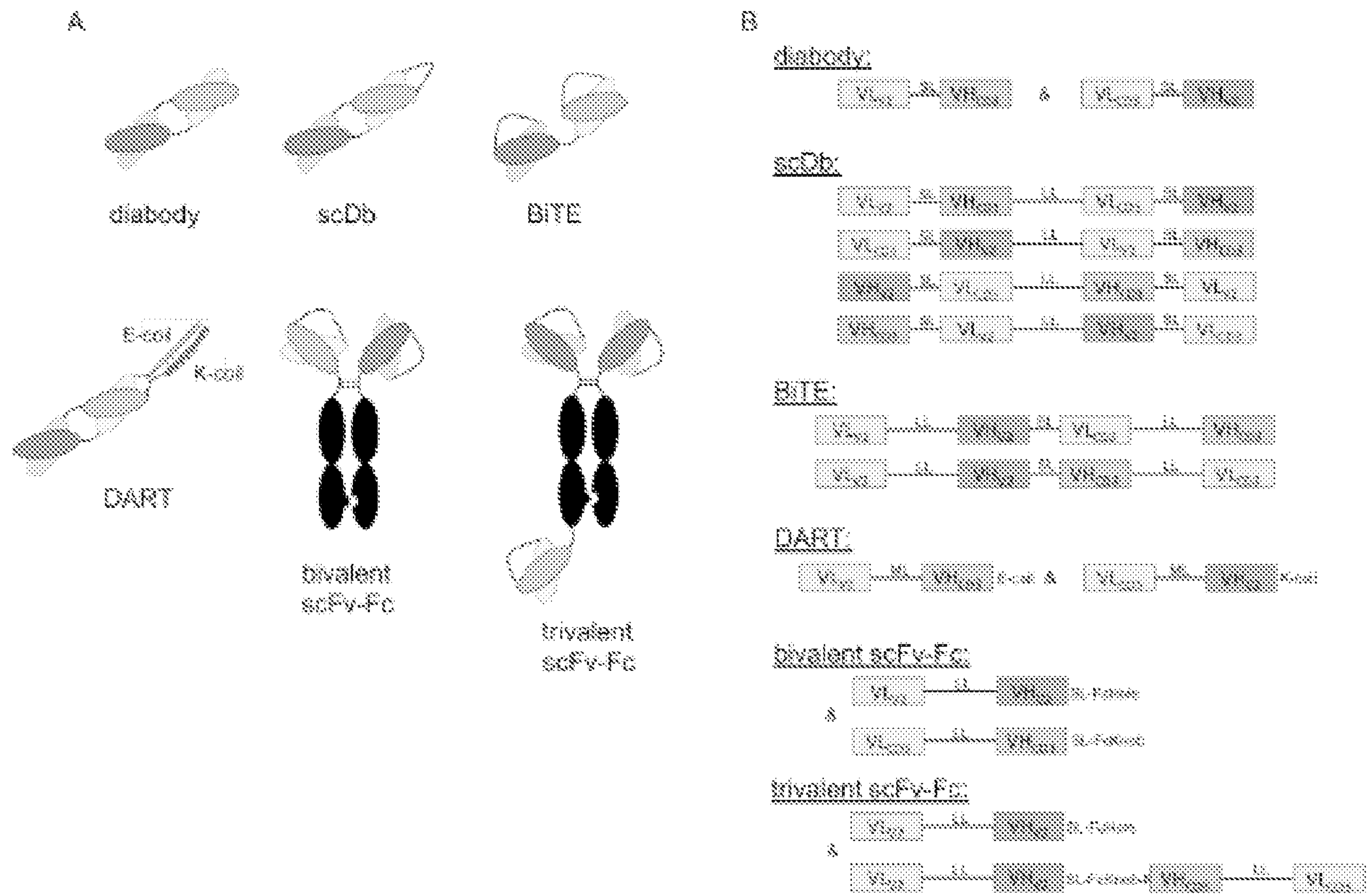


FIG. 49

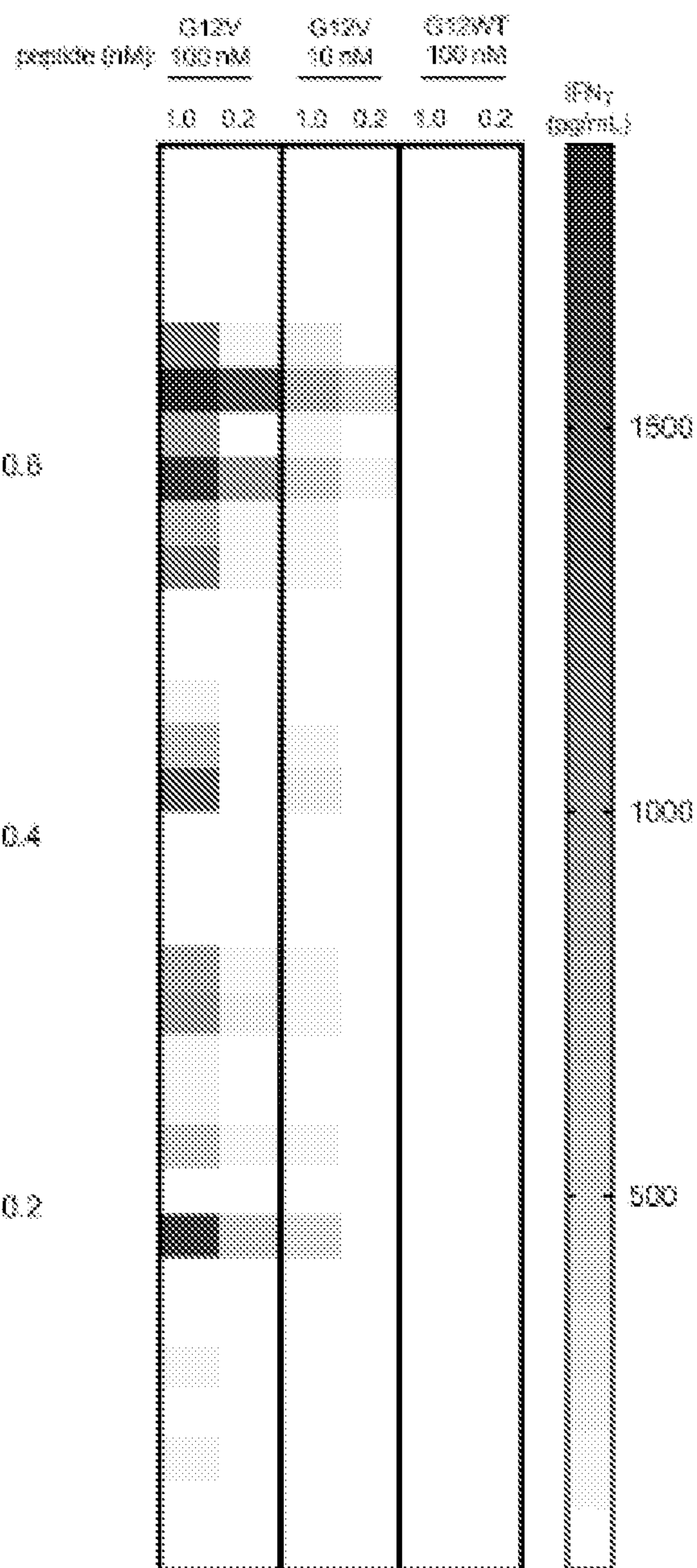


FIG. 49 (continued)

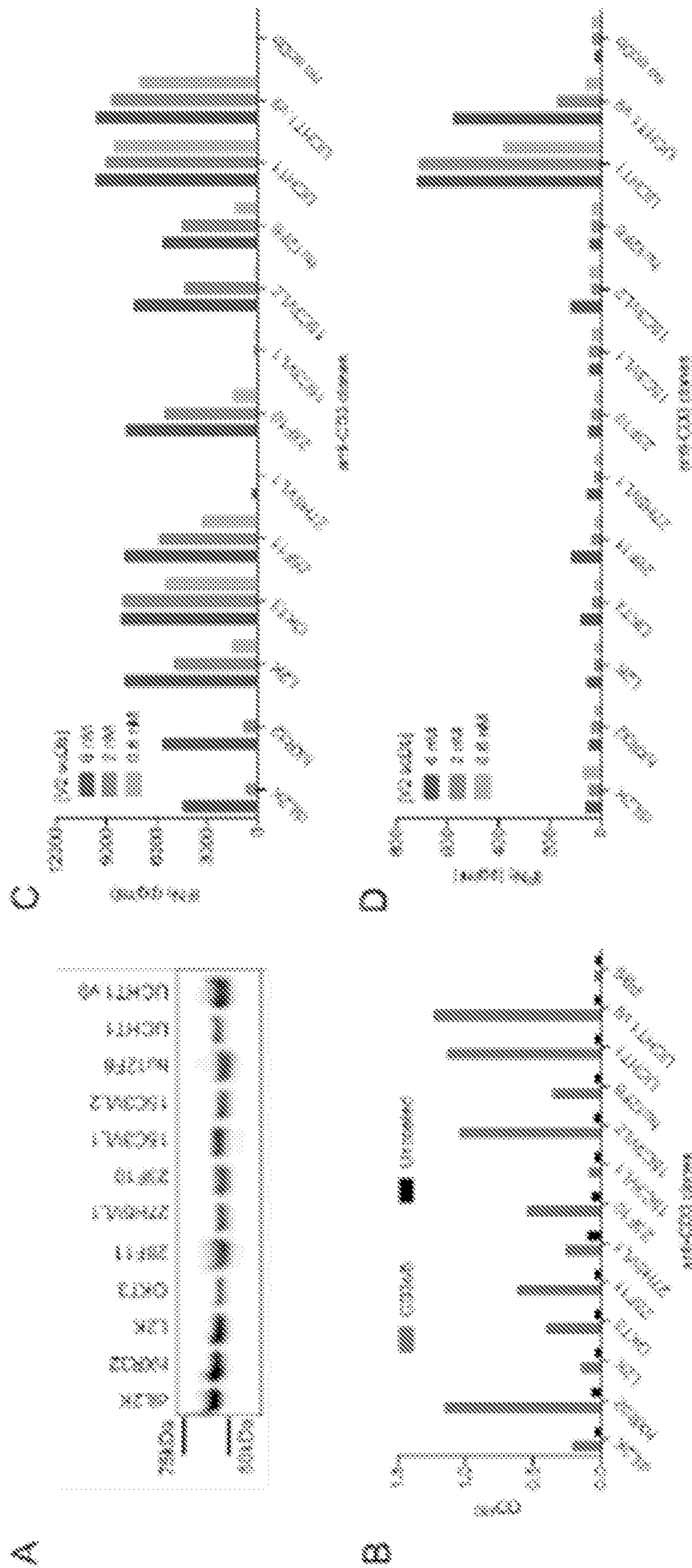


FIG. 50

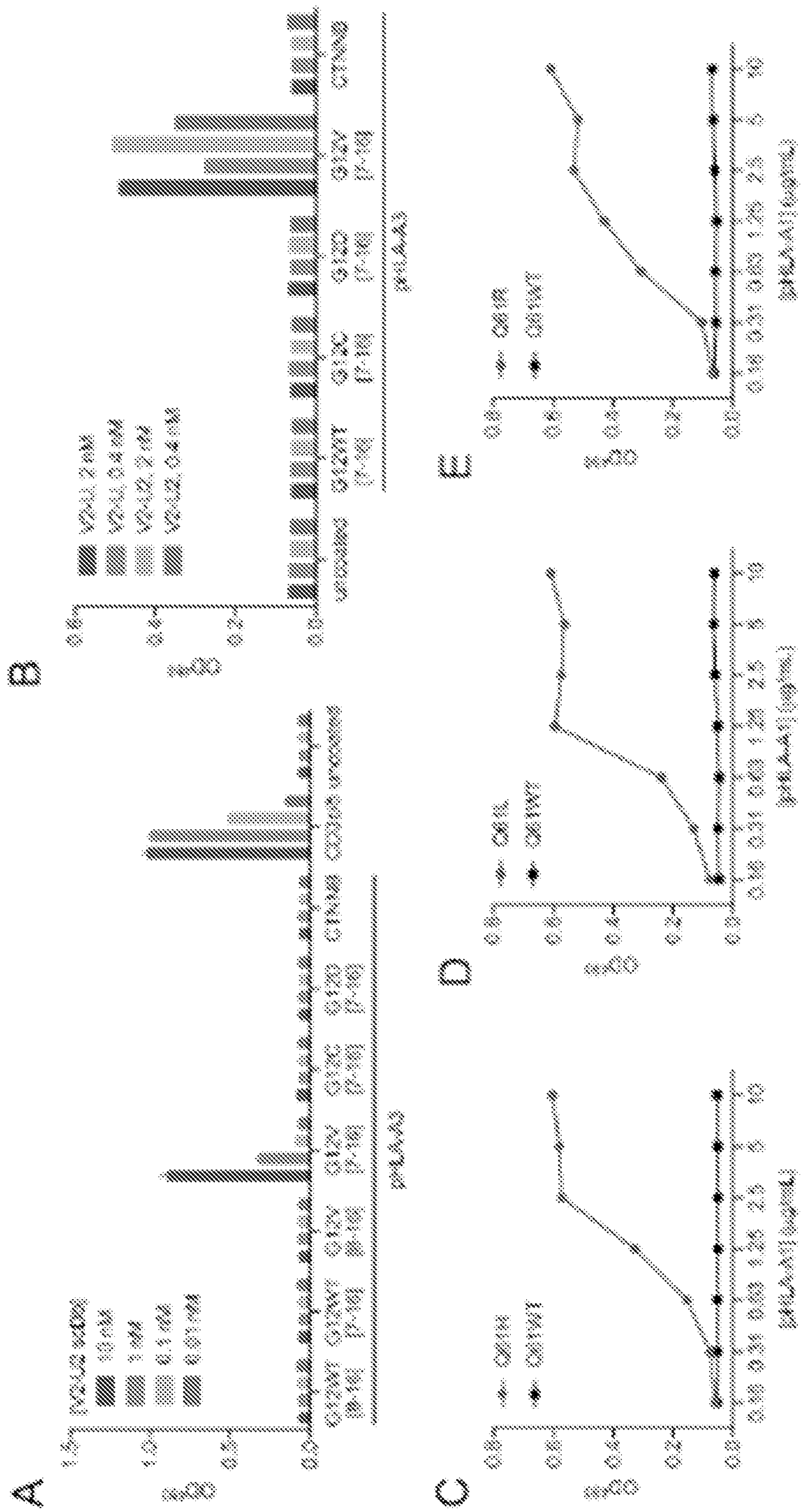


FIG. 51

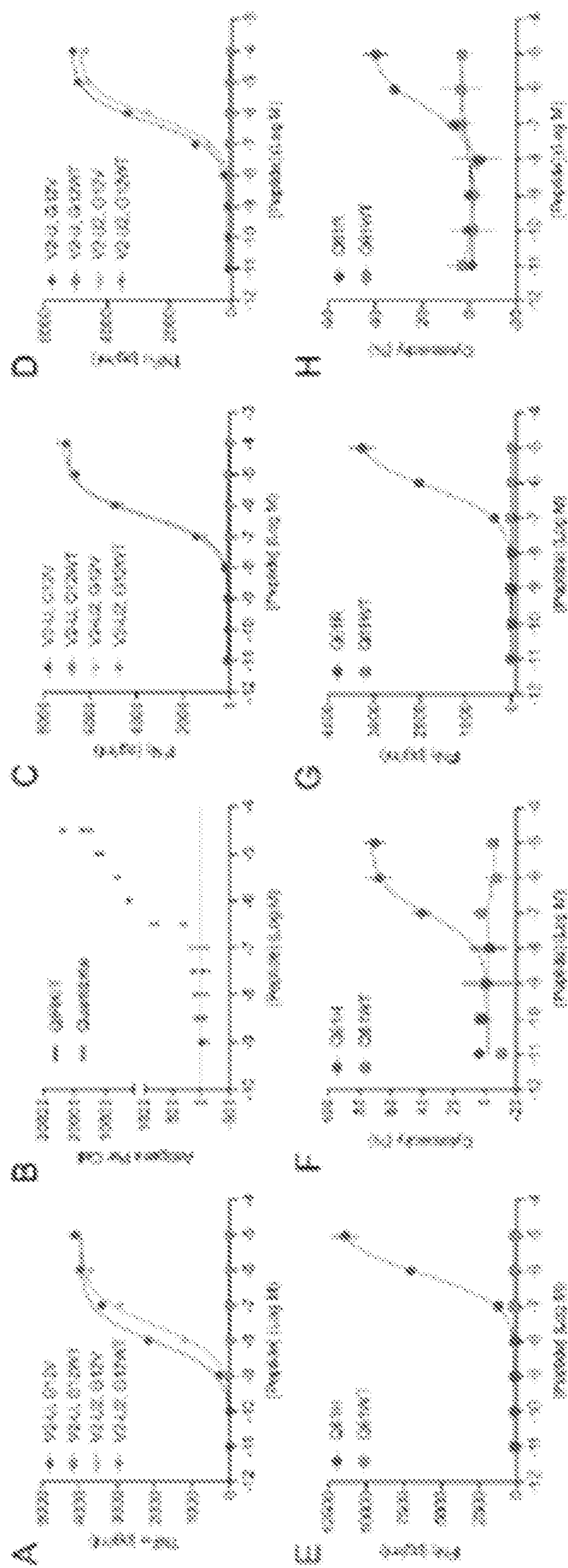


FIG. 52

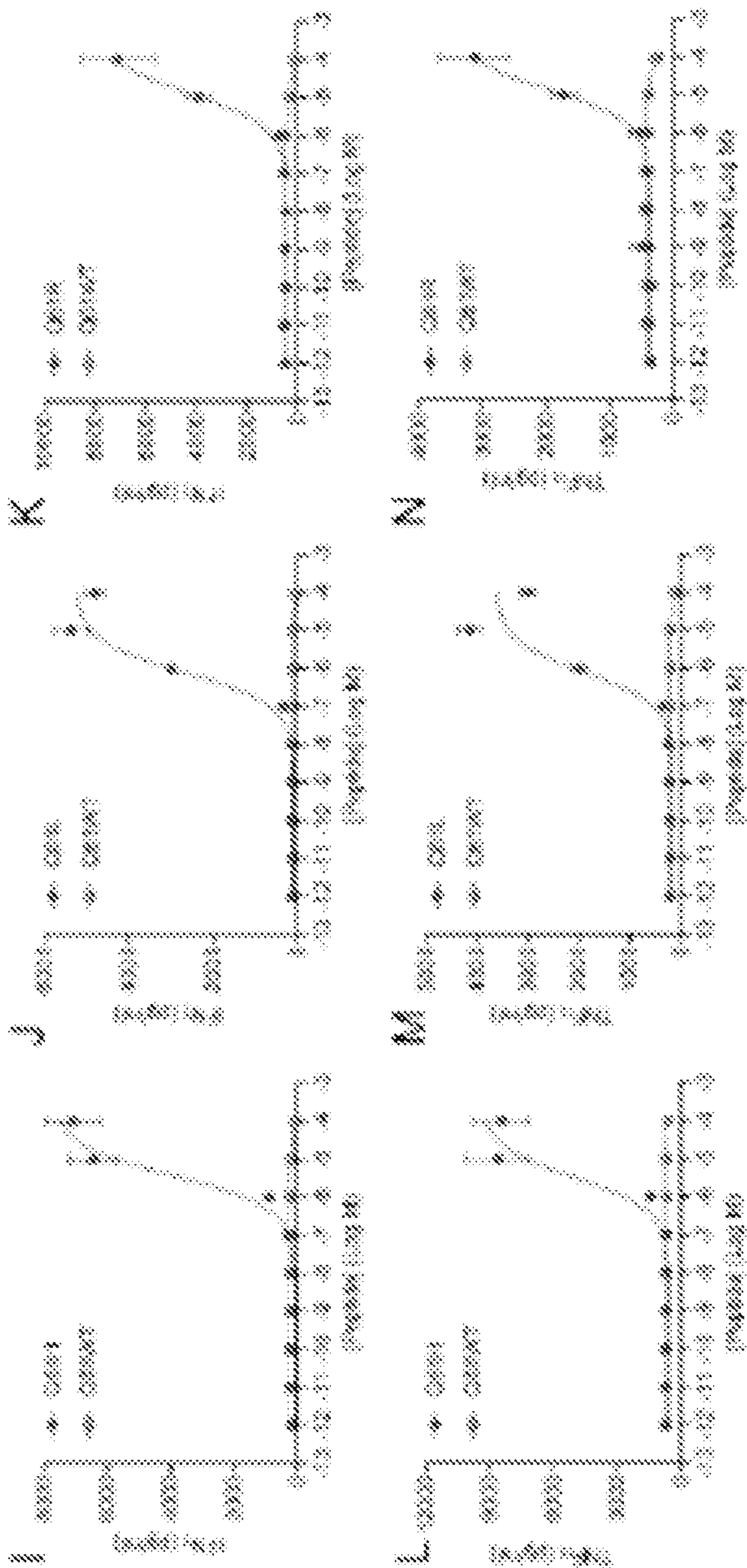


FIG. 52 (continued)

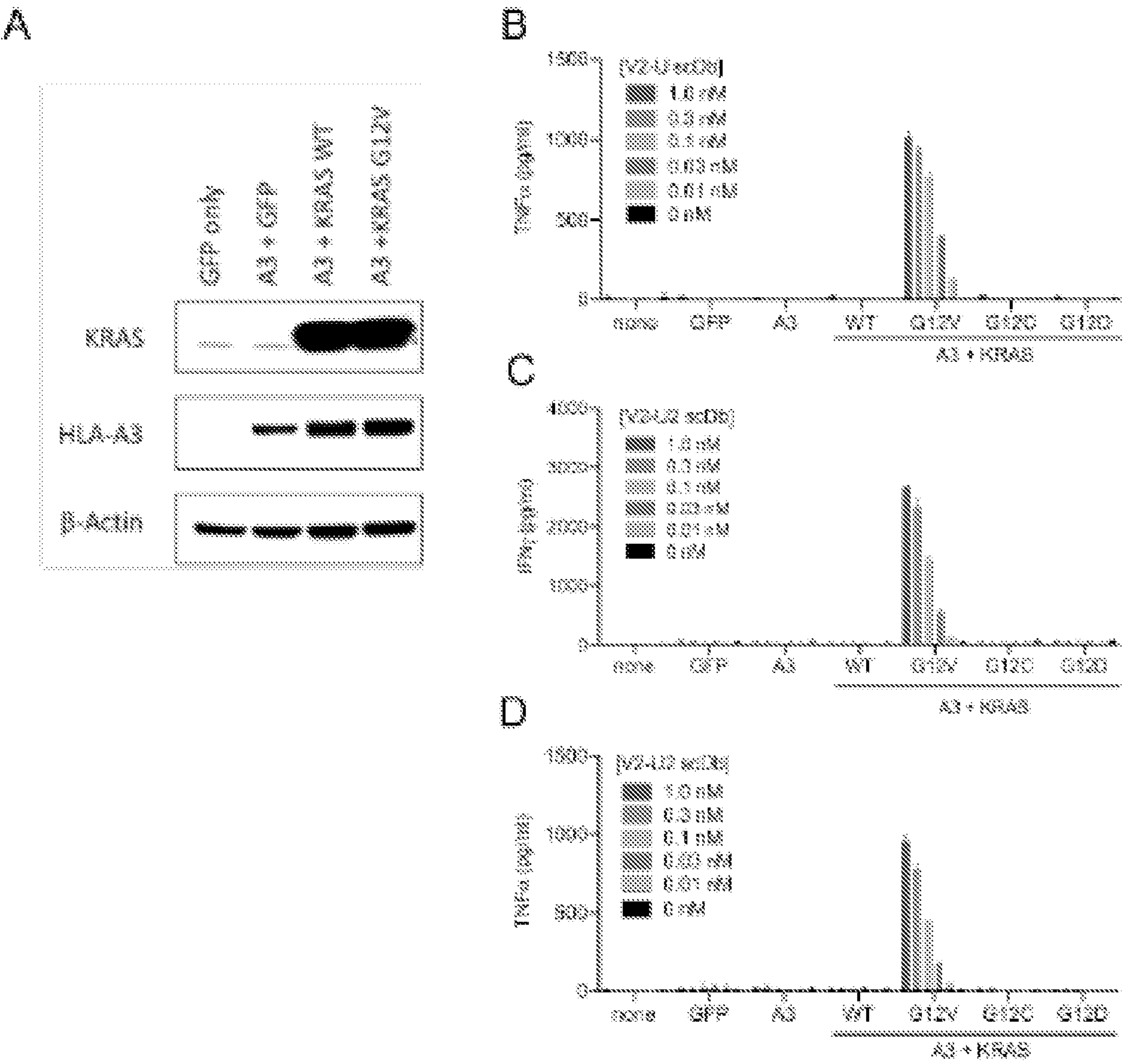


FIG. 53

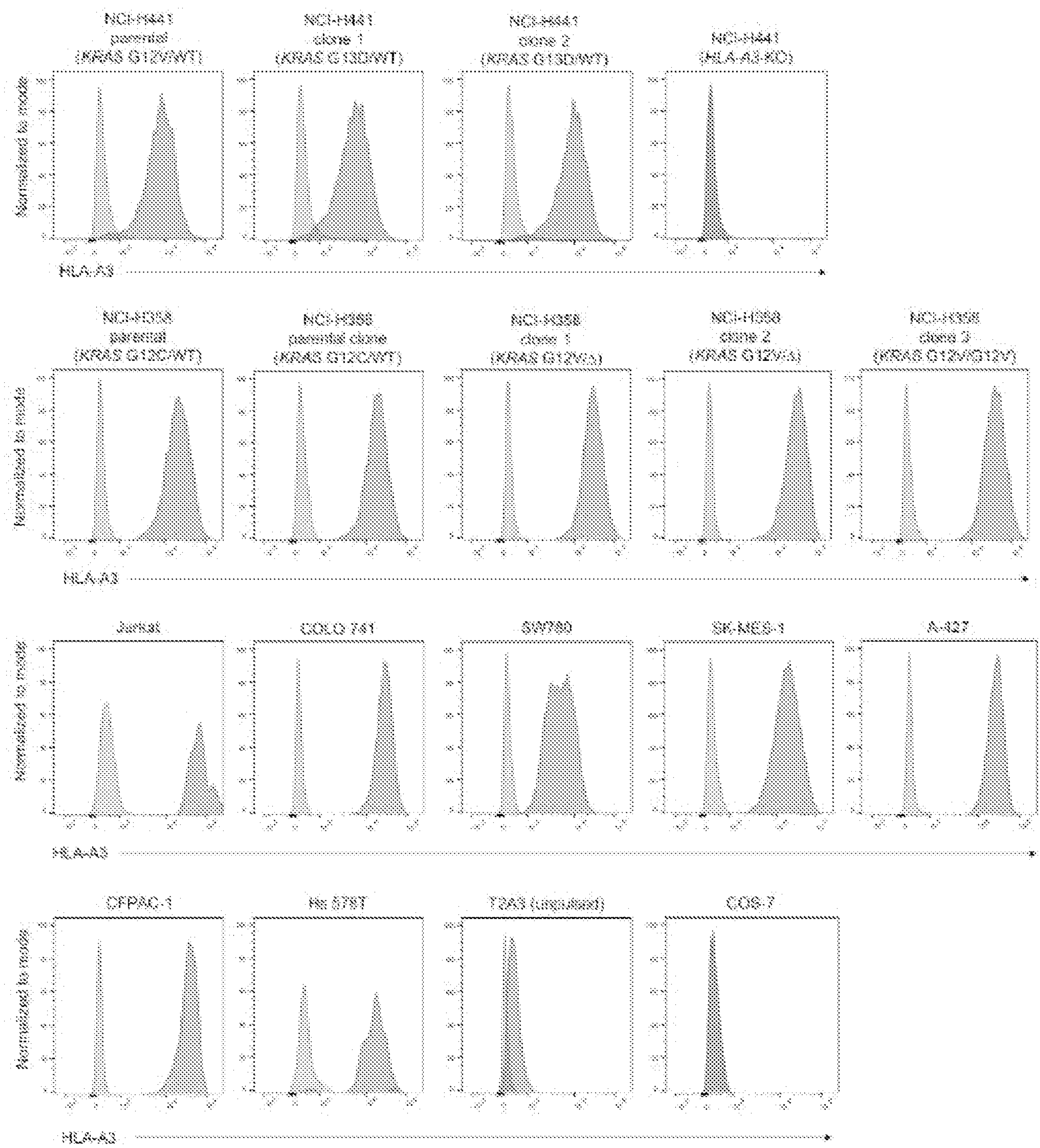


FIG. 54

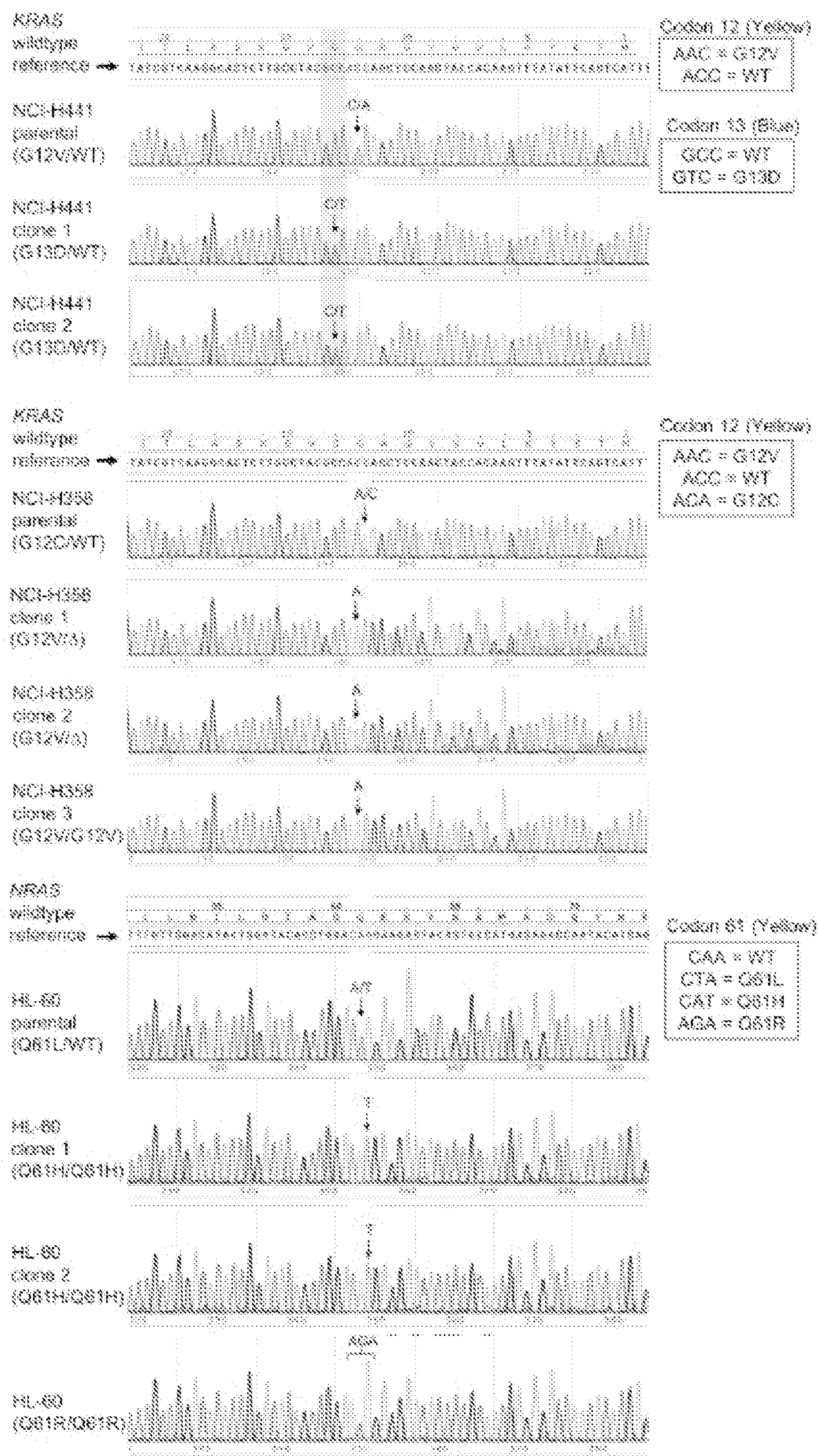


FIG. 55

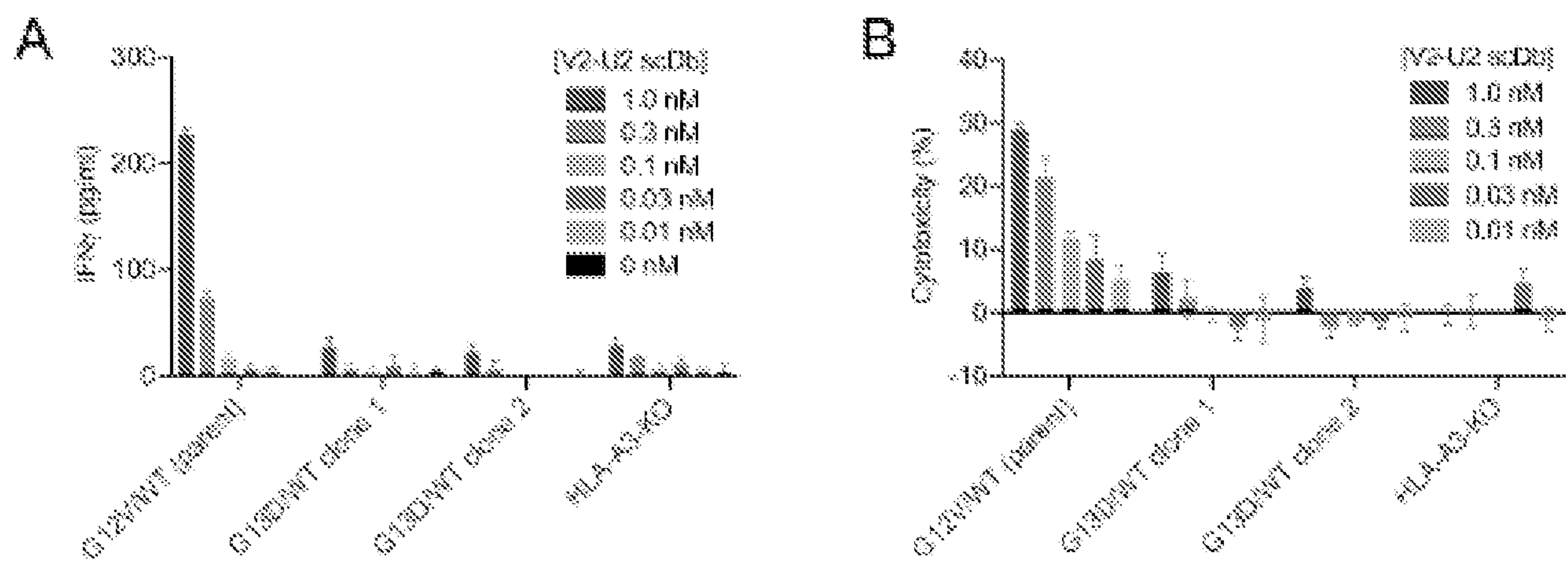


FIG. 56

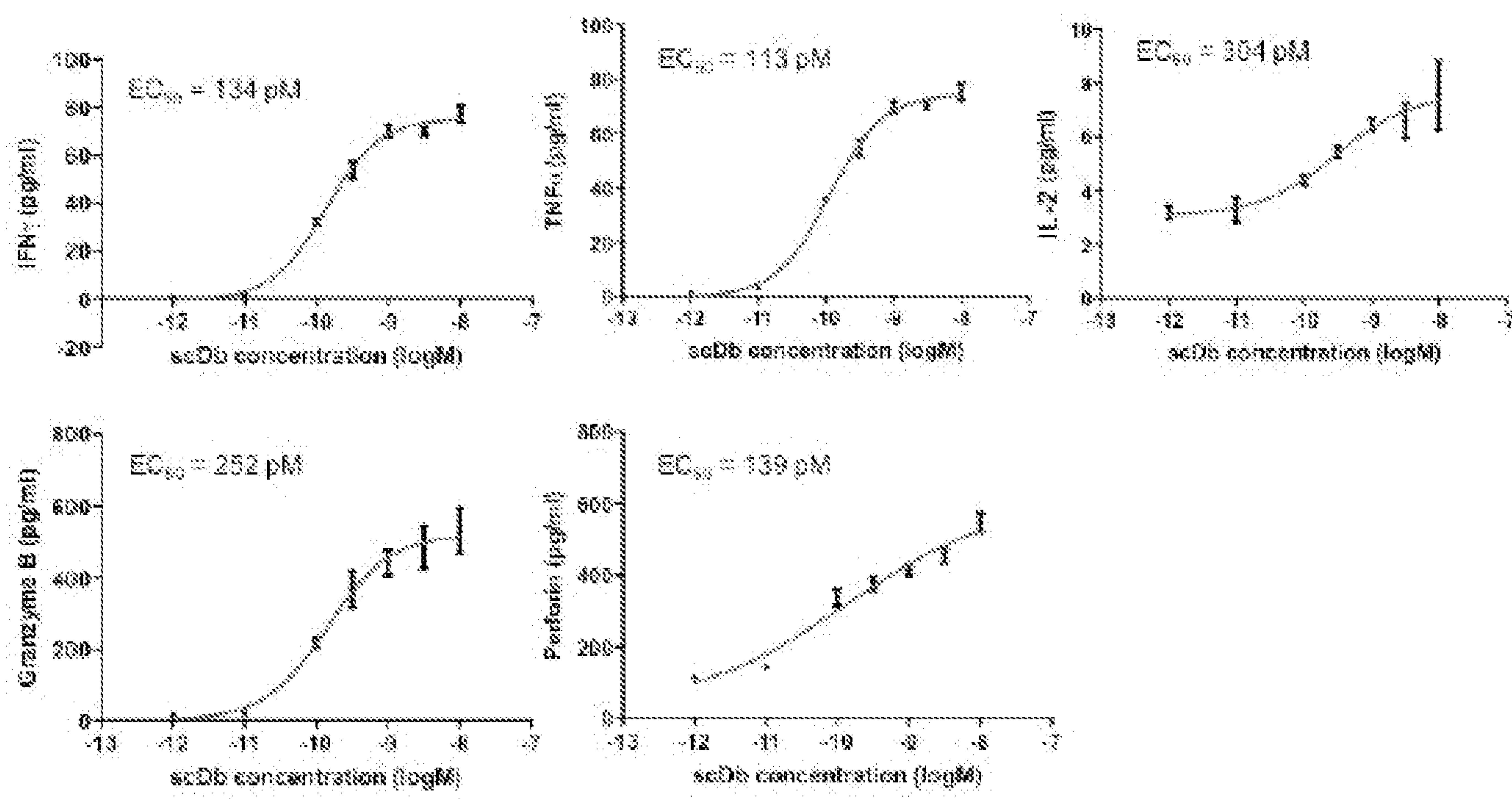


FIG. 57

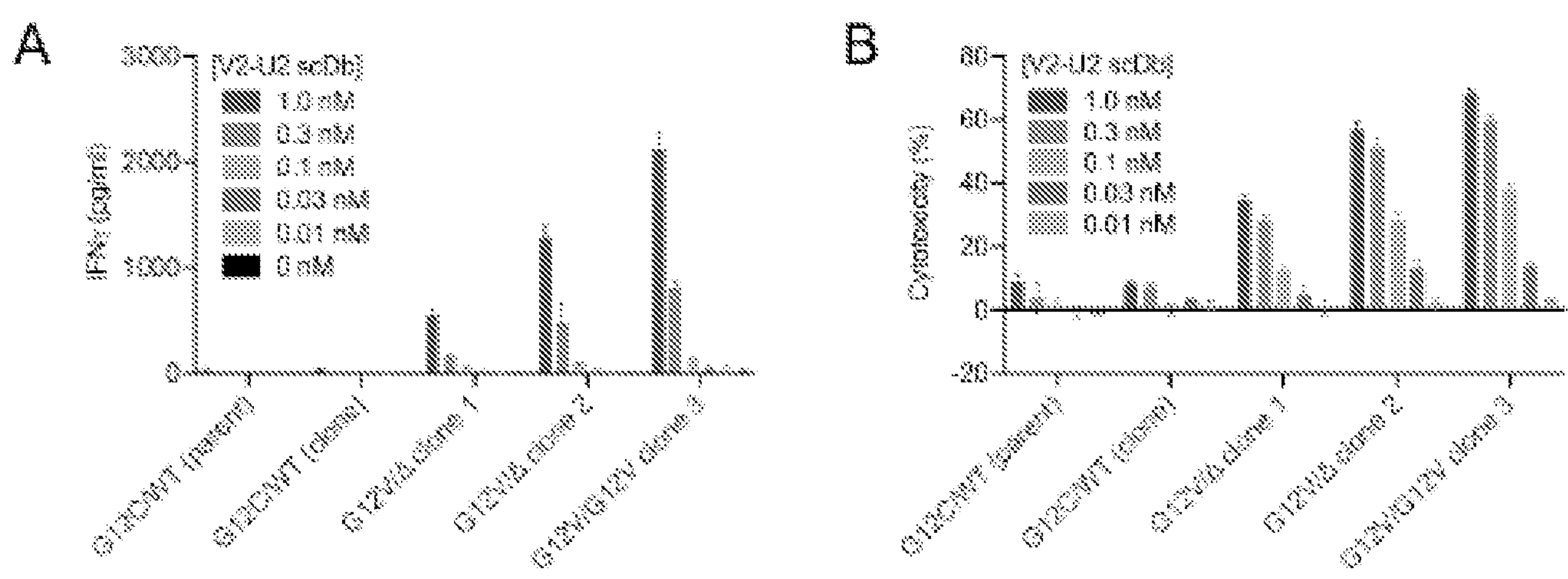


FIG. 58

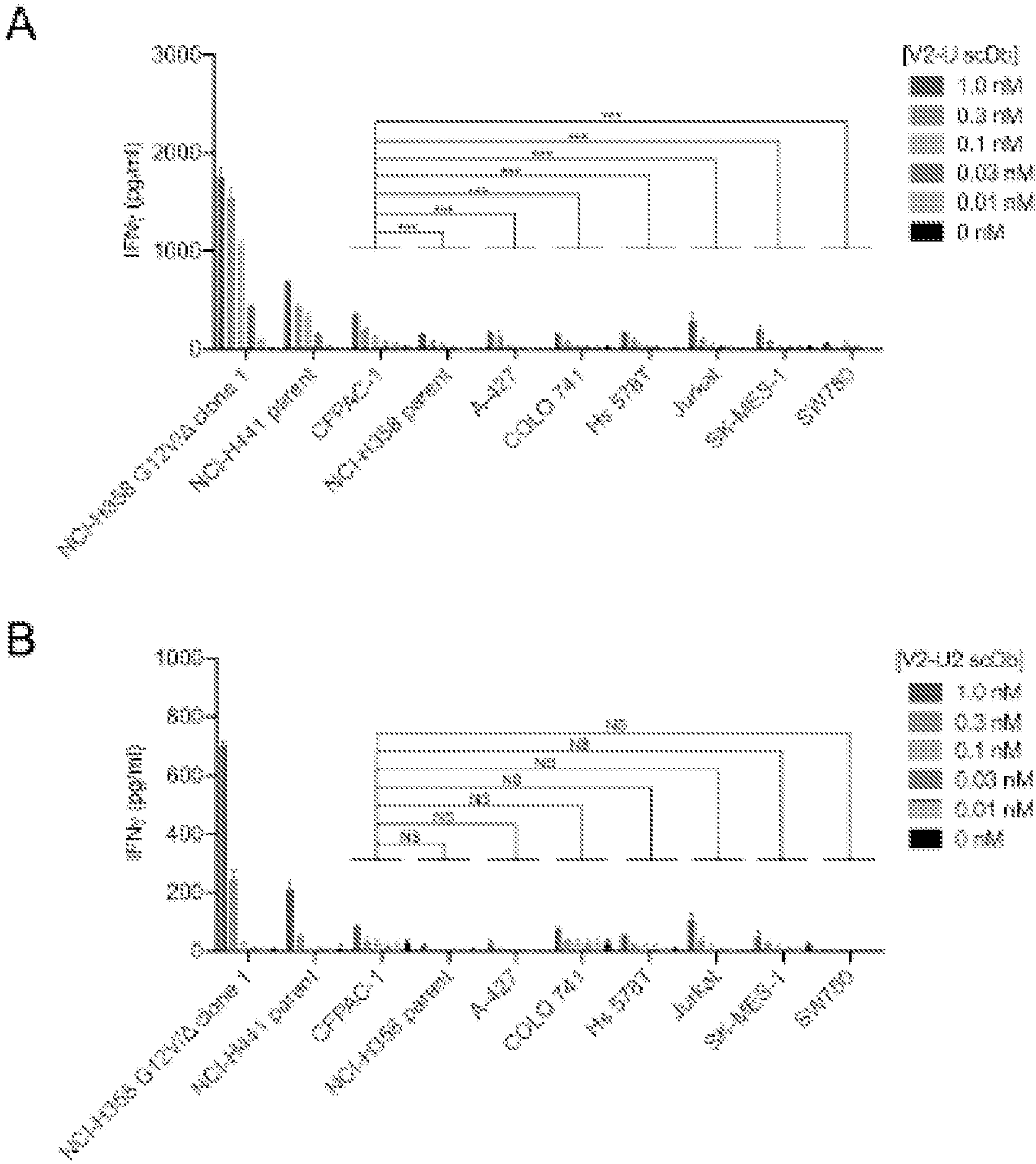


FIG. 59

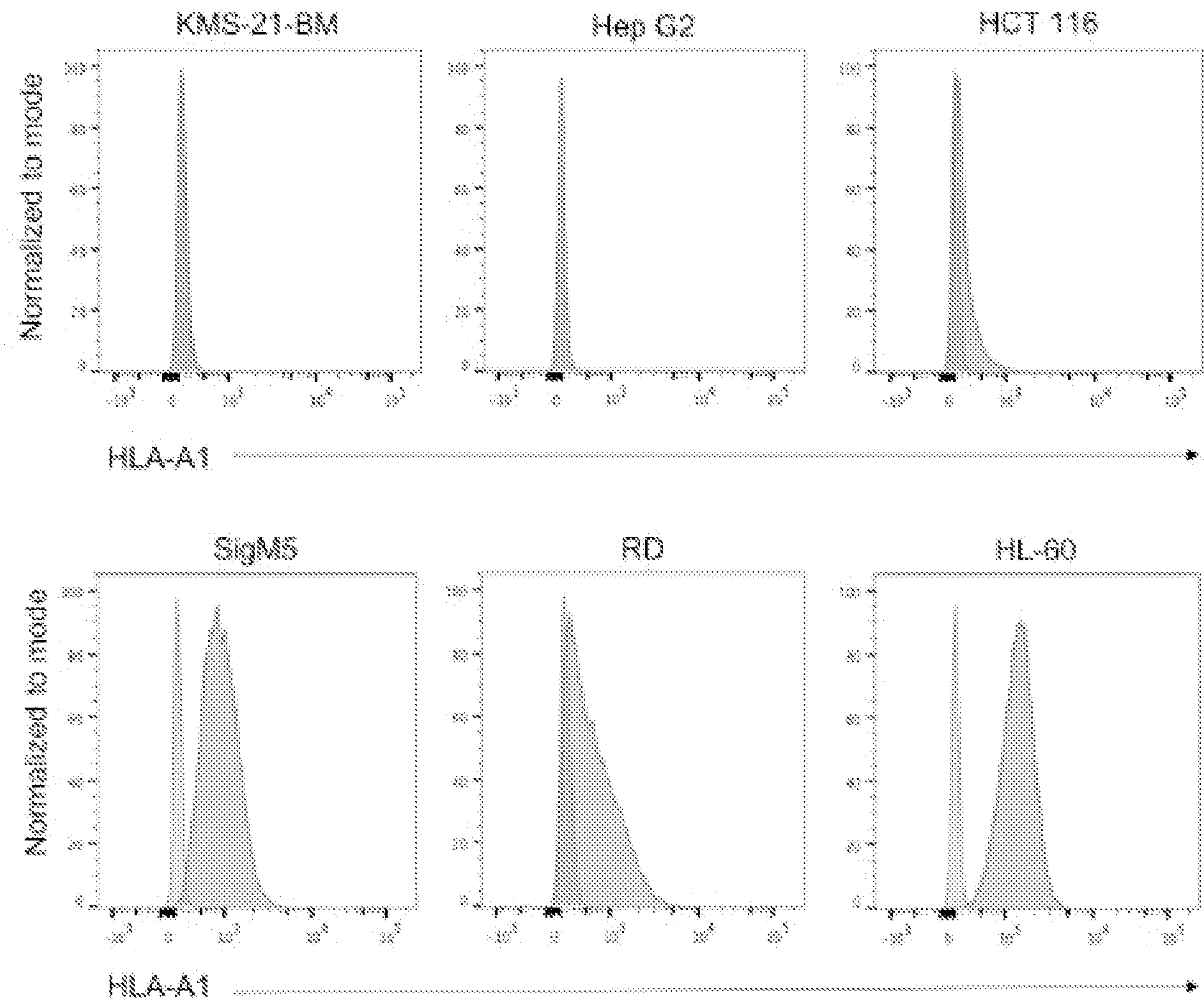


FIG. 60

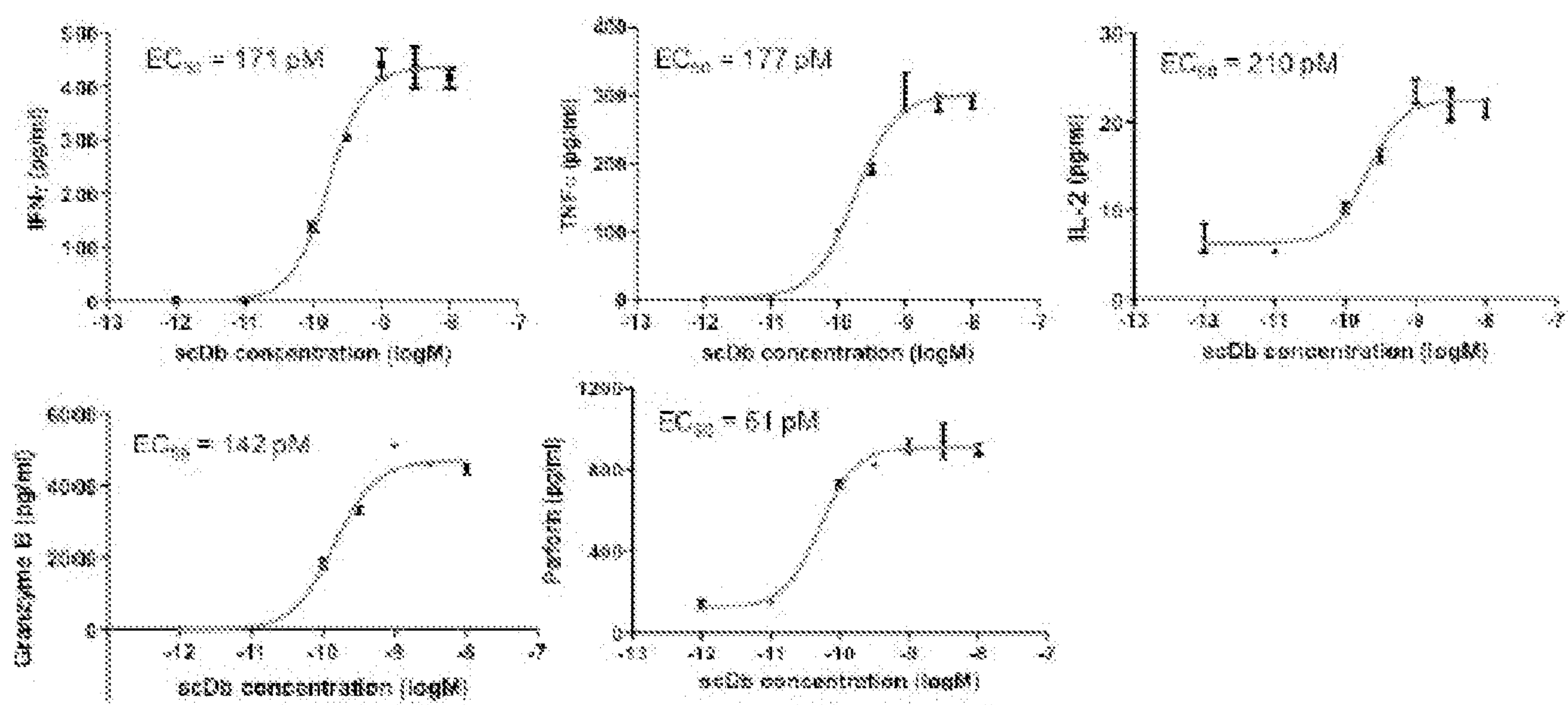


FIG. 61

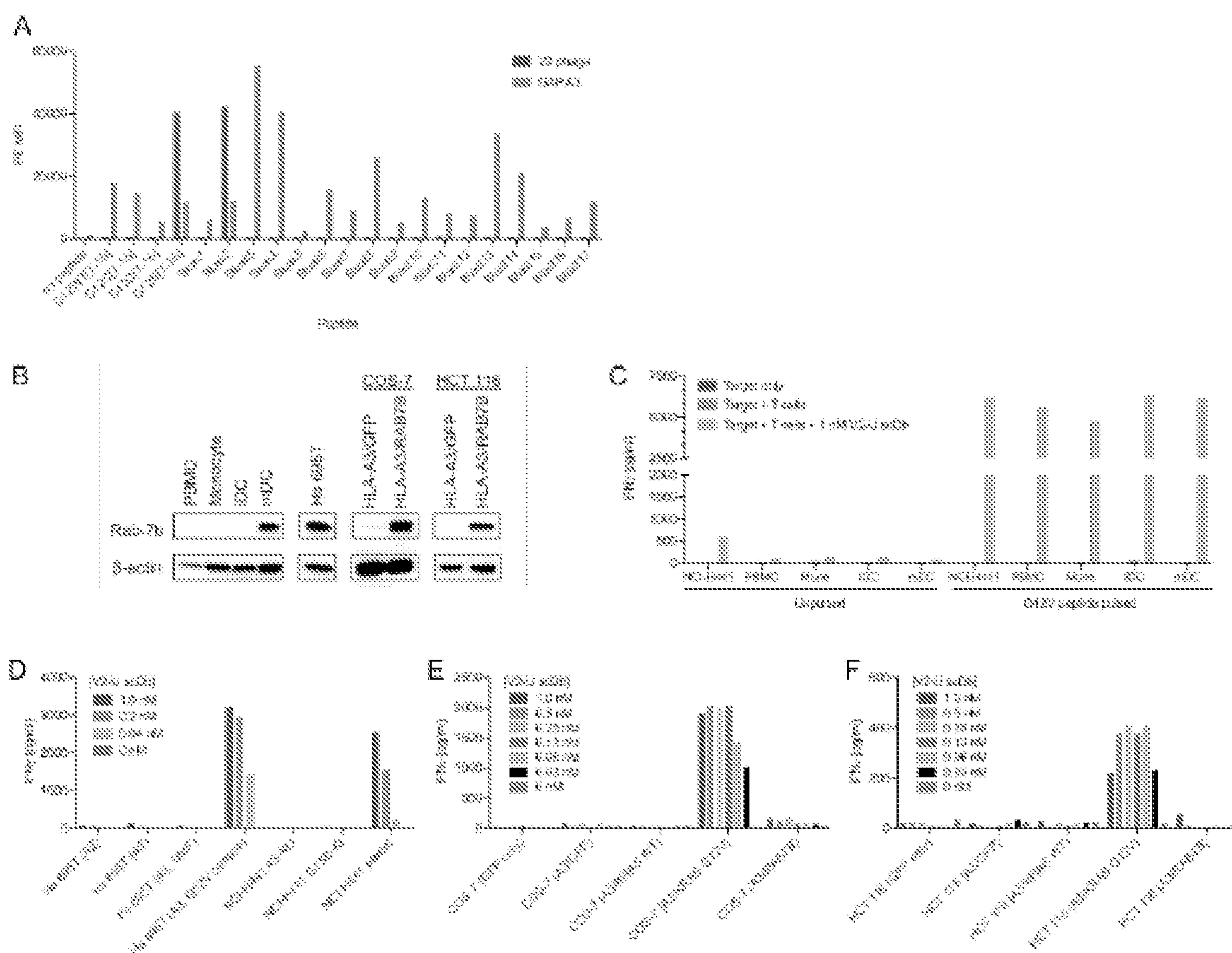


FIG. 62

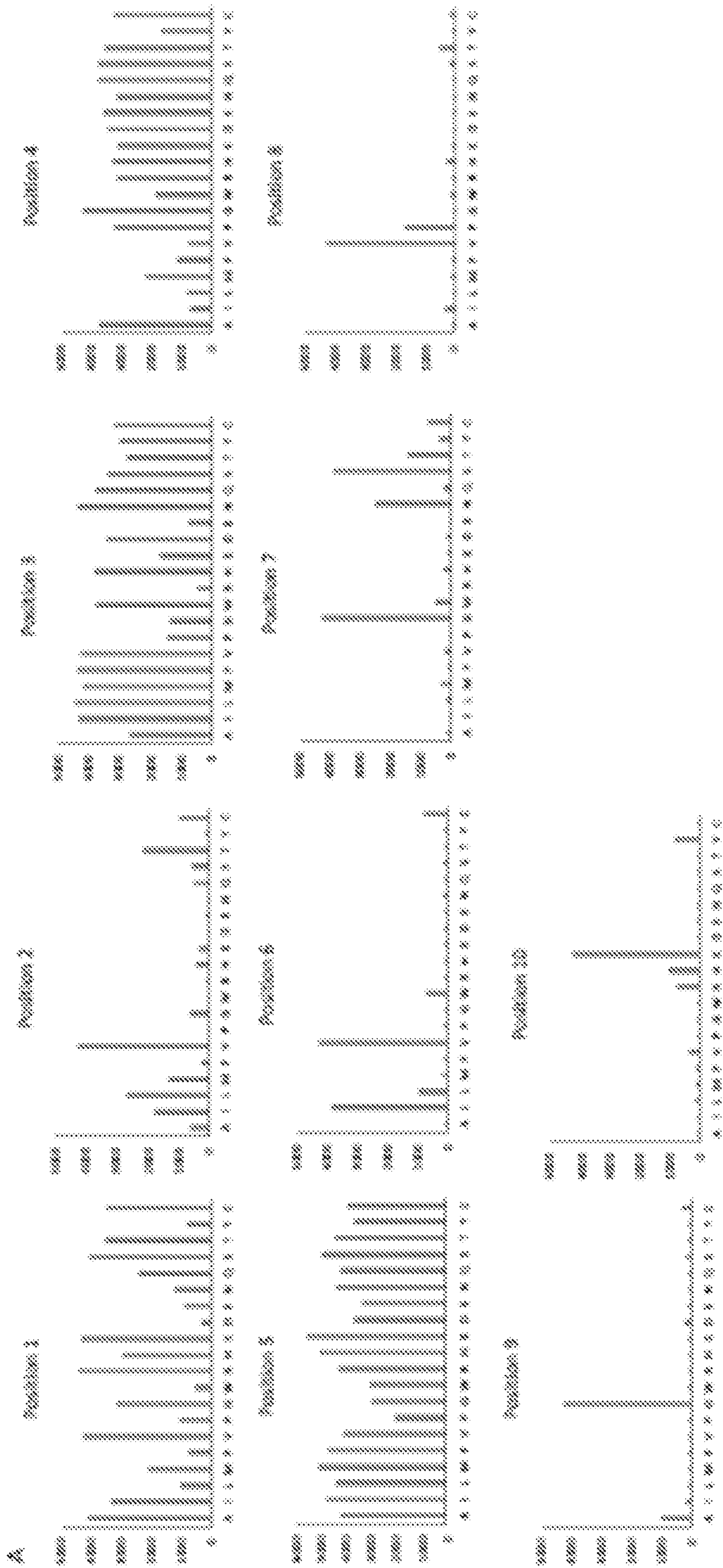


FIG. 63

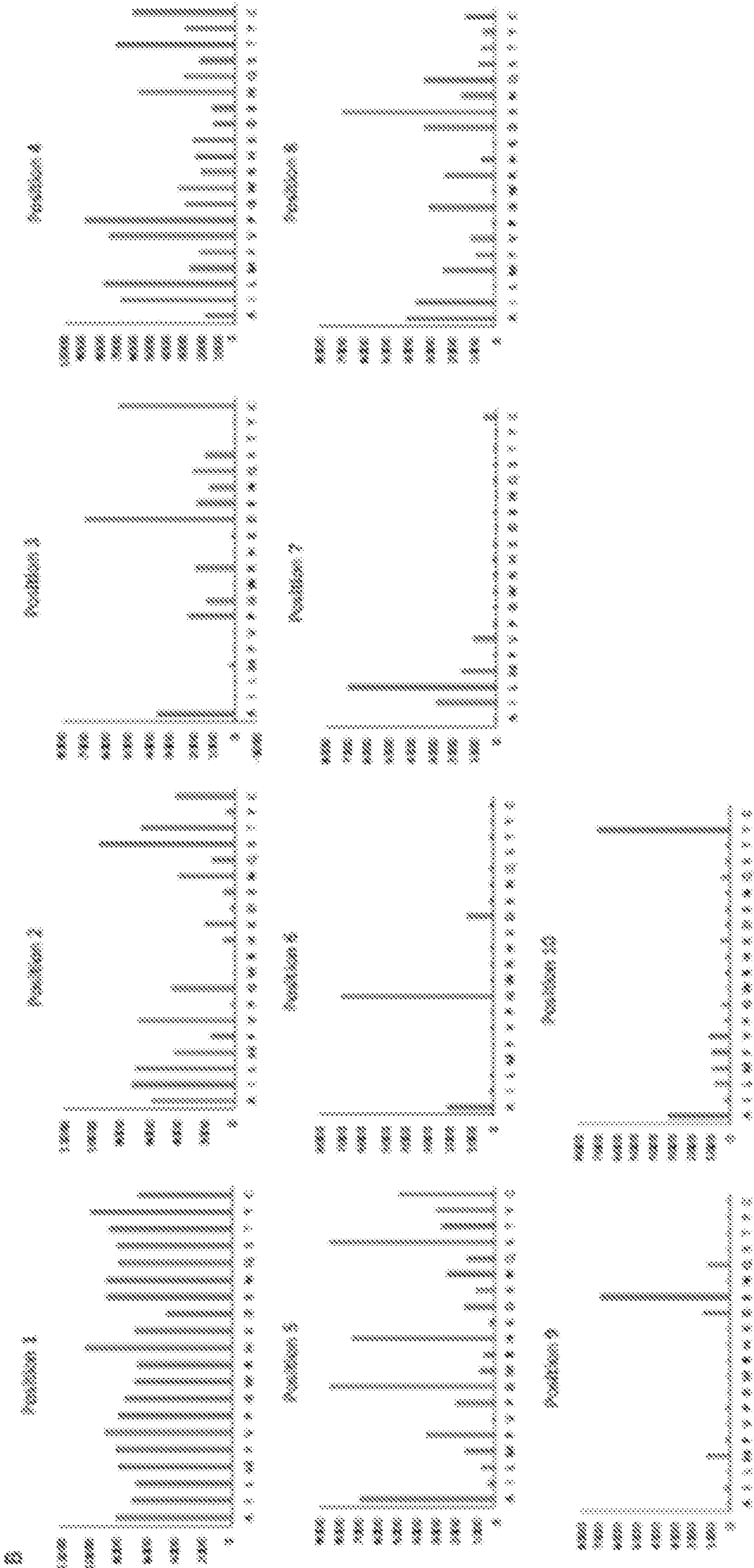


FIG. 63 (continued)

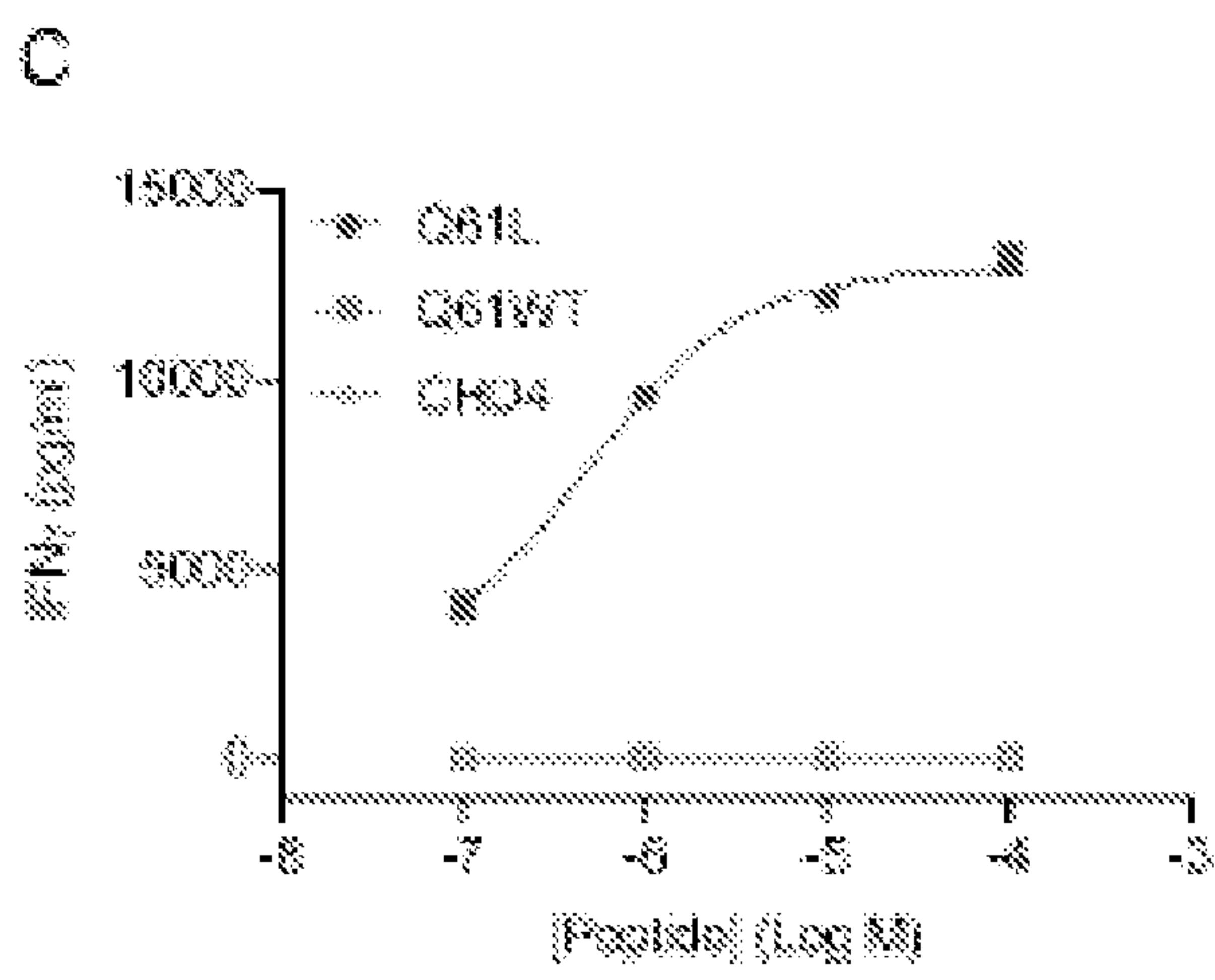


FIG. 63 (continued)

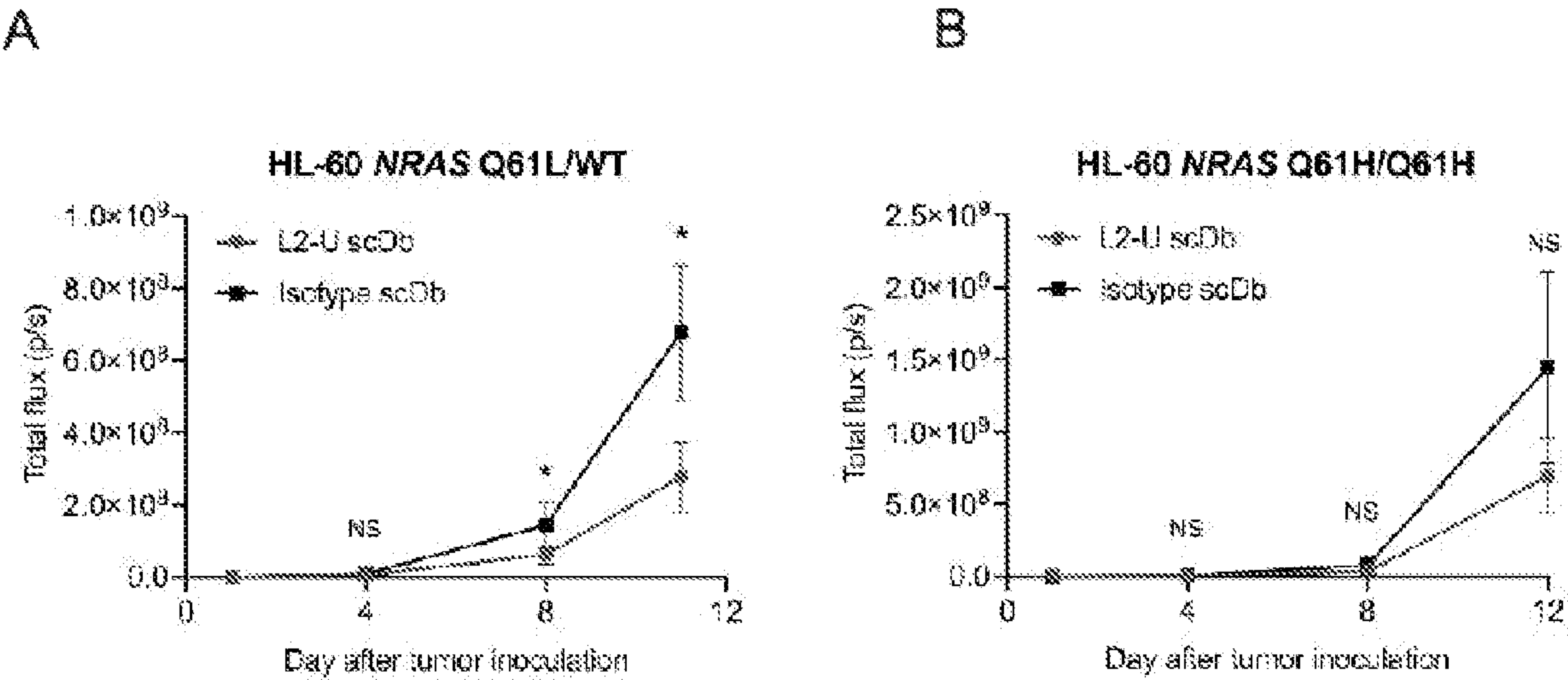


FIG. 64

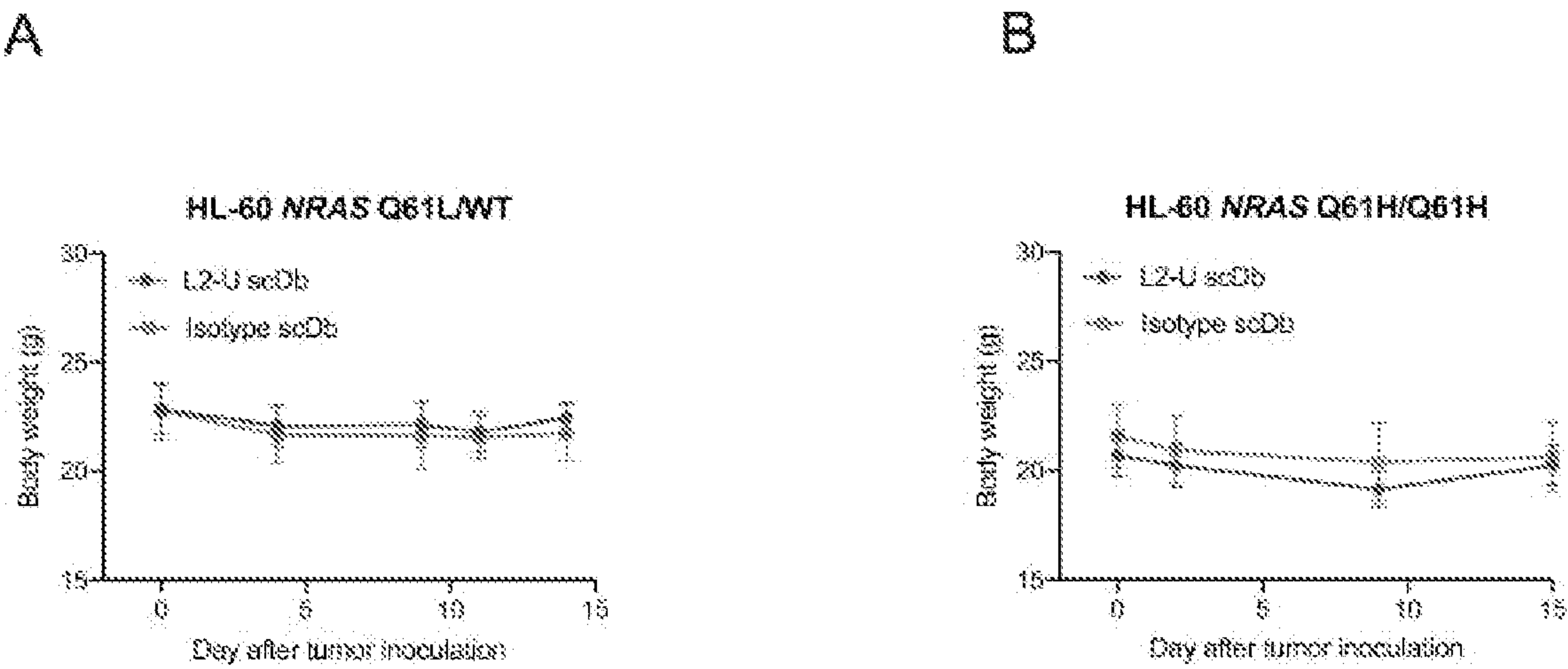


FIG. 65

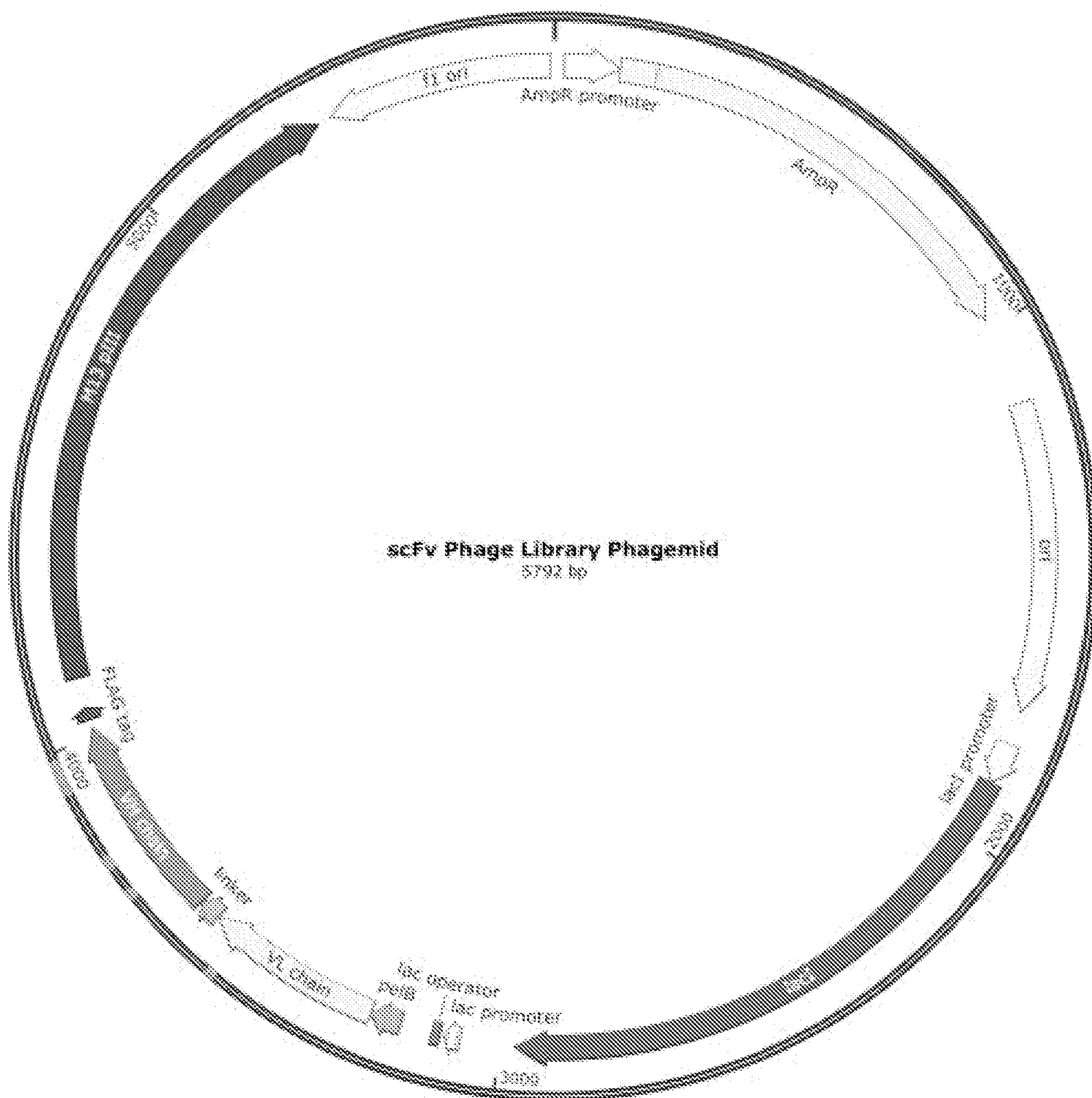


FIG. 66

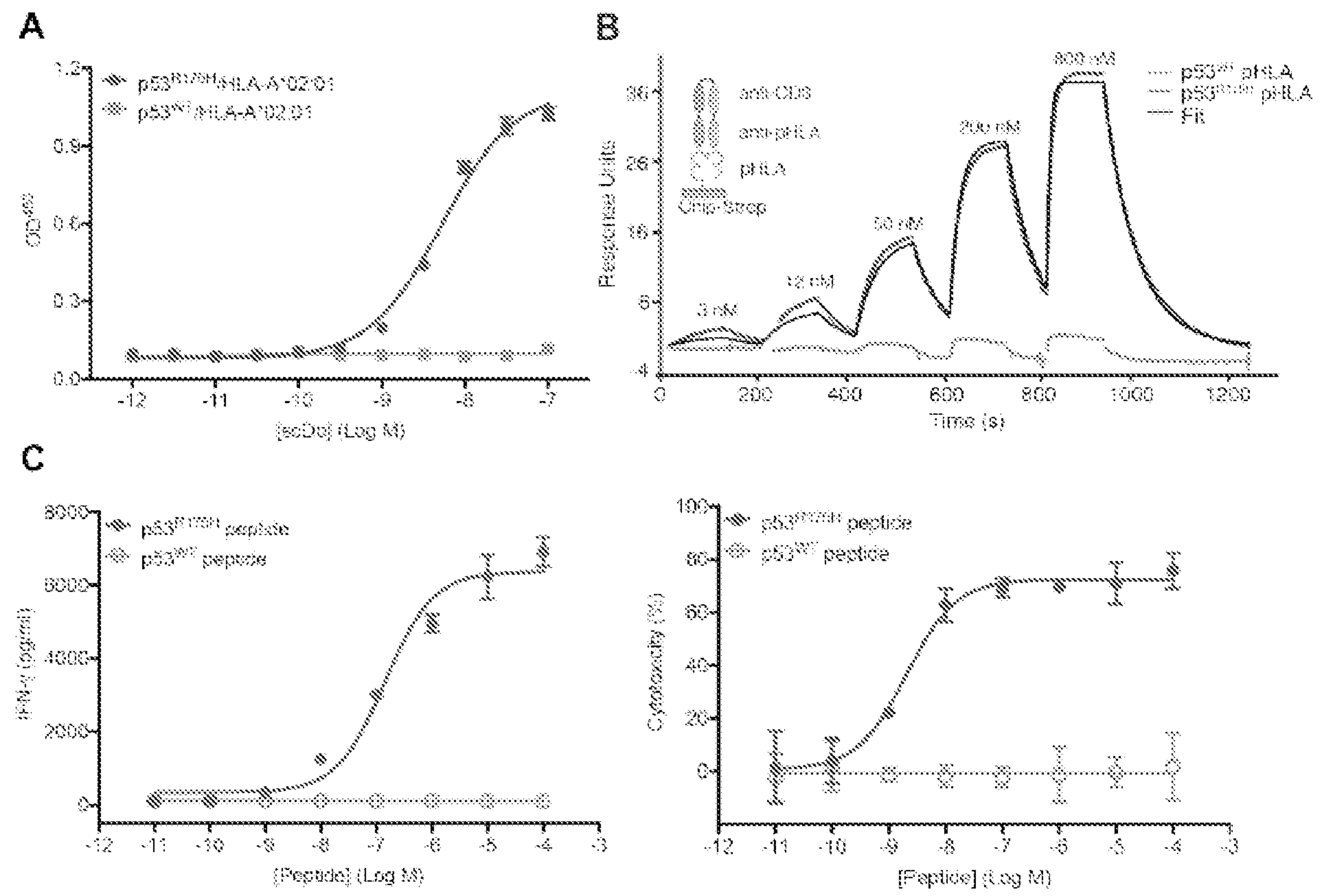


FIG. 67

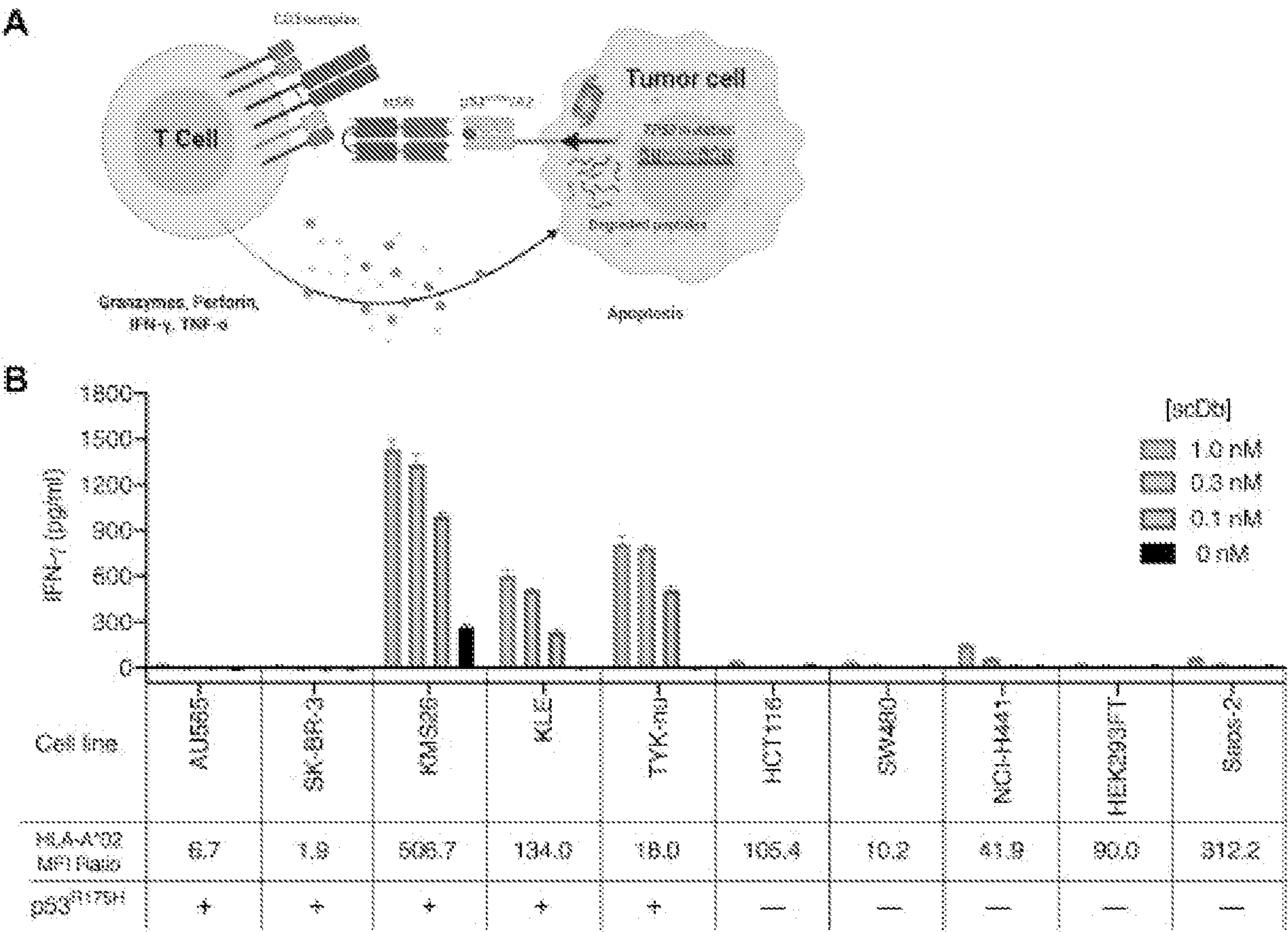


FIG. 68

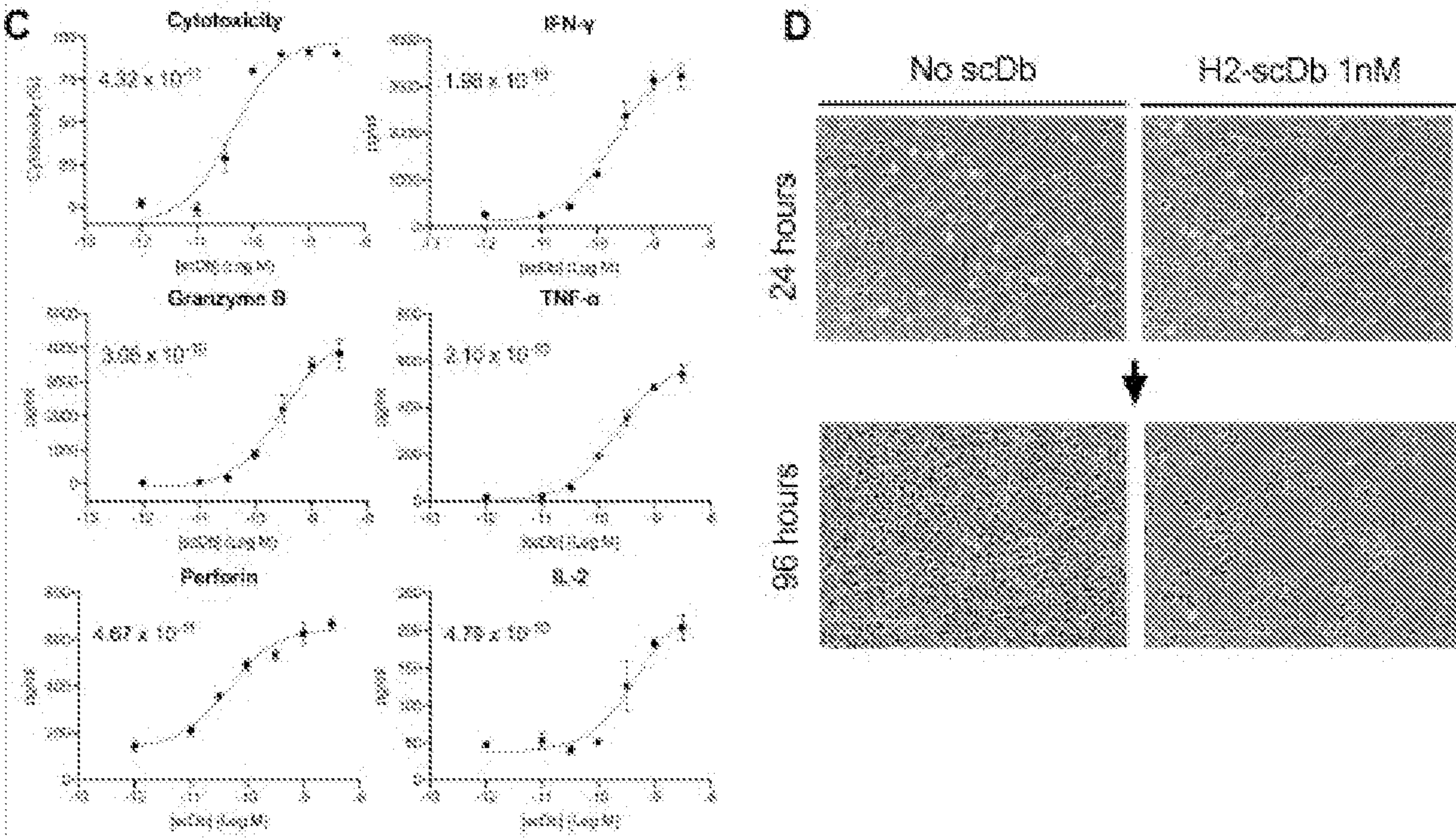


FIG. 68 (cont.)

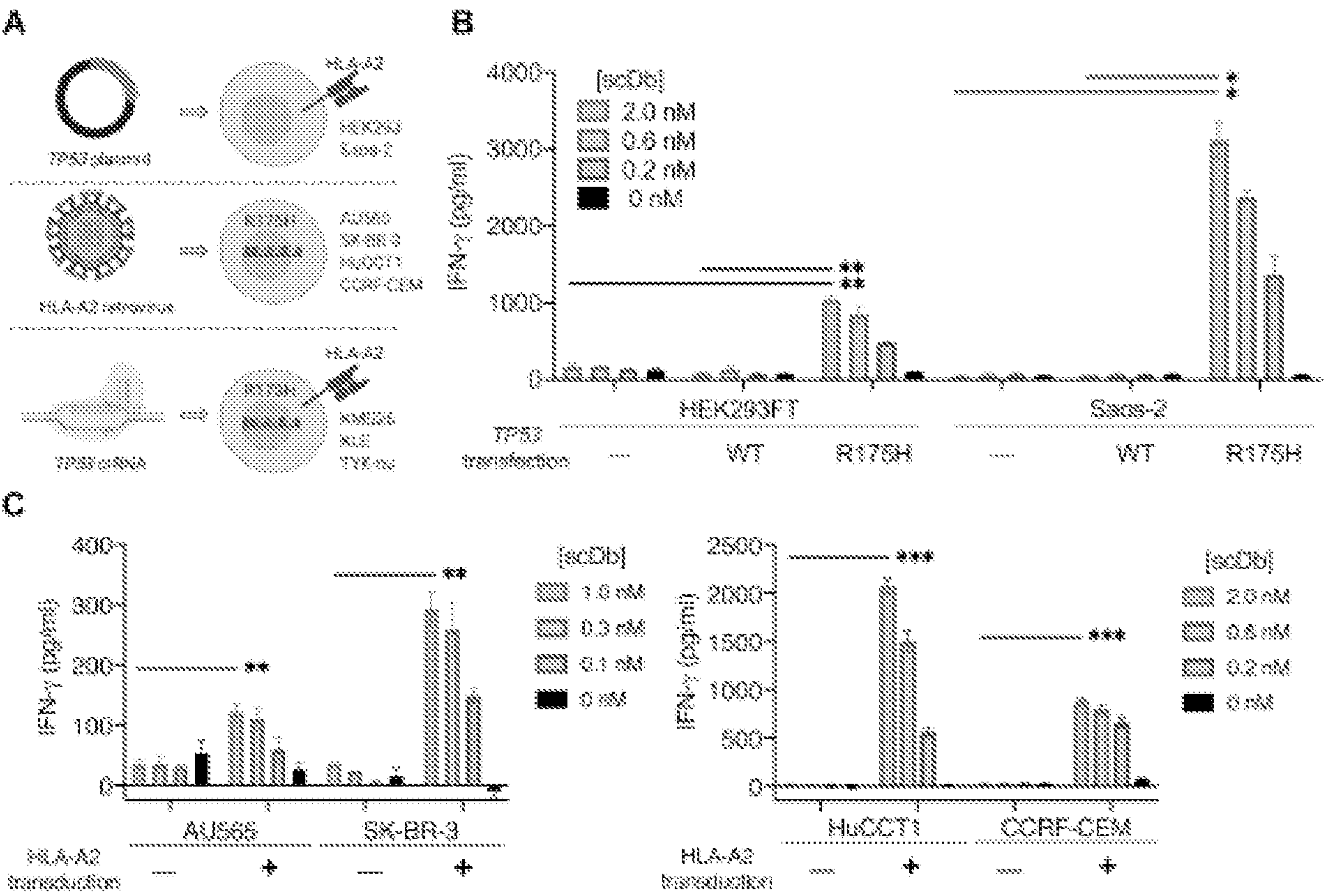


FIG. 69

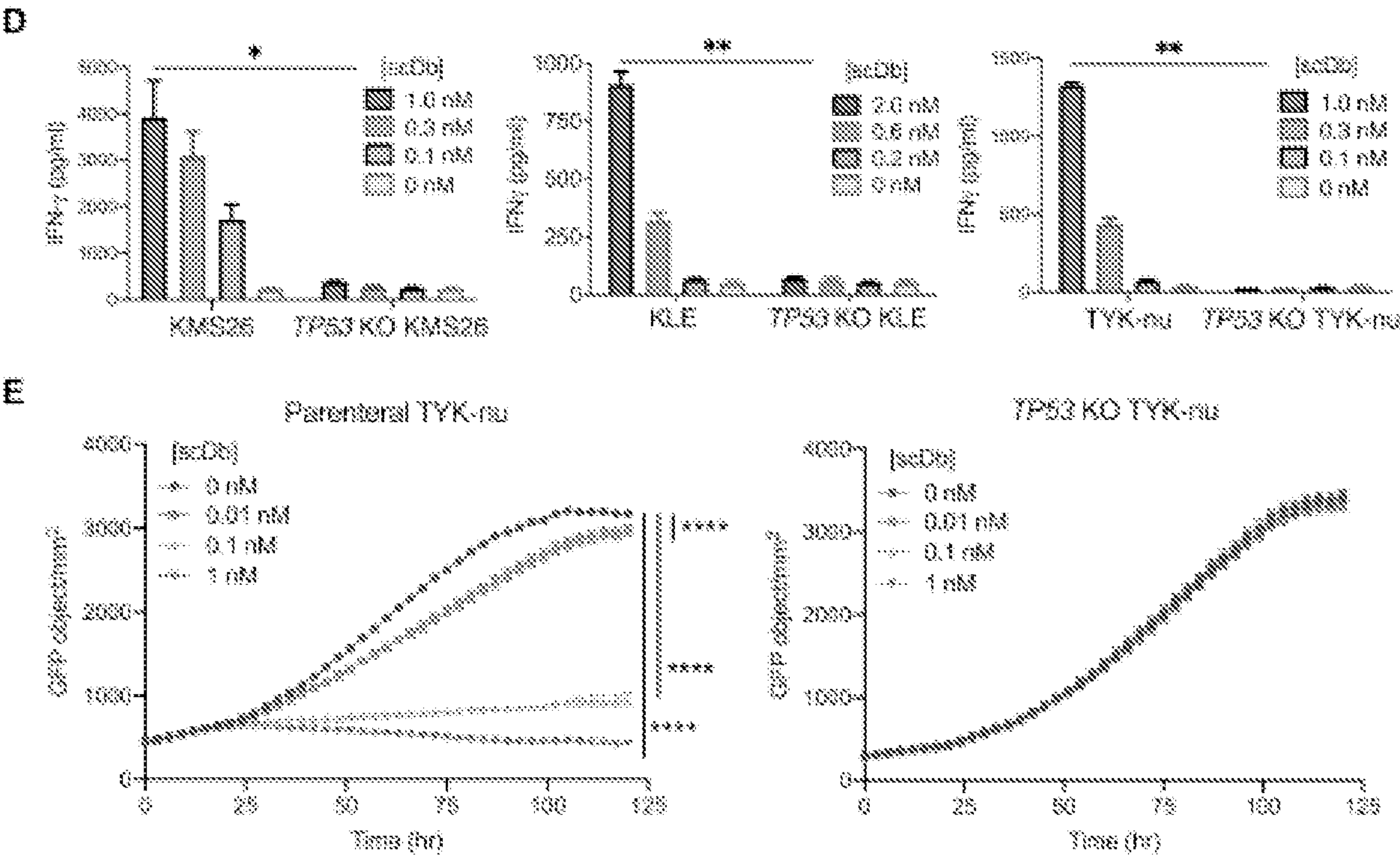


FIG. 69 (cont.)

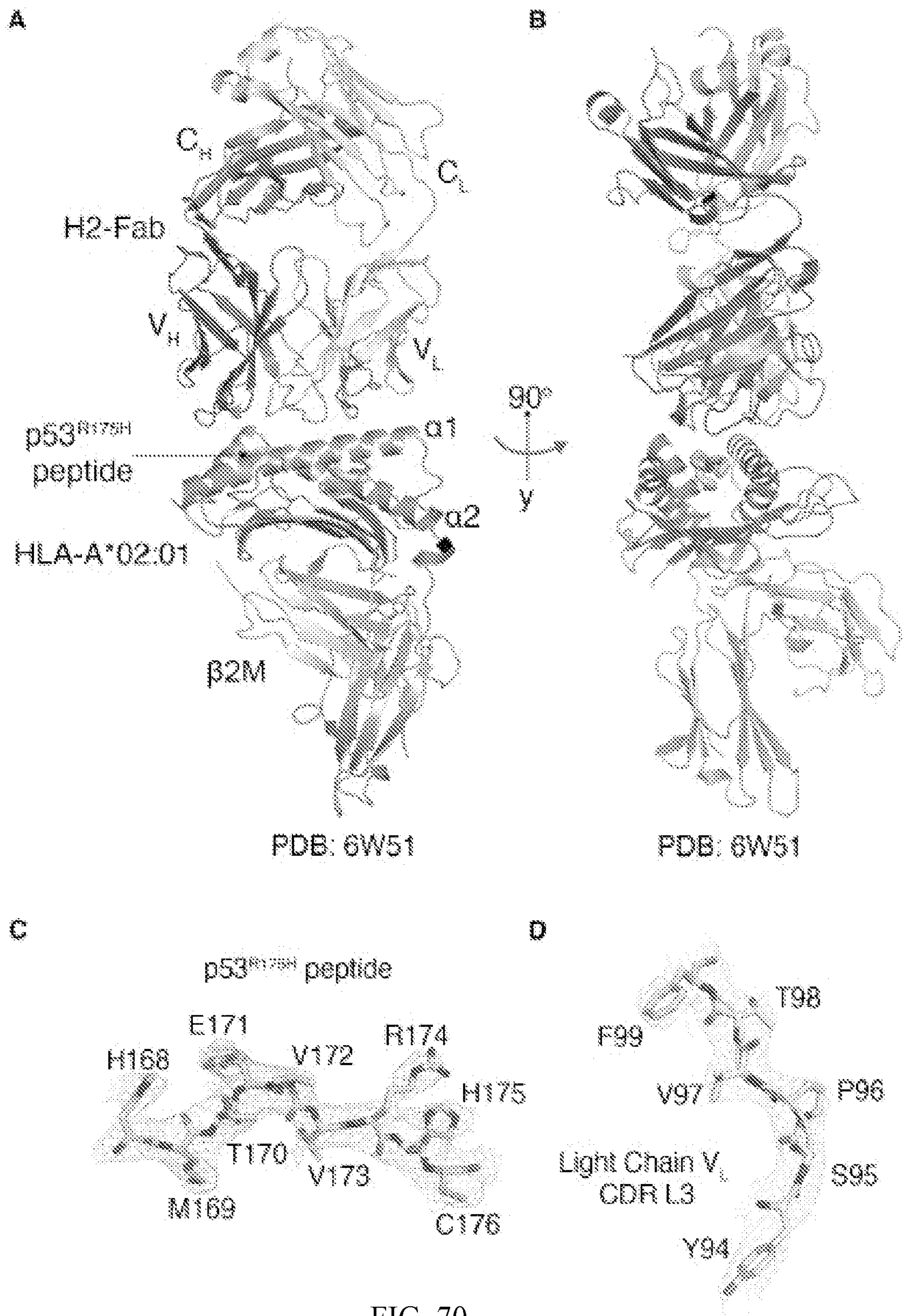


FIG. 70

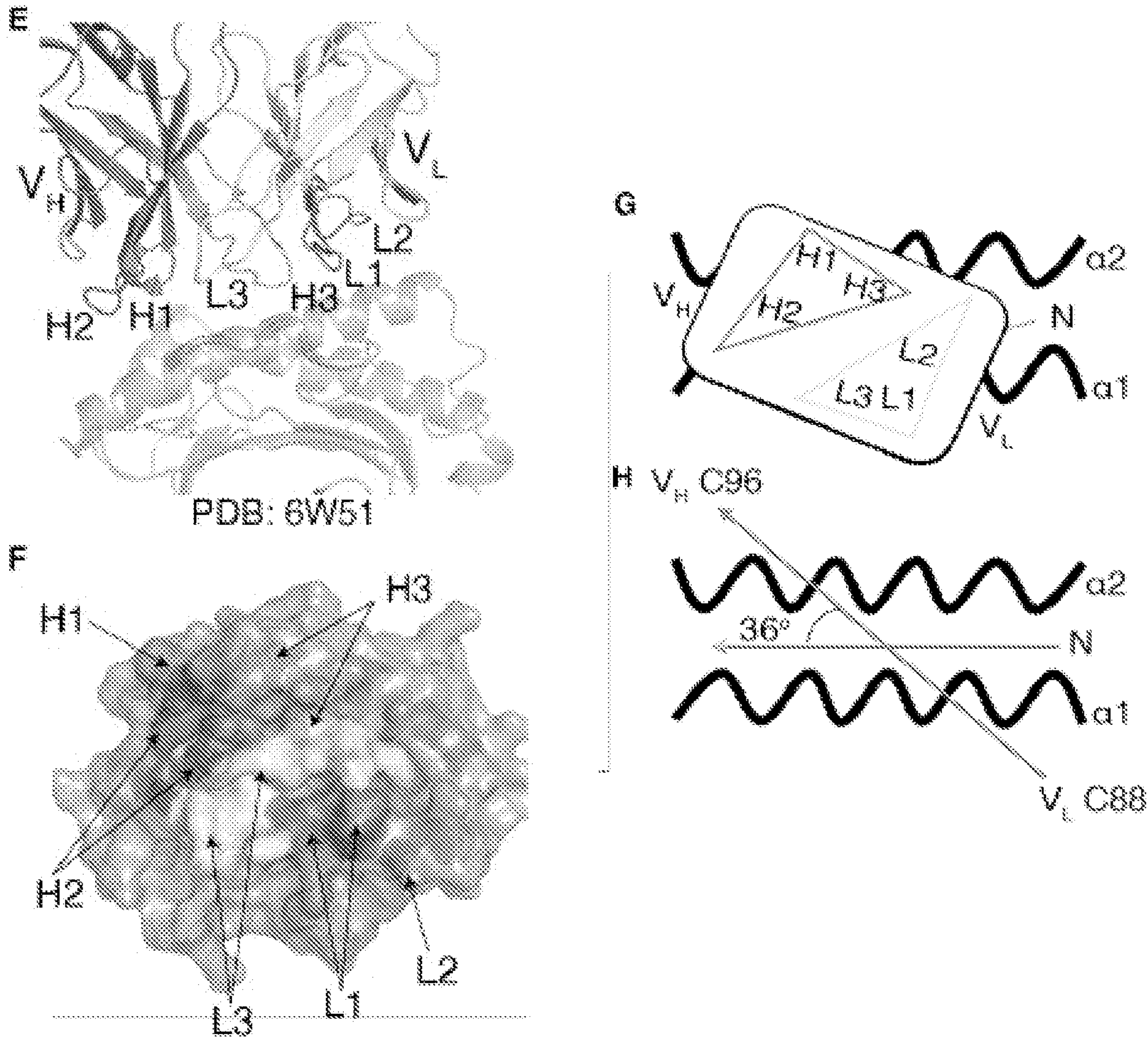


FIG. 70 (cont.)

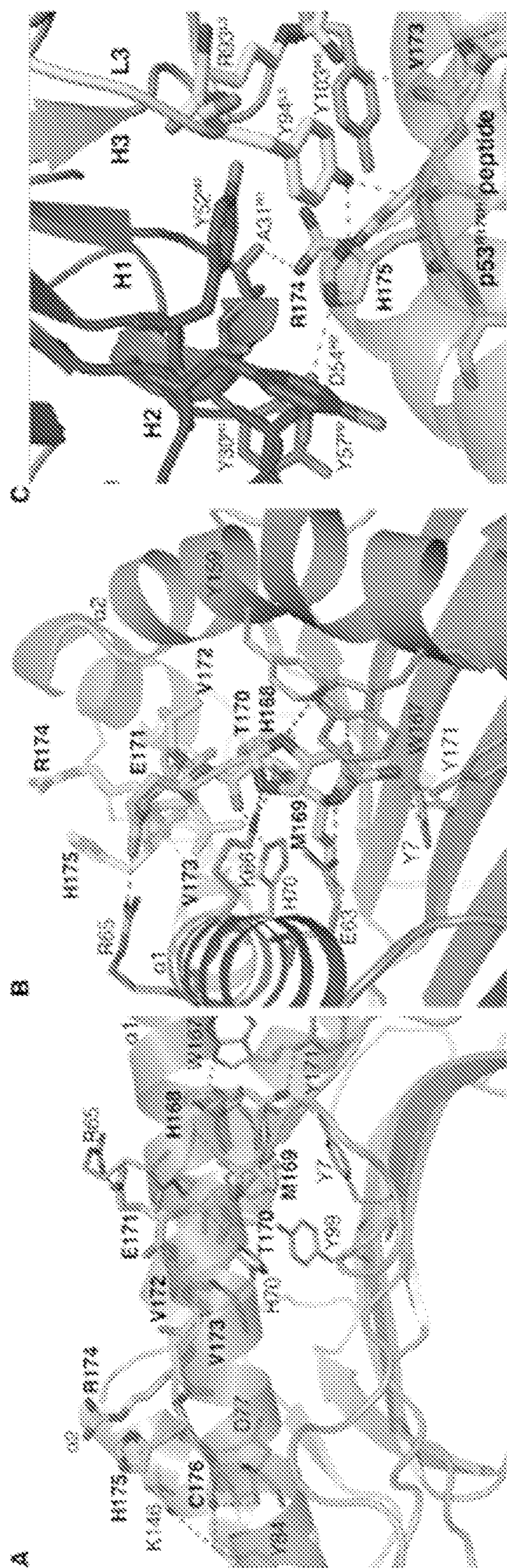


FIG. 71

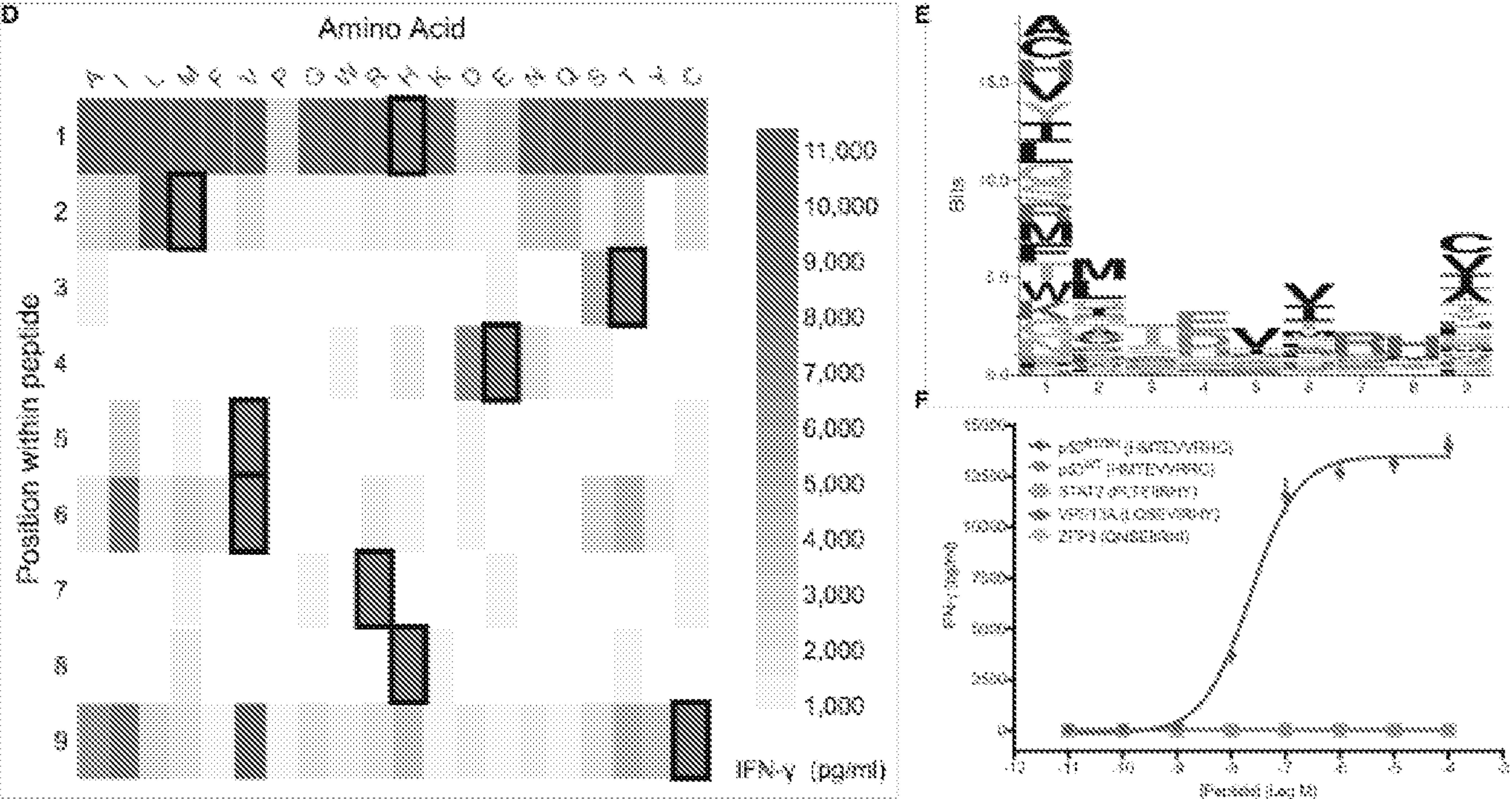


FIG. 71 (cont.)

A

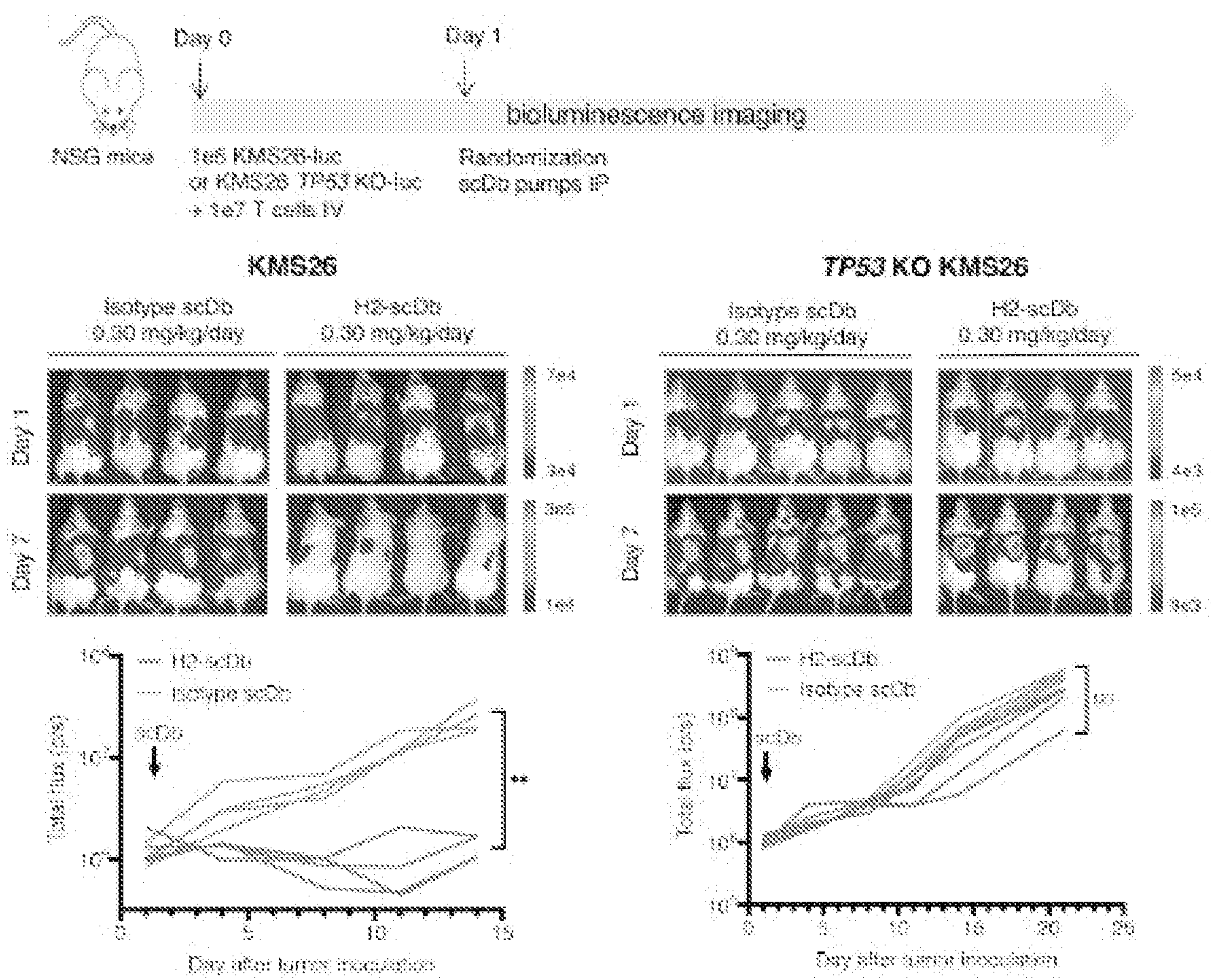


FIG. 72

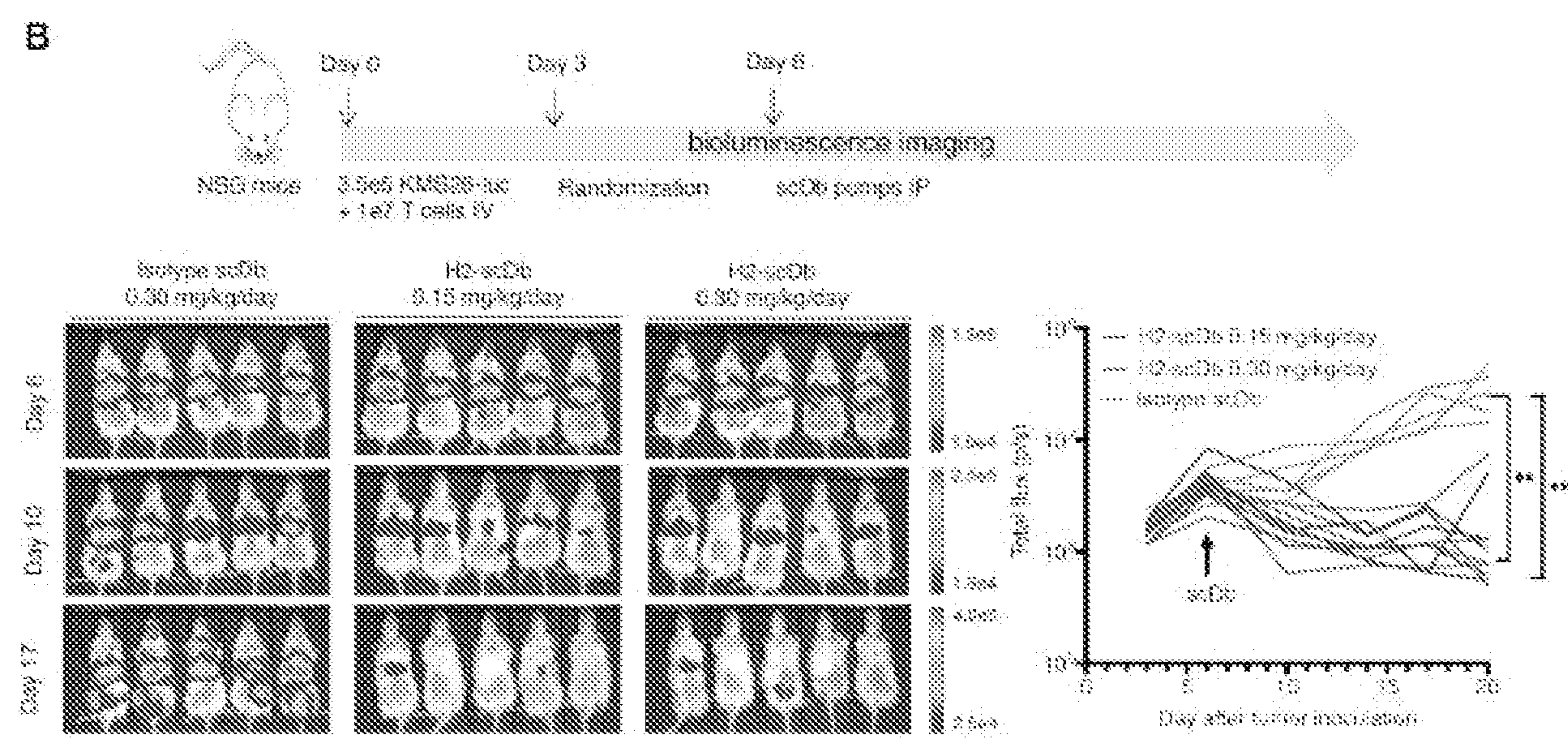


FIG. 72 (cont.)

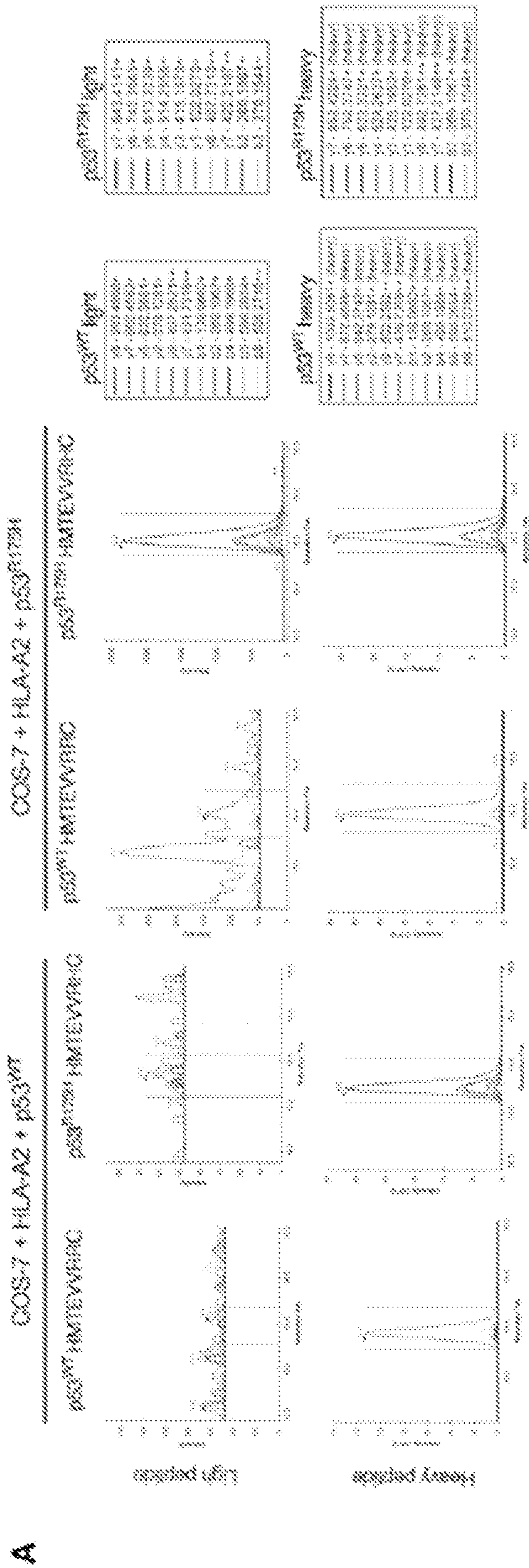


FIG. 73

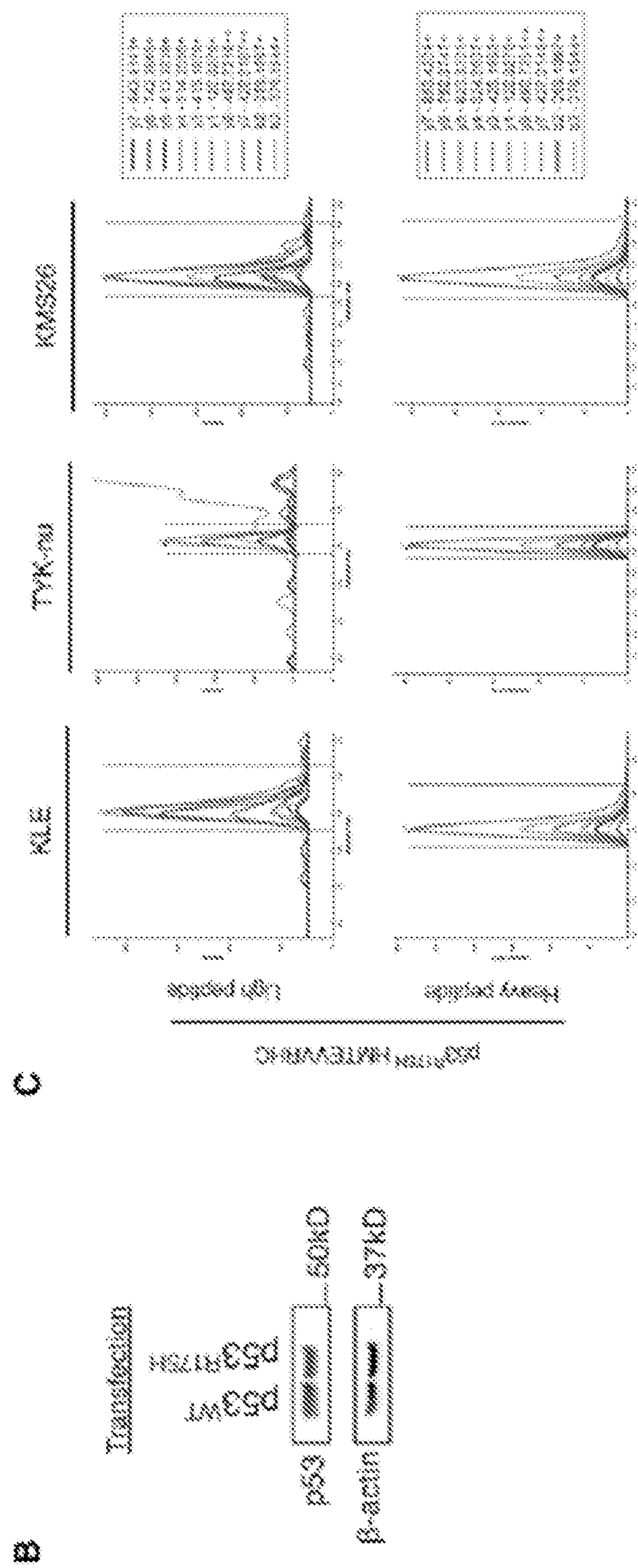


FIG. 73 (cont.)

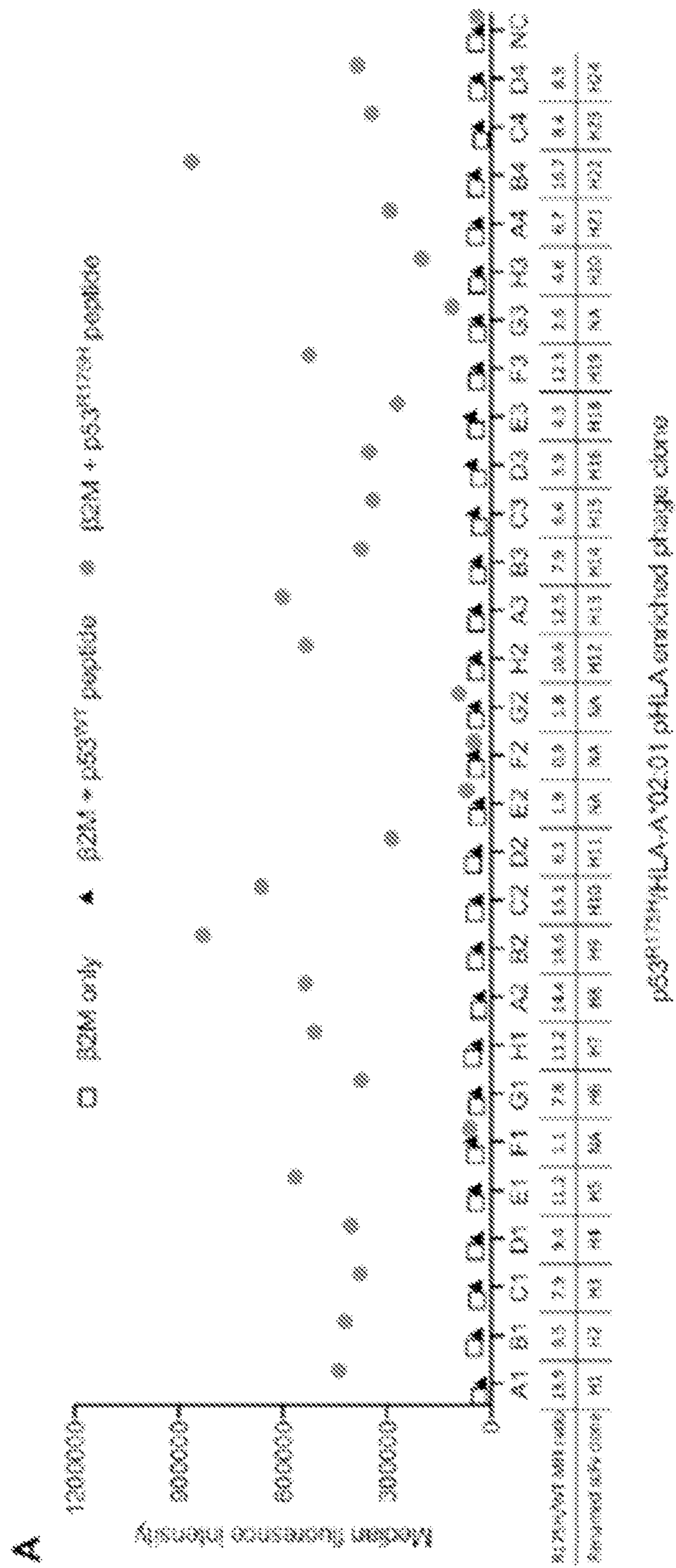


FIG. 74

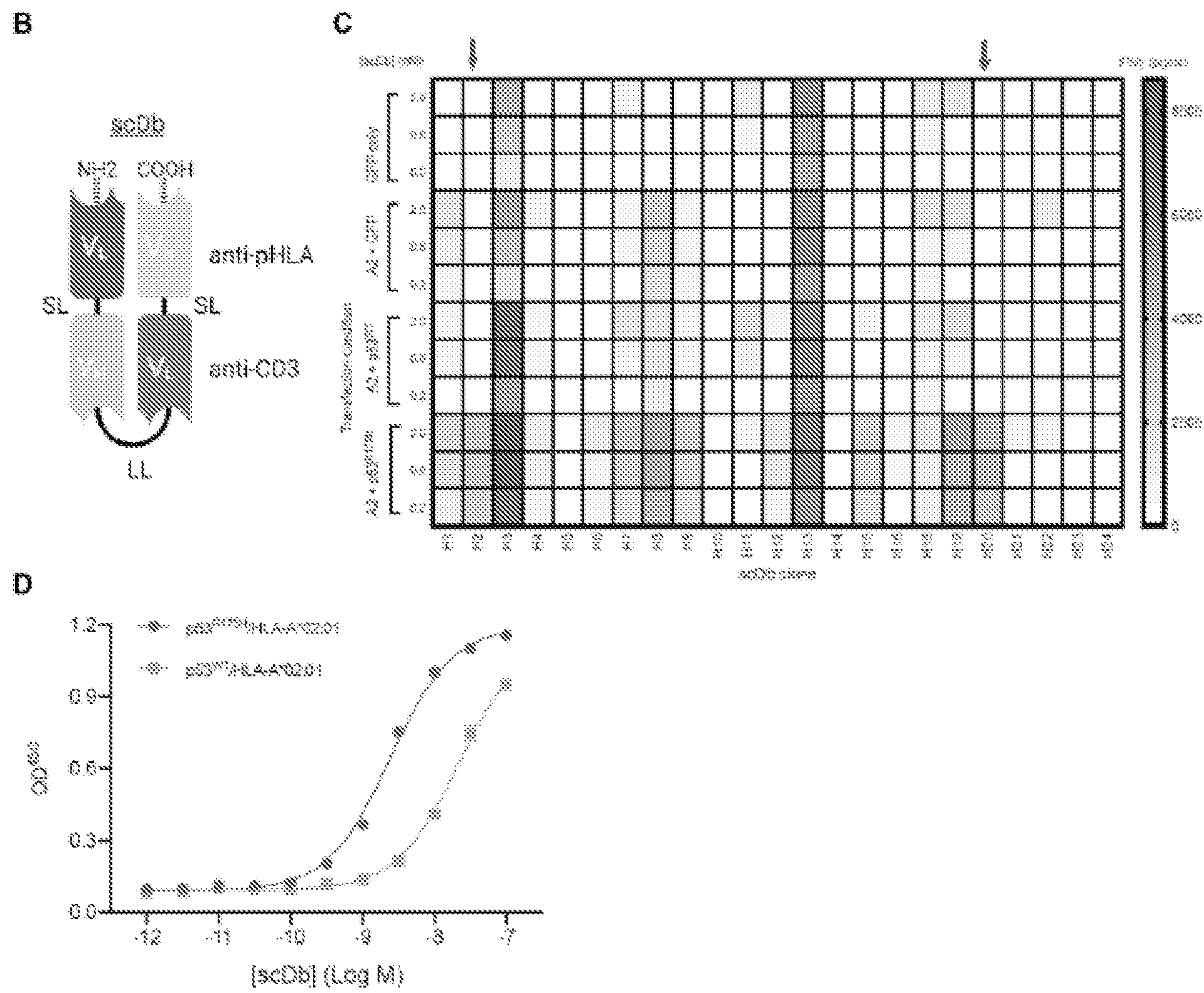


FIG. 74 (cont.)

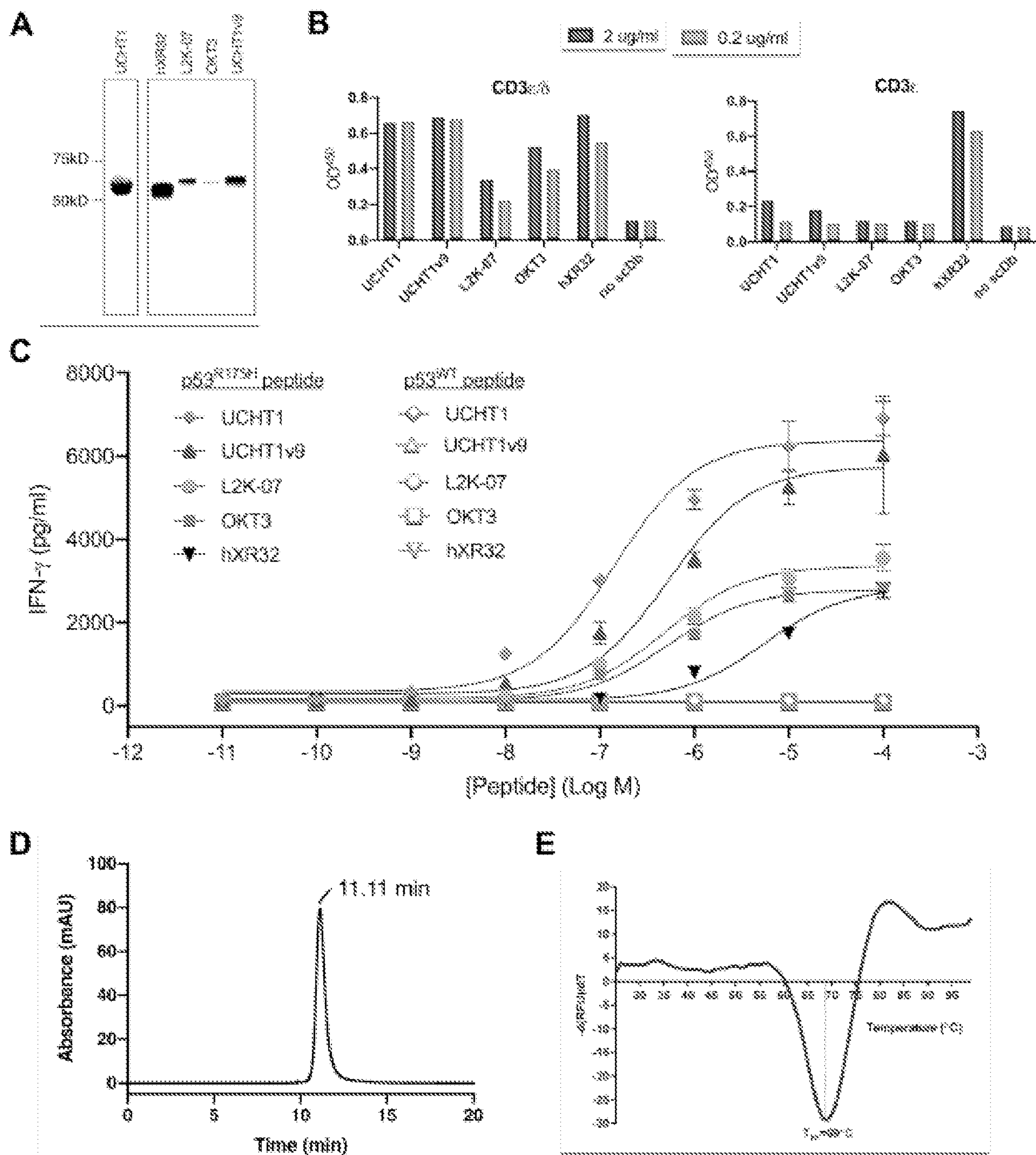


FIG. 75

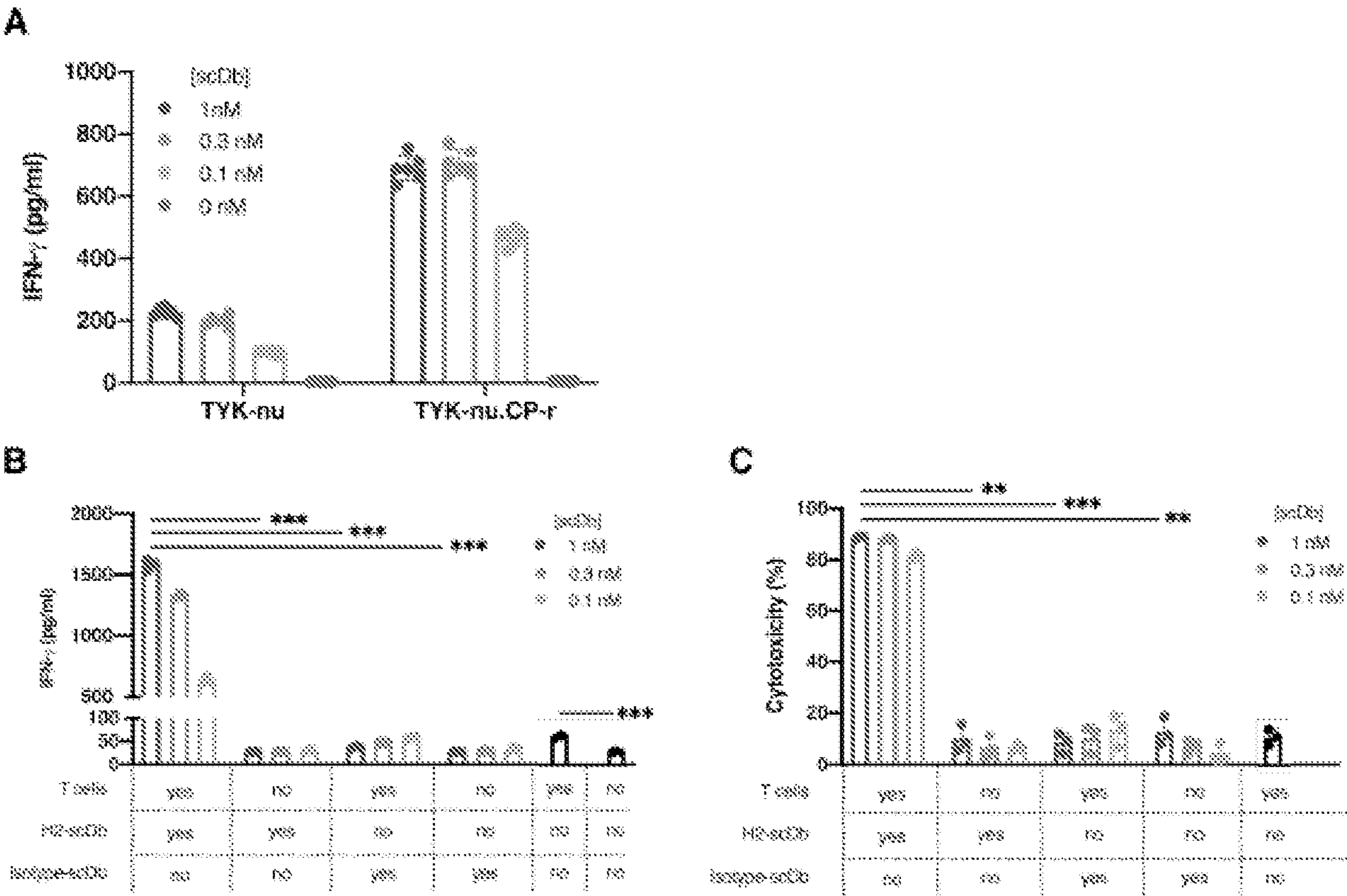
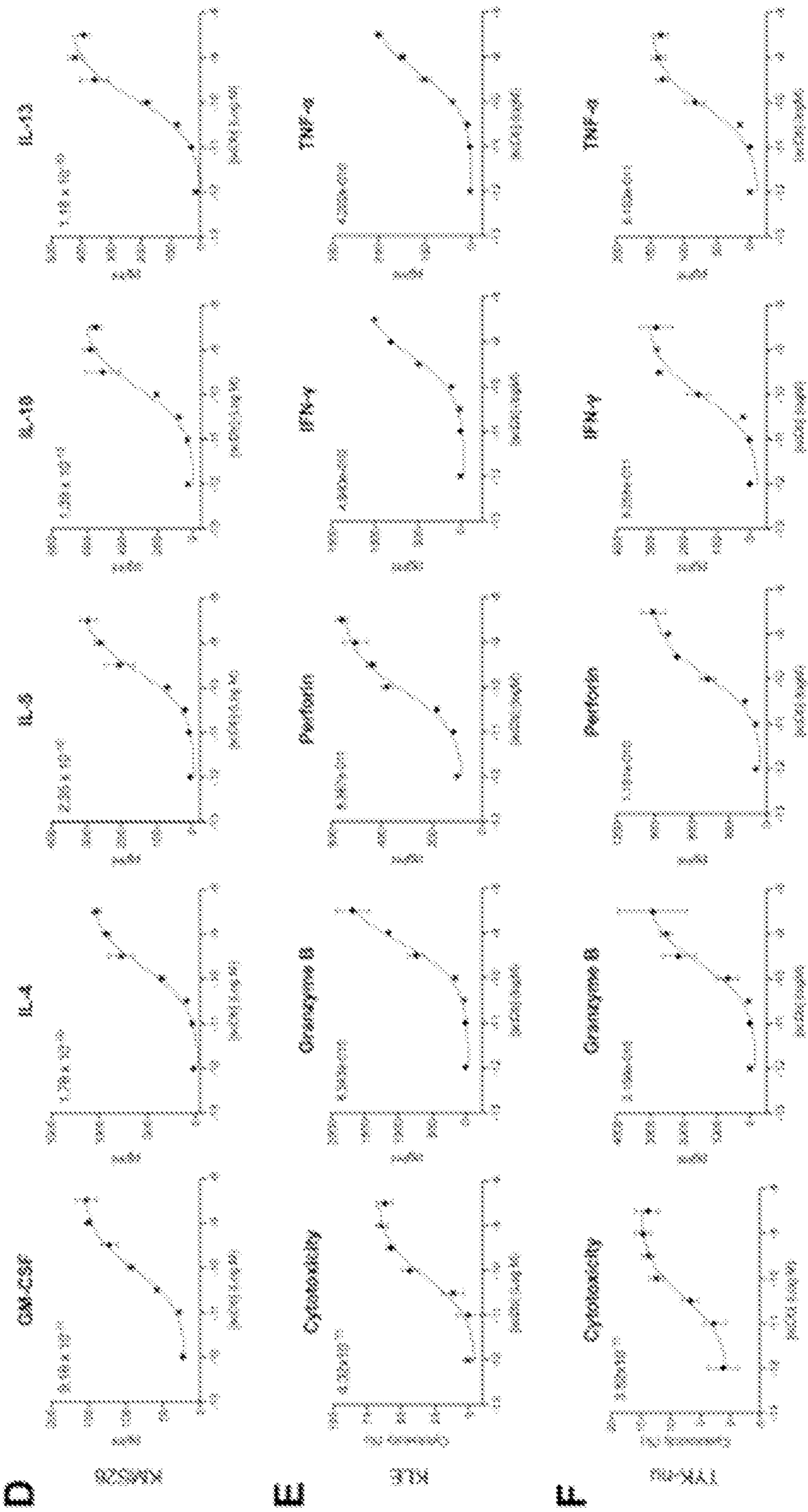


FIG. 76



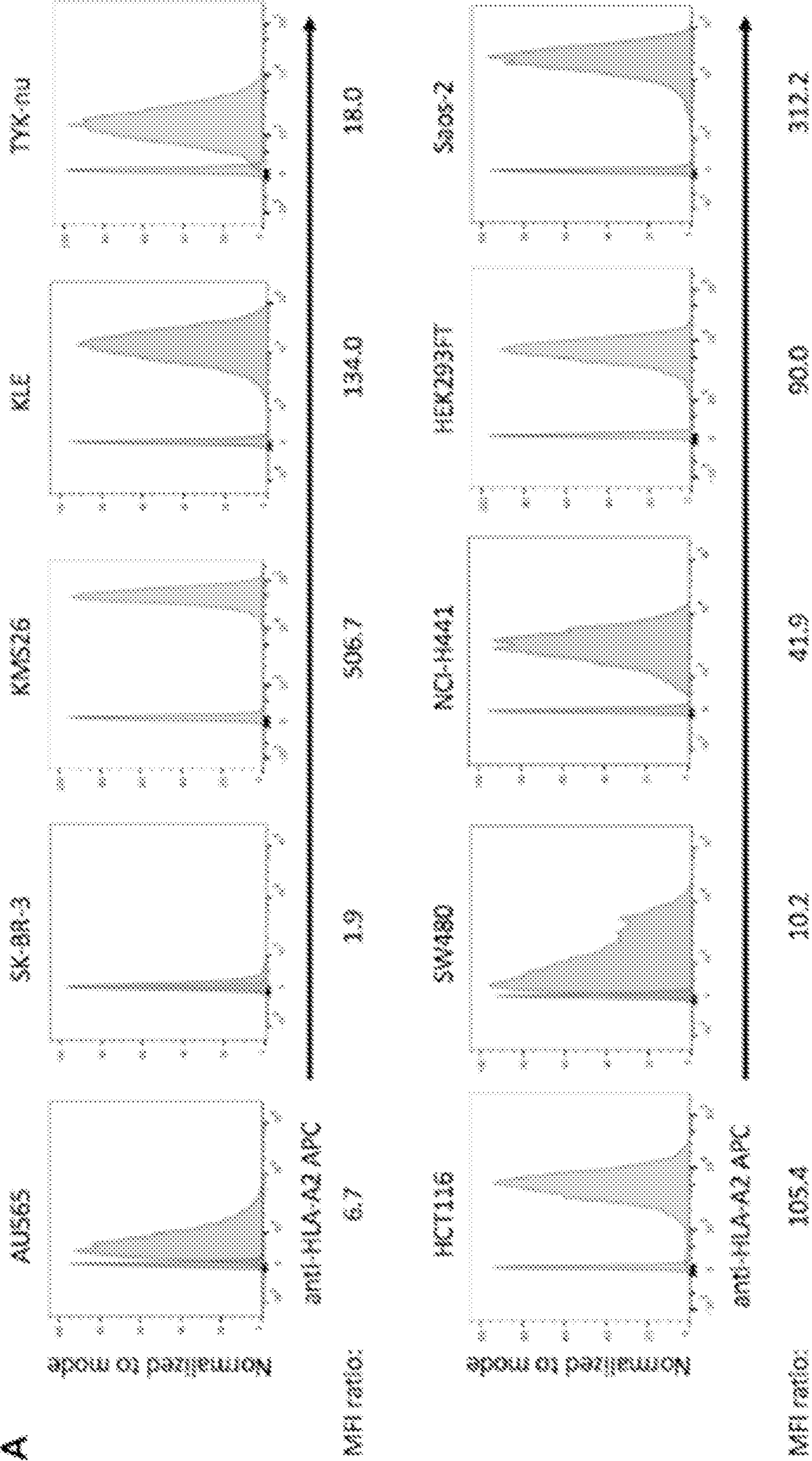


FIG. 77

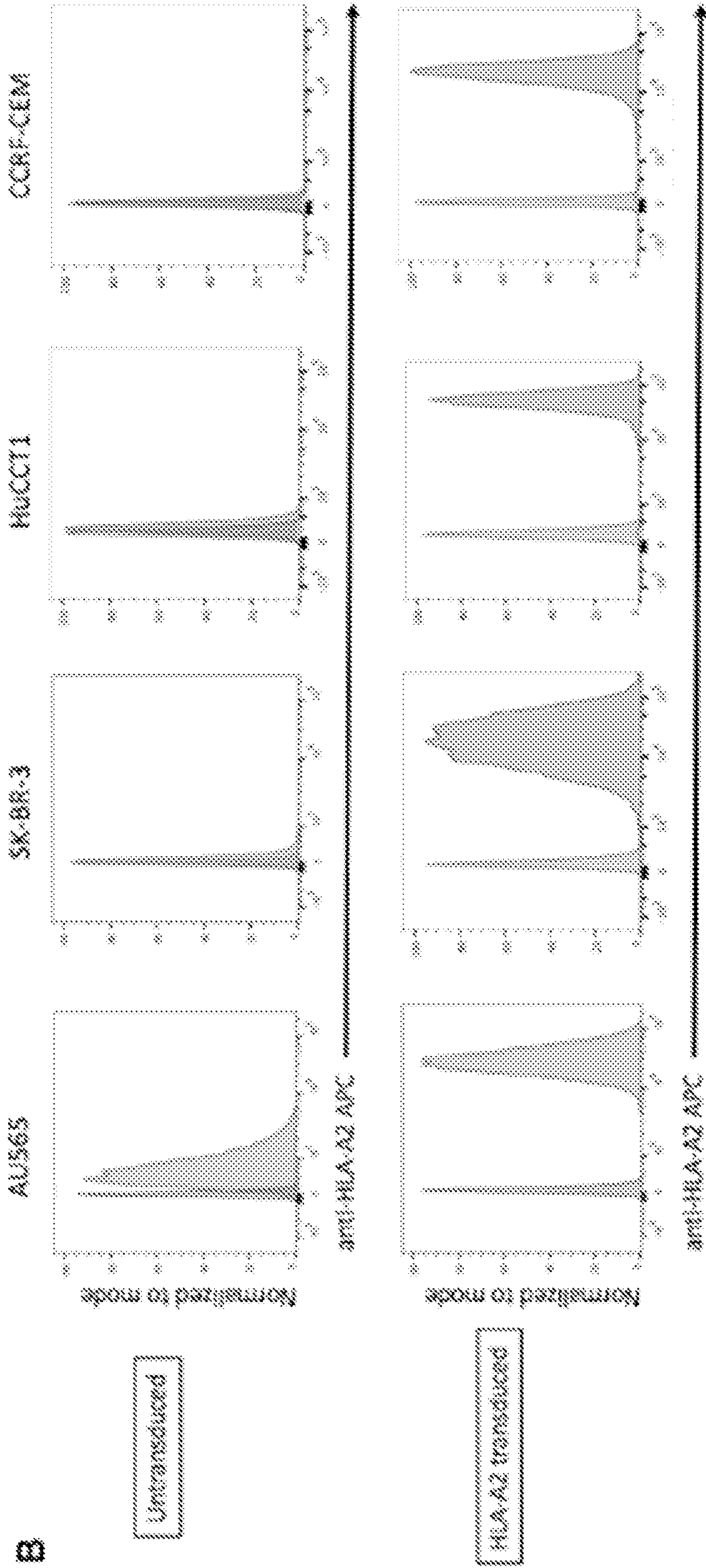


FIG. 77 (cont.)

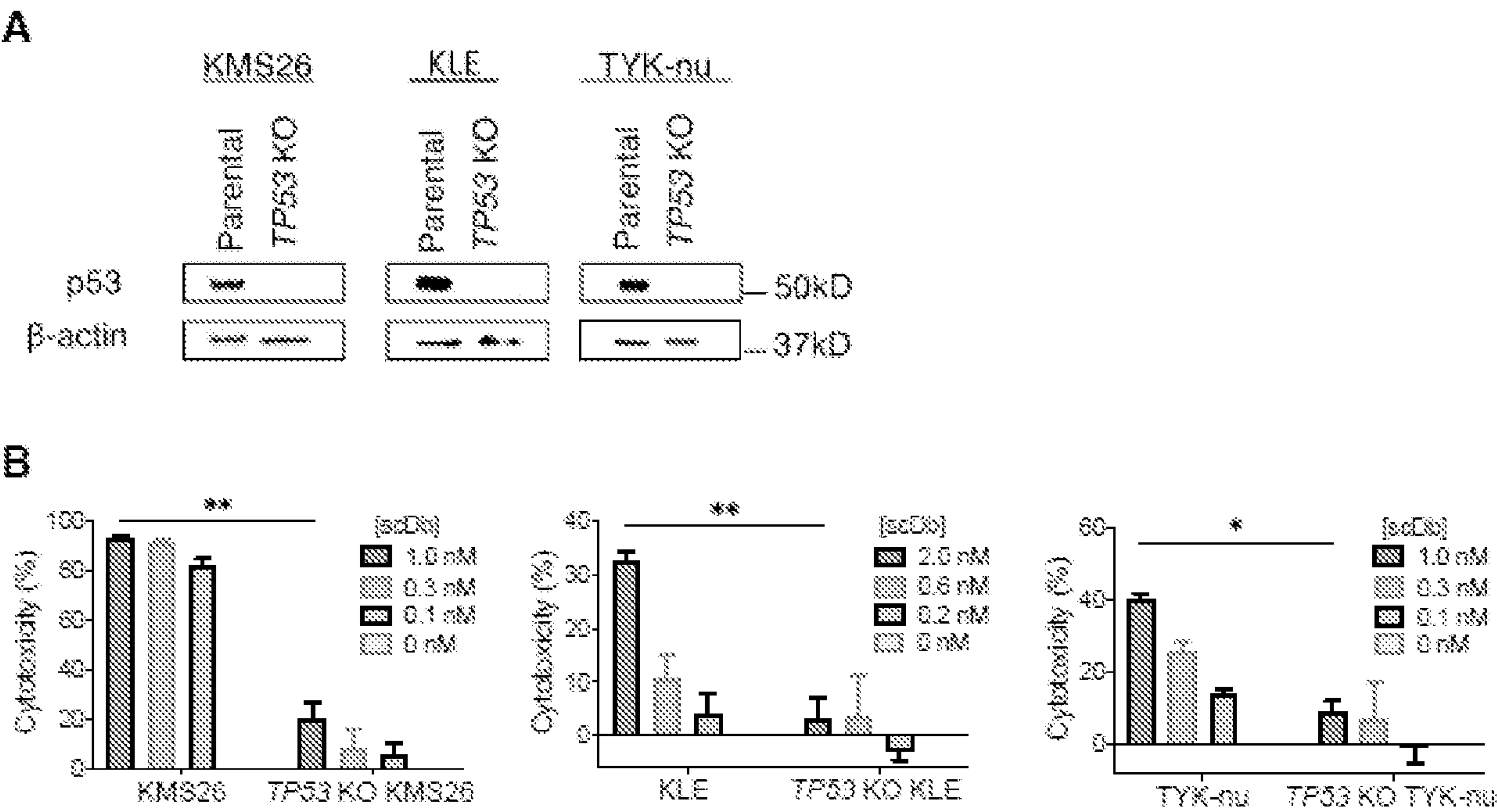


FIG. 78

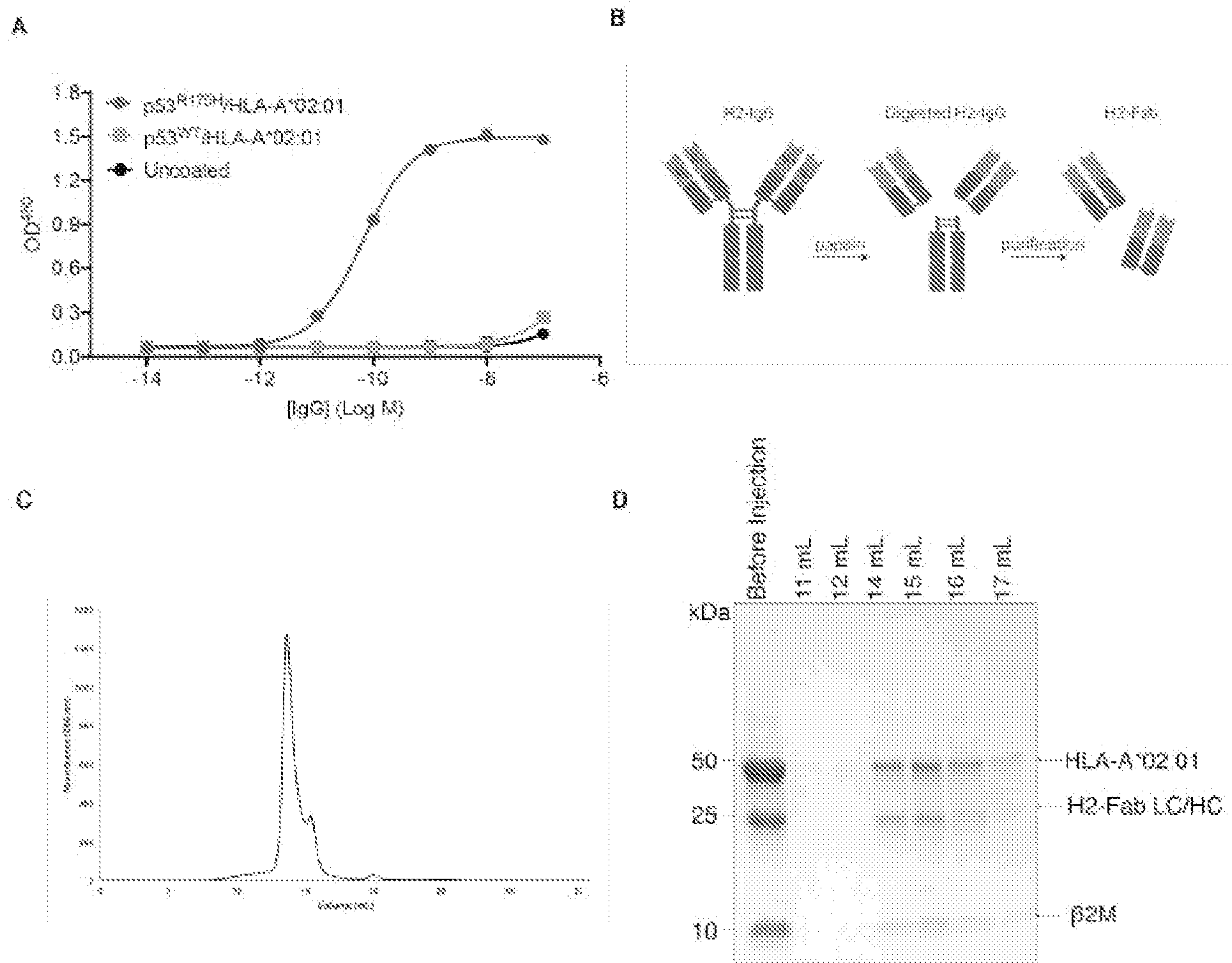


FIG. 79

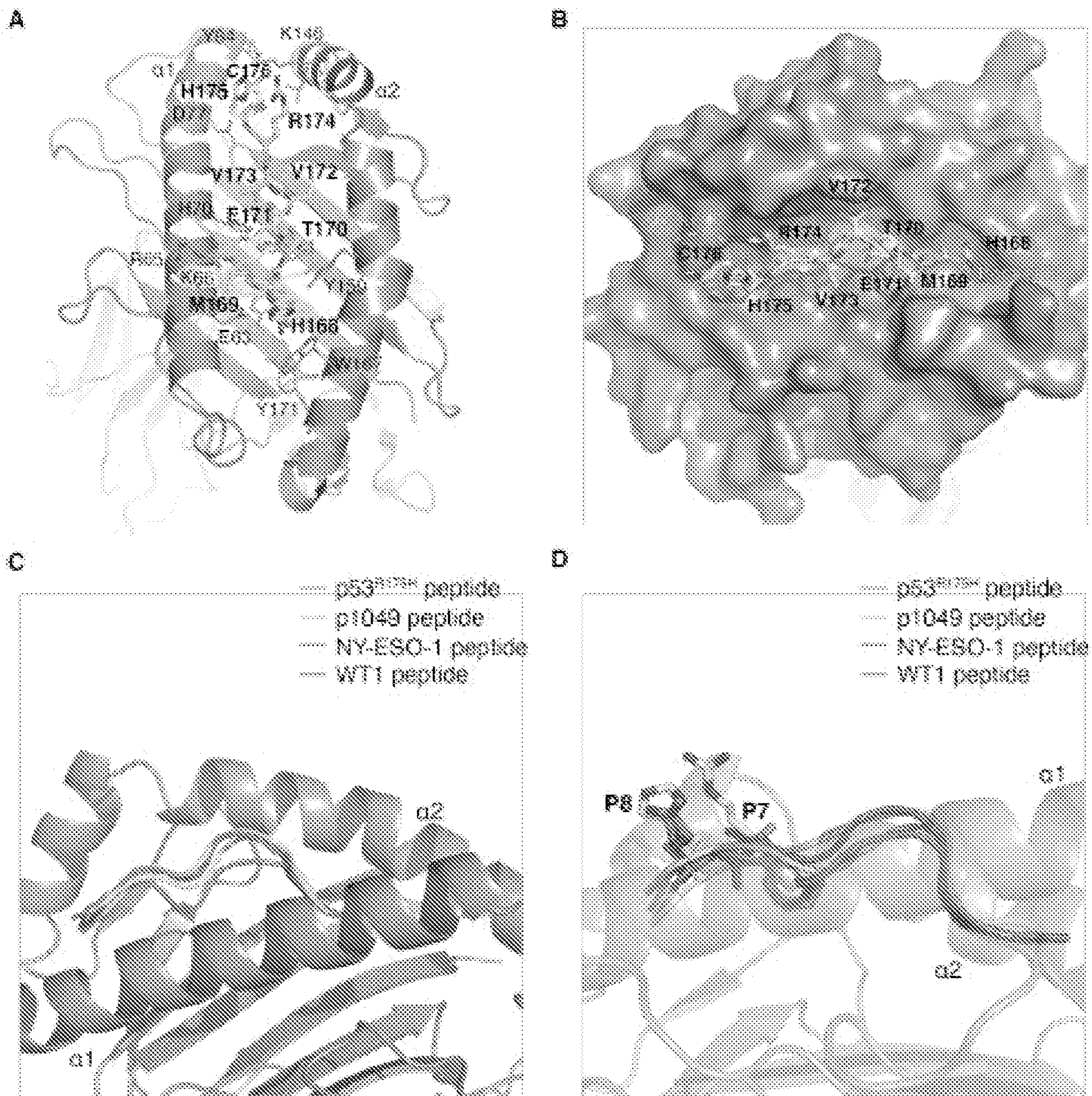


FIG. 80

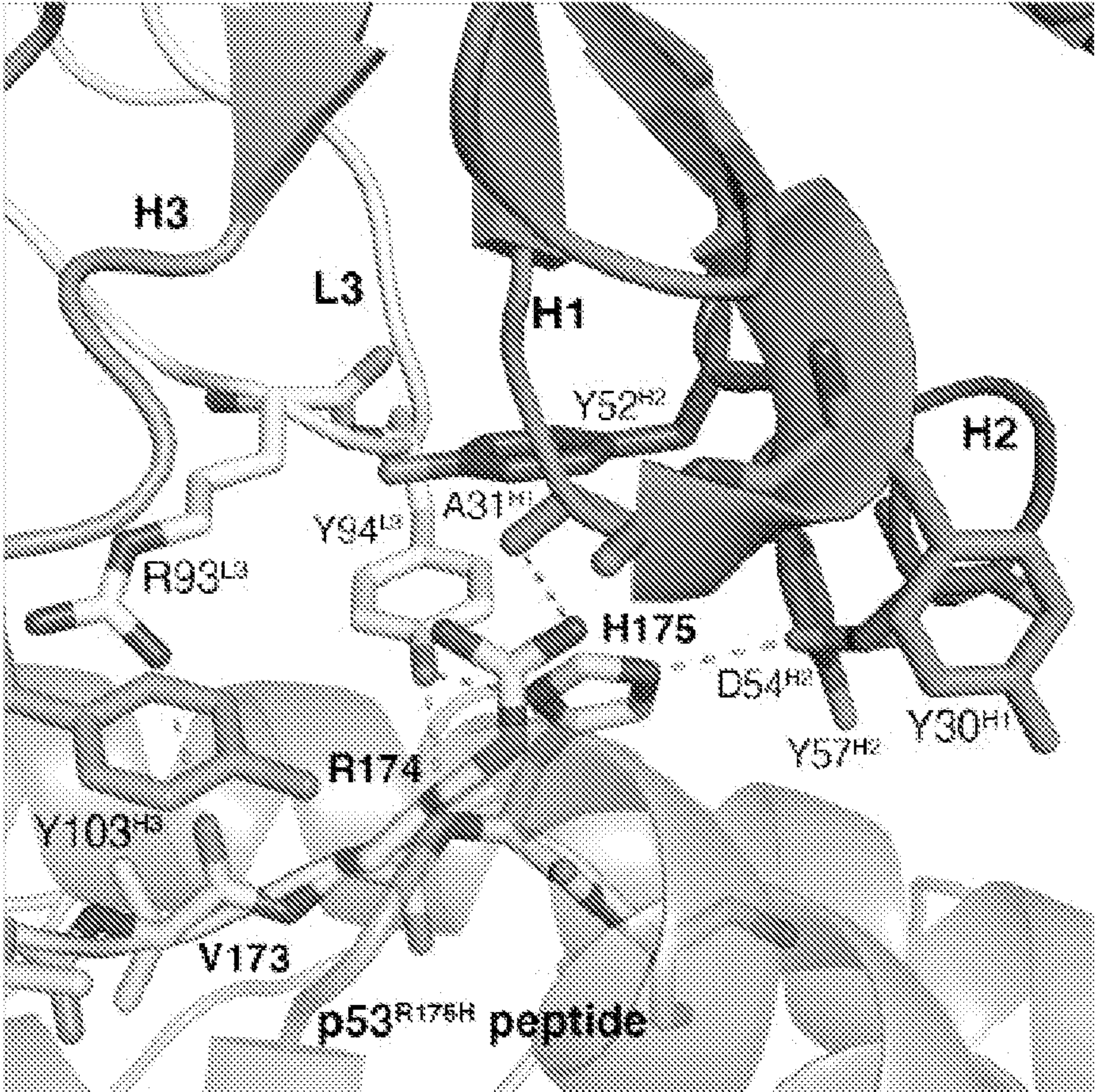


FIG. 81

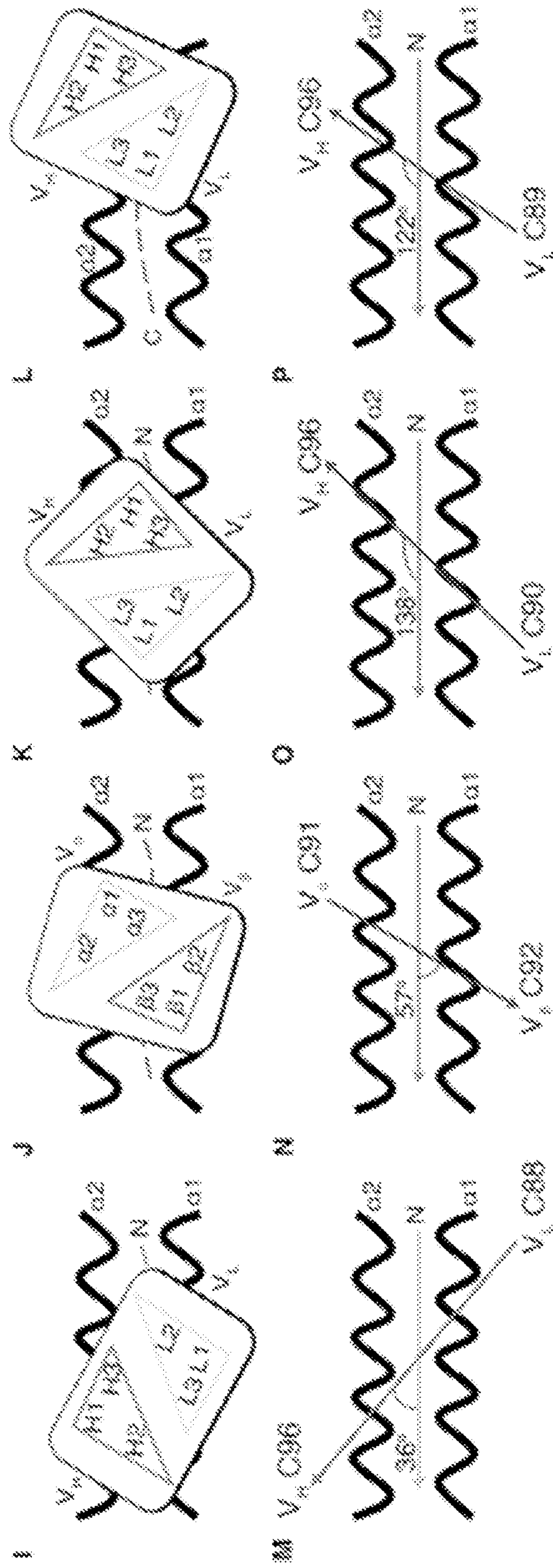


FIG. 82 (cont.)

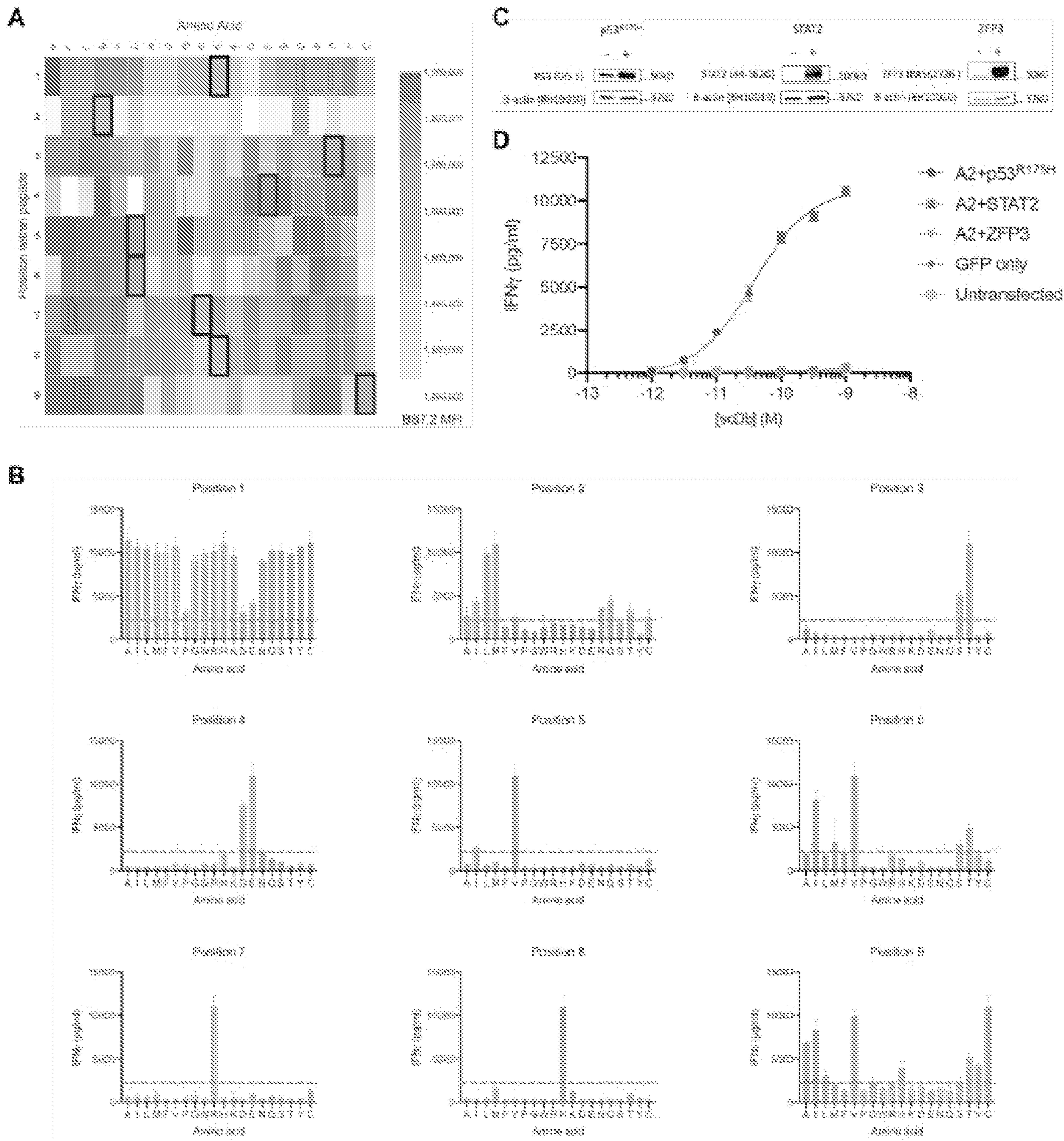


FIG. 83

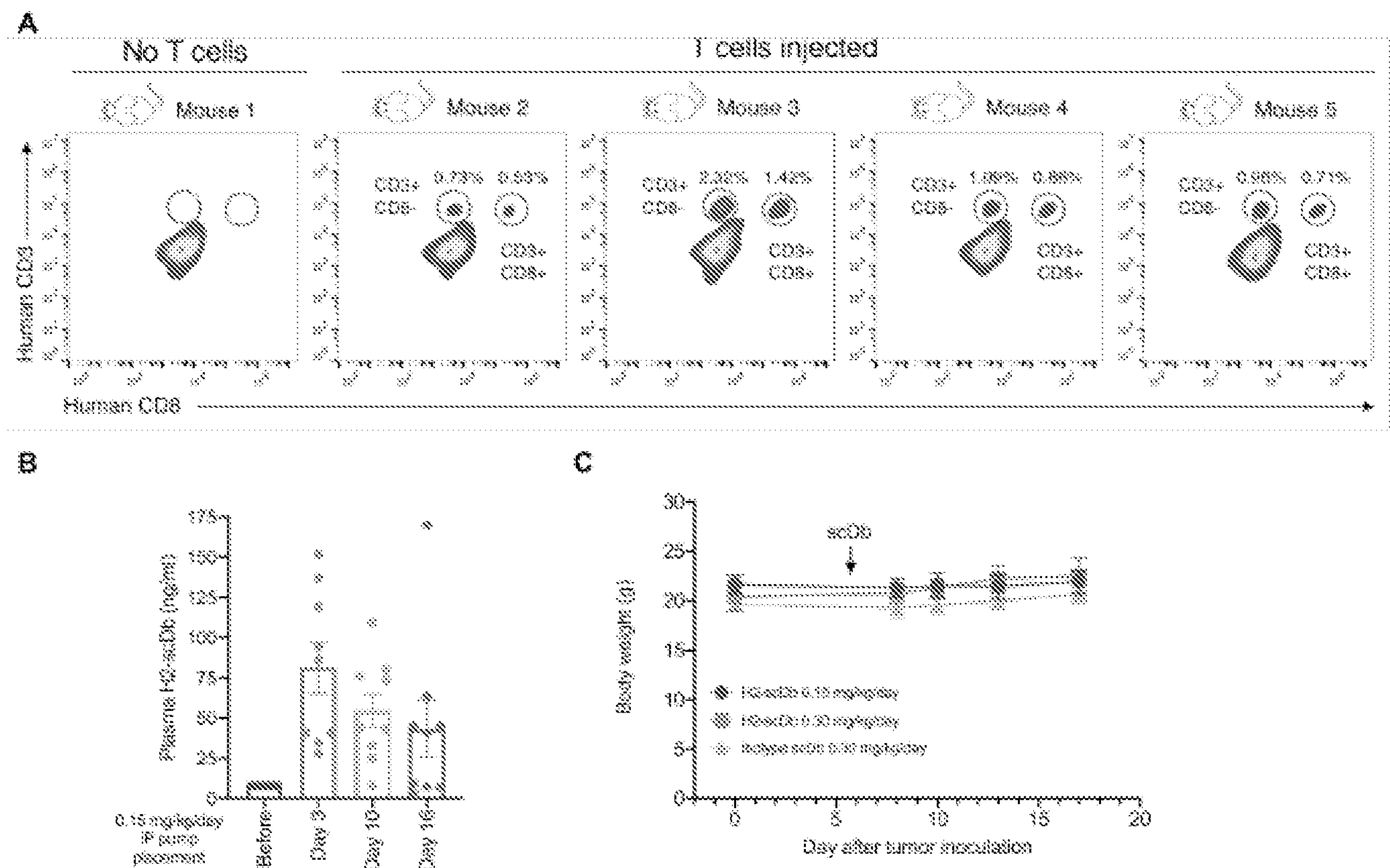


FIG. 84

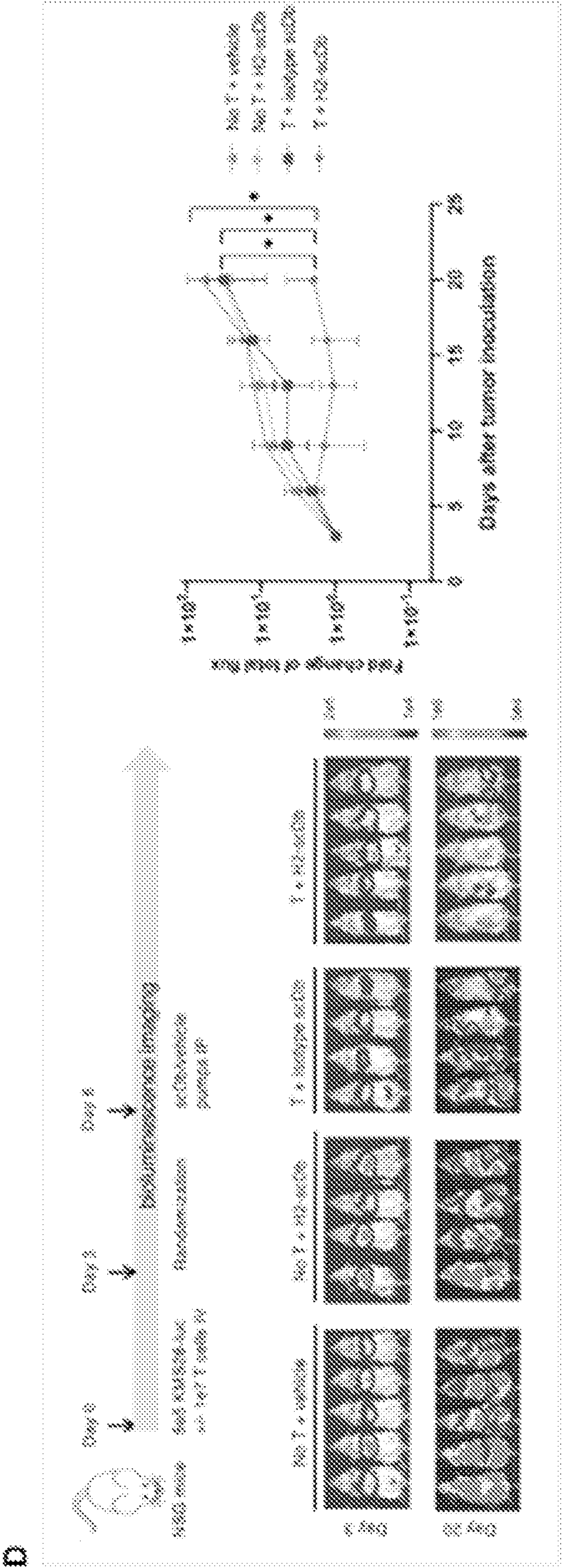


FIG. 84 (cont.)

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.

☐

Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:
2.

☐

Claims Nos.:

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3.

☒

Claims Nos.: 7, 8, 17, 18

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.

☐

As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.

☐

As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3.

☐

As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.

☐

No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

- Remark on Protest

☐

The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.

☐

The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT		International application No. PCT/US 2020/065617
A. CLASSIFICATION OF SUBJECT MATTER C07K 14/82 (2006.01) C07K 16/32 (2006.01) A61K 39/395 (2006.01) A61P 35/00 (2006.01) According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07K 14/82, C07K 16/32, A61K 39/395, A61P 35/00 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EAPATIS, Espacenet, PatSearch (RUPTO internal), USPTO, PATENTSCOPE, NCBI, EMBL-EBI, PubMed, Google		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2018/213467 A1 (THE JOHNS HOPKINS UNIVERSITY et al.) 22.11.2018, claims, abstract, p. 3, lines 14-21, p. 5, lines 8-12, p. 6, lines 6-18, table 1	1-3, 6, 9-12, 16, 19-21
Y		4
A		5, 13-15
Y	GenBank: AAH03596.1. Tumor protein p53 [Homo sapiens] 09.06.2008, section «Origin», sequence	4
Y	CHEN Chao et al. A Comprehensive Survey of Genomic Alterations in Gastric Cancer Reveals Recurrent Neoantigens as Potential Therapeutic Targets. BioMed Research International, 2019 Nov 5, Vol. 2019, Article ID 2183510, 10 pages, <doi: 10.1155/2019/2183510>, abstract, p. 5, column 1, paragraph 3 – column 2, paragraph 1	4
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents:		“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
“A” document defining the general state of the art which is not considered to be of particular relevance		“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
“D” document cited by the applicant in the international application		“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
“E” earlier document but published on or after the international filing date		“&” document member of the same patent family
“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
“O” document referring to an oral disclosure, use, exhibition or other means		
“P” document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 03 March 2021 (03.03.2021)	Date of mailing of the international search report 01 April 2021 (01.04.2021)	
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	Authorized officer N. Romanova Telephone No. +7 (495) 531-64-81	