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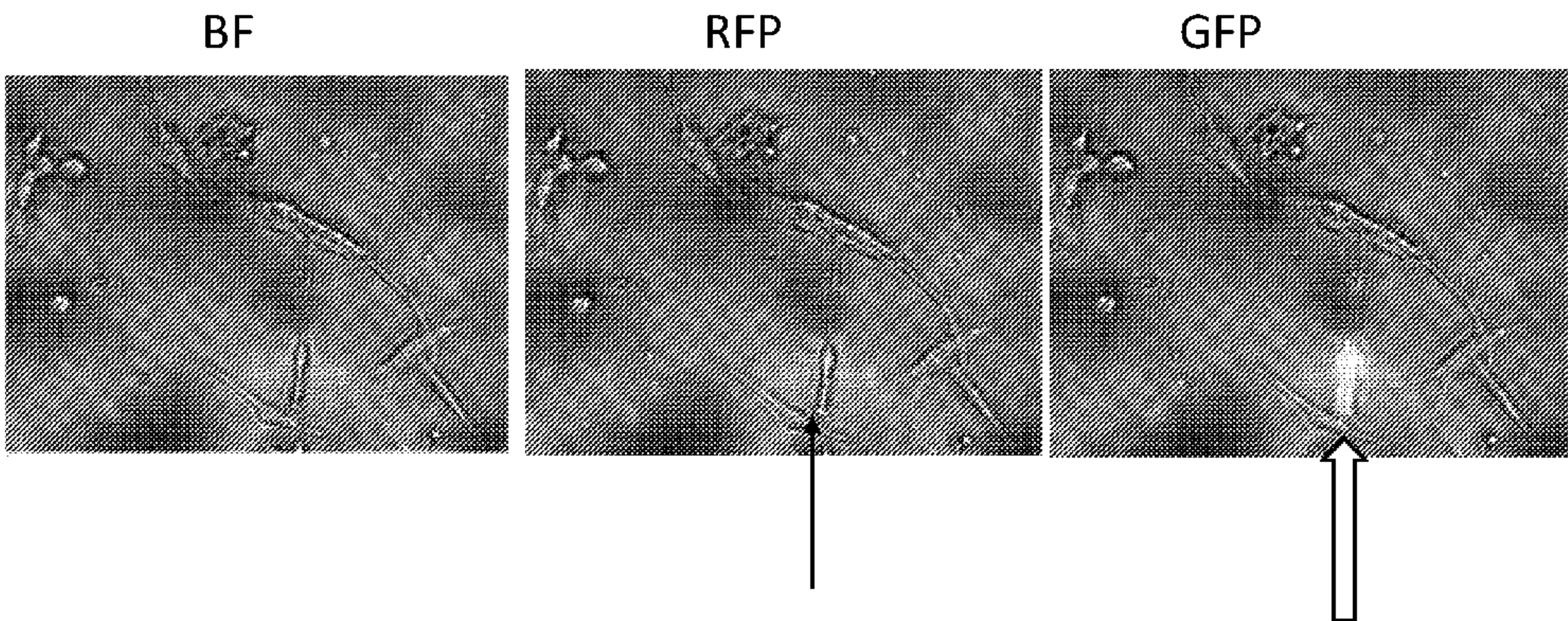


Figure 9C

(57) Abstract: Modified cells comprising an exogenous non-cytotoxic therapeutic proteinaceous agent are provided. Non-cytotoxic chimeric polypeptides comprising a lymphocyte lytic granule- secreted protein or a functional fragment thereof and a protein of interest are also provided. Therapeutic compositions, nucleic acid molecules and methods of use related to the modified lymphocytes and chimeric polypeptides of the invention are also provided.

DELIVERY COMPOSITIONS AND METHODS

CROSS REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of priority of U.S. Provisional Patent Application No. 62/956,342, filed January 2, 2020, the contents of which are all incorporated herein by reference in their entirety.

FIELD OF INVENTION

[002] The present invention is in the field of cell-based delivery systems.

BACKGROUND OF THE INVENTION

[003] There is an urgent need for biological delivery systems that can introduce therapeutic agents into the cytoplasm or even the nucleus of target cells. While delivery systems that can bring therapeutic agents to the cell surface of target cells exist, they are only effective if their target is a cell surface receptor, or if the therapeutic agent contains a cell-penetrating moiety. Thus, many otherwise-druggable cytoplasmic targets are not currently amenable to treatment, due to the lack of delivery systems that can reach them.

[004] In recent years, genome-editing technologies have been developed to exhibit high efficiency, specificity and versatility. Nevertheless, the abovementioned limited ability to effectively deliver treatment into cells equally applies to these novel technologies.

[005] The hurdles that impede effective application of gene therapy technologies include immunogenicity, the need for specificity to the target organ, tissue or cell and the lack of adequate composition, packaging, functional characteristics, and stability of delivery vehicles. Current gene therapy and gene-editing delivery systems for systemic gene therapy, whether viral or non-viral, are rapidly cleared from the circulation following systemic administration. In addition, the immunogenicity of viral systems can elicit an acute inflammatory response, which at best limits the effect of subsequent doses and at worst can

be hazardous to the patient. Therefore, there is still a need for efficient and precise gene editing delivery systems.

[006] Leukocytes, and in particular lymphocytes, have been widely investigated and utilized as cytotoxic therapeutic agents. These cells have been perfected by hundreds of millions of years of evolution to surveil, target and potentially kill, when necessary, any cell in the body. Lymphocyte-induced killing is a highly efficient and highly specific process, which occurs with minimal collateral damage, sparing neighboring bystander cells. Several lineages of lymphocytes, such as T cells, NK cells and NKT cells, can directly adhere to target cells and secrete the content of their lytic granules into those cells, and thus subsequently kill them.

[007] Two key proteins are involved in this process – Perforin assembles on the target cell membrane and forms pores, through which Granzyme, the lytic protein, enters the target cell and initiates an apoptotic cascade. Although, delivery of Granzyme has been envisioned as method of killing cancer cells, it has never been used as a delivery/non-cytotoxic option. International Patent Application WO2015/157864, as well as articles Dalken et al., “Targeted induction of apoptosis by chimeric granzyme B fusion proteins carrying antibody and growth factor domains for cell recognition”, Oberoi et al., 2013 “EGFR-targeted granzyme B expressed in NK cells enhances natural cytotoxicity and mediates specific killing of tumor cells”, and Zhao et al., “Secreted antibody/granzyme B fusion protein stimulates selected killing of HER2-overexpressing tumor cells” all make use of cytotoxic cells and cytotoxic granzyme fusions proteins to enhance immune cell killing of target cells. In all of these cases the goal is the death of the target cell, and in all cases the cells natural cytotoxicity is enhanced. A non-cytotoxic method of protein transfer, that hijacks lymphocytes’ natural lytic pathway to deliver therapeutic agents, in particular gene therapies, would provide a formidable solution to the therapeutic delivery problem. Converting this cell killing pathway to a delivery pathway provides a novel solution that heretofore was not envisioned.

SUMMARY OF THE INVENTION

[008] The present invention provides modified cells comprising an exogenous non-lytic therapeutic proteinaceous agent. Non-lytic chimeric polypeptides comprising a lymphocyte lytic granule-secreted protein, or a functional fragment thereof, and a protein of interest are also provided. Therapeutic compositions comprising the modified cells, nucleic acid molecules encoding the chimeric polypeptides and methods of use are also provided.

[009] According to a first aspect, there is provided a modified lymphocyte or myeloid cell, wherein the lymphocyte or myeloid cell comprises an exogenous non-cytotoxic therapeutic proteinaceous agent.

[010] According to some embodiments, the lymphocyte or myeloid cell does not comprise the non-cytotoxic therapeutic proteinaceous agent within the lymphocyte or myeloid cell's cellular membrane.

[011] According to some embodiments, the agent comprises a therapeutic portion that is not any of:

- a. a naturally secreted protein,
- b. a membranal protein expressed in the membrane of the modified cell,
- c. a surface receptor-binding agent,
- d. a viral penetration or envelope protein, and
- e. a nanoparticle conjugated or encapsulated agent.

[012] According to some embodiments, the lymphocyte is selected from a T cell and a natural killer (NK) cell, or the myeloid cell is a macrophage.

[013] According to some embodiments, the lymphocyte is a non-cytotoxic lymphocyte.

[014] According to some embodiments, the lymphocyte or myeloid cell is a cell of a cell line or a primary cell.

[015] According to some embodiments, the therapeutic proteinaceous agent is in a secretory granule of the lymphocyte.

[016] According to some embodiments, the therapeutic portion is a cytoplasmic or nuclear protein.

[017] According to some embodiments, the therapeutic proteinaceous agent does not comprise a signal peptide.

[018] According to some embodiments, the therapeutic proteinaceous agent is an RNA-protein complex.

[019] According to some embodiments, the therapeutic proteinaceous agent comprises a molecular weight of at least 50 kDa.

[020] According to some embodiments, the therapeutic proteinaceous agent comprises a chimeric protein comprising a lymphocyte lytic granule-secreted protein or a functional fragment thereof or variant thereof and a therapeutic polypeptide.

[021] According to some embodiments, the lymphocyte lytic granule-secreted protein is directly conjugated to the therapeutic polypeptide by a peptide bond or is indirectly conjugated by a protein linker.

[022] According to some embodiments, the linker is a cleavable linker.

[023] According to some embodiments, the cleavable linker is cleaved in acidic pH.

[024] According to some embodiments, the therapeutic polypeptide comprises a nuclear localization sequence (NLS).

[025] According to some embodiments, the lymphocyte lytic granule-secreted protein is selected from the group consisting of: granzyme A, granzyme B, granzyme H, granzyme K, granzyme M, Granulysin, Serglycin, and Perforin.

[026] According to some embodiments, the lymphocyte lytic granule-secreted protein is granzyme B.

[027] According to some embodiments, the lymphocyte lytic granule-secreted protein is non-cytotoxic or inactivated.

[028] According to some embodiments, the therapeutic proteinaceous agent is a genome-editing agent.

[029] According to some embodiments, the genome-editing agent comprises CRISPR associated protein 9 (Cas9).

[030] According to some embodiments, the genome-editing agent comprises a meganuclease.

[031] According to another aspect, there is provided a therapeutic composition comprising a modified lymphocyte of the invention.

[032] According to some embodiments, the therapeutic composition of the invention is formulated for administration to a subject, and comprises a pharmaceutically acceptable carrier, excipient, or adjuvant or both.

[033] According to another aspect, there is provided a non-cytotoxic chimeric polypeptide comprising a lymphocyte lytic granule-secreted protein or a functional fragment thereof and a protein of interest.

[034] According to some embodiments, the protein of interest does not bind a cell surface receptor in the target cell.

[035] According to some embodiments, the lymphocyte lytic granule-secreted protein is directly conjugated to the protein of interest by a peptide bond or is indirectly conjugated by a protein linker.

[036] According to some embodiments, the linker is a cleavable linker, optionally wherein cleavable linker is cleaved in the secretory granule.

[037] According to some embodiments, the protein of interest comprises at least one NLS.

[038] According to some embodiments, the lymphocyte lytic granule-secreted protein is selected from the group consisting of: granzyme A, granzyme B, granzyme H, granzyme K, granzyme M, Granulysin, Serglycin, and Perforin.

[039] According to some embodiments, the lymphocyte lytic granule-secreted protein is granzyme B.

[040] According to some embodiments, the protein of interest is a genome-editing agent.

[041] According to some embodiments, the genome-editing agent is CRISPR associated protein 9 (Cas9).

[042] According to some embodiments, the genome-editing agent is a meganuclease.

[043] According to another aspect, there is provided a polynucleotide encoding a chimeric polypeptide of the invention.

[044] According to some embodiments, the polynucleotide is an expression vector capable of expressing the chimeric polypeptide in a lymphocyte or myeloid cell.

[045] According to another aspect, there is provided a method of delivering a non-lytic therapeutic proteinaceous agent to a target cell, the method comprising contacting the target cell with any one of:

- a. a modified lymphocyte or myeloid cell of the invention;
- b. a therapeutic composition of the invention;
- c. a modified lymphocyte or myeloid cell expressing a chimeric polypeptide of the invention; and
- d. a modified lymphocyte or myeloid cell expressing a polynucleotide of the invention;

thereby delivering a non-cytotoxic therapeutic proteinaceous agent to a target cell.

[046] According to some embodiments, the target cell is in a subject in need of treatment with the non-cytotoxic therapeutic proteinaceous agent.

[047] According to some embodiments, the modified lymphocyte or myeloid cell is autologous or allogeneic to the subject.

[048] According to some embodiments, the method of the invention comprises extracting lymphocytes or myeloid cells from the subject, expressing in the lymphocytes or myeloid cells a non-cytotoxic therapeutic proteinaceous agent to produce modified lymphocytes or myeloid cells and returning the modified lymphocytes or myeloid cells to the subject.

[049] By another aspect, there is provided, the modified lymphocytes or myeloid cells of the invention or the pharmaceutical compositions of the invention for use in treating a subject in need thereof.

[050] According to another aspect, there is provided a method of delivering a non-lytic therapeutic protein of interest into a target cell, the method comprising contacting the target cell with a modified leukocyte wherein the modified leukocyte comprises reduced

cytotoxicity as compared to a non-modified leukocyte and comprises the non-lytic therapeutic protein of interest, thereby delivering a non-lytic therapeutic protein of interest into a target cell.

[051] According to another aspect, there is provided a method of delivering a genome editing protein into a target cell, the method comprising contacting the target cell with a modified leukocyte wherein the modified leukocyte comprises the genome editing protein, thereby delivering a non-lytic therapeutic protein of interest into a target cell.

[052] According to some embodiments, the non-lytic therapeutic protein of interest or genome editing protein is delivered to the cytoplasm or nucleus of the target cell.

[053] According to some embodiments, the modified leukocyte is capable of forming an immune synapse with the target cell.

[054] According to some embodiments, the modified leukocyte comprises the non-lytic therapeutic protein of interest or genome editing protein within a secretory lysosome.

[055] According to some embodiments, the modified leukocyte does not comprise the non-lytic therapeutic protein of interest or genome editing protein within or conjugated to the modified cell's cellular membrane.

[056] According to some embodiments, the modified leukocyte is selected from a modified T cell, modified natural killer (NK) cell and a modified myeloid cell.

[057] According to some embodiments, the modified leukocyte is a modified non-cytotoxic leukocyte, or wherein the modified leukocyte has been further modified to reduce cytotoxicity.

[058] According to some embodiments, the modified leukocyte comprises a knockout or knockdown or at least one endogenous cytotoxic protein, optionally wherein the endogenous cytotoxic protein is Granzyme B.

[059] According to some embodiments, the modified leukocyte comprises a mutation in at least one endogenous cytotoxic protein and wherein the mutation decreases cytotoxicity of the endogenous cytotoxic protein.

[060] According to some embodiments, the modified leukocyte comprises an anti-sense mediated reduction of at least one endogenous cytotoxic protein and wherein the anti-sense mediated decreases cytotoxicity of the endogenous cytotoxic protein.

[061] According to some embodiments, the non-lytic therapeutic protein of interest does not comprise any of:

- a. a naturally secreted protein;
- b. a membranal protein expressed in the membrane of the modified leukocyte;
- c. a surface receptor-binding protein;
- d. a viral penetration or envelope protein; and
- e. a nanoparticle conjugated or encapsulated protein.

[062] According to some embodiments, the non-lytic therapeutic protein of interest or genome editing protein is a cytoplasmic or nuclear protein.

[063] According to some embodiments, the non-lytic therapeutic protein of interest or genome editing protein does not comprise a signal peptide.

[064] According to some embodiments, the non-lytic therapeutic protein of interest or genome editing protein is a ribonuclear-protein (RNP) complex.

[065] According to some embodiments, the non-lytic therapeutic protein of interest comprises a genome editing protein.

[066] According to some embodiments, the genome editing protein modifies a gene within a nucleus of the target cell.

[067] According to some embodiments, the genome editing protein is a meganuclease.

[068] According to some embodiments, the non-lytic therapeutic protein of interest or genome editing protein comprises a molecule weight of at least 50 kDa.

[069] According to some embodiments, the genome editing protein is CRISPR associated protein 9 (Cas9).

[070] According to some embodiments, the non-lytic therapeutic protein of interest is a chimeric protein comprising a lymphocyte lytic granule-secreted protein or a functional fragment or variant thereof and a therapeutic polypeptide or wherein the genome editing protein is a chimeric protein comprising a lymphocyte lytic granule-secreted protein or a functional fragment or variant thereof and the genome editing protein.

[071] According to some embodiments, the lymphocyte lytic granule-secreted protein or a functional fragment or variant thereof comprises a signal peptide, optionally wherein the signal peptide is an N-terminal signal peptide.

[072] According to some embodiments, the lymphocyte lytic granule-secreted protein is directly conjugated to the therapeutic polypeptide or genome editing protein by a peptide bond or is indirectly conjugated by a protein linker.

[073] According to some embodiments, the linker is a cleavable linker, optionally wherein the linker is cleaved in a secretory granule or at acidic pH.

[074] According to some embodiments, the therapeutic polypeptide or genome editing protein comprises a nuclear localization sequence (NLS).

[075] According to some embodiments, the lytic granule-secreted protein is a lytic protein comprising at least one inactivating mutation, wherein the inactivating mutation inhibits the lytic function of the lytic protein.

[076] According to some embodiments, the lymphocyte lytic granule secreted protein is selected from the group consisting of: granzyme A, granzyme B, granzyme H, granzyme K, granzyme M, Granulysin, Serglycin, and Perforin.

[077] According to some embodiments, the lymphocyte lytic granule secreted protein is Granzyme B.

[078] According to some embodiments, the transfer is not mediated by exosomes.

[079] According to some embodiments, delivering to a cytoplasm does not comprise entering an endosome.

[080] According to some embodiments, the method further comprises providing the leukocyte, activating the leukocyte and expressing the non-lytic therapeutic protein of

interest or genome editing protein in the leukocyte after the activating to produce the modified leukocyte.

[081] According to some embodiments, the expression is done not more than 5 days before the contacting.

[082] According to some embodiments, the target cell is in a subject in need of treatment with the non-lytic therapeutic protein of interest or genome editing protein, and the method comprises administering a pharmaceutical composition comprising the modified leukocyte.

[083] According to some embodiments, the subject is in need to gene therapy and the modified leukocyte comprises a genome editing protein.

[084] According to some embodiments, the modified leukocyte is autologous or allogeneic to the subject.

[085] According to some embodiments, the method comprises extracting leukocytes from the subject, activating the leukocytes, expressing the non-cytotoxic therapeutic protein of interest or genome editing protein in the leukocytes after the activating to produce the modified leukocytes and returning the modified leukocytes to the subject.

[086] According to some embodiments, the expressing is done not more than 5 days before the returning.

[087] According to some embodiments, the treatment does not comprise killing the target cell.

[088] According to some embodiments, the subject does not suffer from cancer.

[089] According to another aspect, there is provided a non-lytic chimeric polypeptide comprising a lymphocyte lytic granule-secreted protein or a functional fragment or variant thereof and a protein of interest.

[090] According to some embodiments, the protein of interest does not bind a cell surface receptor.

[091] According to some embodiments, the lymphocyte lytic granule-secreted protein is directly conjugated to the protein of interest by a peptide bond or is indirectly conjugated by a protein linker.

[092] According to some embodiments, the linker is a cleavable linker, optionally wherein cleavable linker is cleaved in a secretory granule or at acidic pH.

[093] According to some embodiments, the protein of interest comprises at least one NLS.

[094] According to some embodiments, the lymphocyte lytic granule-secreted protein is selected from the group consisting of: granzyme A, granzyme B, granzyme H, granzyme K, granzyme M, and Perforin.

[095] According to some embodiments, the lymphocyte lytic granule-secreted protein is granzyme B.

[096] According to some embodiments, the protein of interest is a genome-editing protein.

[097] According to some embodiments, the genome-editing protein is CRISPR associated protein 9 (Cas9).

[098] According to some embodiments, the genome-editing protein is a meganuclease.

[099] According to some embodiments, the protein of interest comprises a molecular weight of at least 50kDa.

[0100] According to another aspect, there is provided a polynucleotide encoding a chimeric polypeptide of the invention.

[0101] According to some embodiments, the polynucleotide is an expression vector capable of expressing the chimeric polypeptide in a lymphocyte or myeloid cell.

[0102] According to another aspect, there is provided a modified leukocyte with reduced cytotoxicity as compared to a non-modified leukocyte, comprising at least one of:

- a. a non-cytotoxic chimeric polypeptide of the invention;
- b. a polynucleotide of the invention; and
- c. a secretory granule comprising a non-cytotoxic therapeutic protein of interest.

[0103] According to some embodiments, the leukocyte is capable of forming an immune synapse with a target cell.

[0104] According to some embodiments, the leukocyte is selected from a T cell, a natural killer (NK) cell, and a myeloid cell.

[0105] According to some embodiments, the modified leukocyte does not comprise the non-cytotoxic therapeutic protein of interest within or conjugated to the modified leukocyte's cellular membrane.

[0106] According to some embodiments, the modified leukocyte is a modified non-cytotoxic leukocyte, or wherein the modified leukocyte comprises a mutation of at least one endogenous cytotoxic protein wherein the mutation decreases the cytotoxicity of the endogenous cytotoxic protein.

[0107] According to some embodiments, the modified cell comprises a knockout or knockdown or at least one endogenous cytotoxic protein, optionally wherein the endogenous cytotoxic protein is Granzyme B.

[0108] According to another aspect, there is provided a therapeutic composition comprising a modified leukocyte of the invention.

[0109] According to some embodiments, the composition is formulated for administration to a subject, and comprising a pharmaceutically acceptable carrier, excipient, or adjuvant or both.

[0110] According to another aspect, there is provided a kit comprising at least one of:

- a. a non-cytotoxic chimeric polypeptide of the invention;
- b. a polynucleotide of the invention;
- c. a modified leukocyte of the invention; and
- d. a therapeutic composition of the invention.

[0111] Further embodiments and the full scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes

and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0112] **Figure 1: Schematic representation of fusion-protein expressing plasmids of the invention.**

[0113] **Figures 2A-E: Granzyme-Cherry protein transfer to K562 target cells.** (2A) Dot plot of size separation of target K562 cells from effector T cells using FSC/SSC gating of total live events. (2B) Histogram of gating of GFP positive target K562 cells after co-culture with Granzyme-Cherry transduced T cells. (2C) Histogram of Cherry fluorescence in GFP positive target K562 cells (GFP+ gate as shown in 2B) after co-culture with either mock electroporated T cells (filled gray histogram) or T cells electroporated with mRNA IVT product of the coding region of plasmid 1_3 described in **figure 1** encoding Granzyme-Cherry (empty black histogram). (2D) Histogram of Cherry fluorescence in GFP positive target K562 cells after co-cultured with either mock electroporated YTS cells (filled gray histogram) or YTS cells electroporated with mRNA IVT product of the coding region of plasmid 1_3 shown in **Figure 1** encoding Granzyme-Cherry (empty black histogram). (2E) Histogram of Cherry fluorescence in Tag-it labeled K562 cells after co-culture with Cherry expressing GZMB-KO YTS cells (used as negative control).

[0114] **Figures 3A-D: Granzyme knock-out cells with functionality.** (3A) Histogram of GZMB expression in parental (grey) and GZMB-KO (black line) YTS cells. (3B) Histogram of the size, as measured by forward-scatter of K562 cells co-cultured with YTS cells expressing endogenous GZMB (light grey) and K562 cells co-cultured with YTS GZMB-KO cells (dark grey). (3C) Histograms of mCherry expression in K562 cells co-cultured with YTS cells expressing endogenous GZMB (left histogram) mock electroporated (shaded) or electroporated with GZMB-crmCherry plasmid (grey outline) and with YTS GZMB-KO cells (right histogram) mock electroporated (shaded) or electroporated with GZMB-crmCherry plasmid (black outline). (3D) Histogram presentation of live/dead staining of K562 cells alone or after co-cultured with parental YTS cells or GZMB KO YTS.

[0115] **Figures 4A-D:** (4A) Representative micrographs for primary T cells electroporated with pMAX_hGZMB_HA-Cas9_P2A_crmCherry plasmid. Bright-field (BF), mCherry, Cas-9, Granzyme B (GZMB) and a merge of all the channels is shown. (4B) Histogram of gates used to define Tag-it labeled target K562 cells and unlabeled Tag-it negative effector YTS cells. (4C) Photograph of western blot detection of Cas9 in YTS cells over-expressing mRNA IVT product of hGZMB_Cas9 (first lane) and in K562 target cells co-cultured with the GZMB-CAS9 expressing YTS cells (second lane). YTS cells and K562 cells were sorted by Tag-it expression using the gate shown in 4B. The gray arrow indicates the Cas9 band in the K562 cells. A ladder is provided for size comparison and shows that the lower of the two bands observed in the YTS cells corresponds to the molecular weight of Cas9. (4D) Histogram of GZMB-Cas9 as detected by intra-cellular staining of Granzyme B in K562 target cells following co-culture with GZMB-KO YTS cells expressing GZMB-CAS9 or mock treated GZMB KO YTS cell.

[0116] **Figures 5A-D: Editing in target cells after Granzyme mediated CAS9 and Meganuclease transfer.** (5A-B) Micrographs of melanoma cells after co-culture with (5A) YTS cells and (5B) T cells electroporated with GZMB_Cas9 mRNA. RFP cells are shown (black arrow) and GFP positive cells indicate successful genome editing (white arrow). (5C) Histogram of GFP expressing cells in the RFP positive population of K562 target cells following transfer of GZMB-CAS9 from YTS cells electroporated with GZMB-CAS9 mRNA. (5D) Histogram of GFP expressing cells in the RFP positive population of K562 target cells following transfer of GZMB-Meganuclease from YTS cells electroporated with GZMB-Meganuclease mRNA.

[0117] **Figure 6A-C: K562 myeloid cell line functions as an effector cell capable of cargo delivery.** (6A) Histogram of mCherry signal in Tag-it positive melanoma target cells after co-culture with K562 cells electroporated with GZMB-crmCherry mRNA IVT product of the coding region of plasmid 1_3 shown in **Figure 1**. (6B) Histogram presentation of GFP signal in Tag-it positive melanoma target cells after co-culture with K562 cells electroporated with GZMB-Meganuclease-GFP mRNA IVT product of the coding region of plasmid 2_6 shown in **Figure 1**. (6C) Histogram of GFP expressing cells in the RFP positive population of melanoma target cells following transfer of GZMB-CAS9 from K562 cells electroporated with GZMB-CAS9 mRNA.

[0118] **Figures 7A-D: Cas9-GFP RNP transfer from T cells to P815 target cells. (7A-C)** Histograms of (7A) GFP expression in Mock and Cas9-GFP RNA-Protein complex electroporated T cells, (7B) gates used for CD8 positive and negative differentiation between target P815 cells and CD8 positive T cells in co-culture experiments with Mock or Cas9-GFP transduced T cells, and (7C) GFP fluorescence in CD8 negative target P815 cells (CD8-gate as shown in 7B) co-cultured with either mock electroporated T cells or Cas9-GFP transduced T cells. Mock is shown as filled gray histograms and Cas9-GFP RNA-Protein complex transduced T cells shown as empty black histograms. (7D) Representative micrograph of co-cultured Cas9-GFP RNA-Protein complex transduced T cells and P815 cells. Cas9-GFP can be seen as green. CD8 positive T cells are shown as light blue. Cells that are only green and not blue are target cells that have received the RNA-Protein complex, some are marked by arrows.

[0119] **Figures 8A-I: Cas9-GFP RNP transfer from YTS cells to K562 and MCF7 target cells.** Histograms of (8A) Cas9-GFP fluorescence in mock electroporated and Cas9-GFP RNA-protein complex transduced YTS cells, (8B) gates used to define Tag-it labeled target K562 cells and unlabeled Tag-it negative effector YTS cells, (8C) Cas9-GFP RNA-Protein complex derived fluorescence in Tag-it positive target K562 cells (Tagit+ gate as shown in 8B) co-cultured with either mock electroporated YTS cells or Cas9-GFP transduced YTS cells, (8D) gates used to define Tag-it labeled target MCF7 cells and unlabeled Tag-it negative effector YTS cells, and (8E) Cas9-GFP RNA-Protein complex derived fluorescence in Tag-it positive target MCF7 cells (Tagit+ gate as shown in 8D) co-cultured with either mock electroporated YTS cells or Cas9-GFP transduced YTS cells. Mock electroporation experiments are shown as filled gray histograms and Cas9-GFP RNA-Protein complex electroporation experiments are shown as empty black histograms. (8F) Histograms of GFP signal in K562 cells incubated with the supernatant of the co-culture between Cas9-GFP transduced YTS cells and K562 cells. (8G) GFP signal of melanoma cells incubated with the supernatant of the co-culture between Cas9-GFP transduced YTS cells and melanoma cells. (8H) Cas9-GFP RNA-Protein complex derived fluorescence in Tag-it positive target K562 cells co-cultured with either mock electroporated YTS cells or Cas9-GFP transduced YTS cells (supernatant of this co-culture was used in figure 8F). (8I) Cas9-GFP RNA-Protein complex derived fluorescence in Tag-it positive target melanoma

cells co-cultured with either mock electroporated YTS cells or Cas9-GFP transduced YTS cells (supernatant of this co-culture was used in figure 8G).

[0120] **Figures 9A-C: Editing in target cells after CAS9 RNP transfer. (9A-C)** Micrographs of melanoma cells after co-culture with (9A) YTS cells transduced with CAS9 RNPs, (9B) YTS Granzyme B Knockout cells transduced with CAS9 RNPs and (9C) K562 myeloid cells transduced with CAS9 RNPs. RFP cells are shown (black arrow) and GFP positive cells indicate successful genome editing (white arrow).

DETAILED DESCRIPTION OF THE INVENTION

[0121] The present invention, in some embodiments, provides modified cells comprising an exogenous therapeutic agent. Chimeric polypeptides comprising a protein of interest and a lymphocyte lytic granule-secreted protein, a variant thereof or a fragment thereof, are also provided. Therapeutic compositions comprising the modified cells, nucleic acid molecules encoding the chimeric polypeptide and methods of use of the modified cells, pharmaceutical compositions and chimeric polypeptides of the invention are also provided.

[0122] The instant invention is based on the surprising finding that leukocytes, and in particular lymphocytes such as T cells and natural killer (NK) cells, can be used as delivery systems for therapeutic agents. In particular, it has been found that, though these cells are naturally cytotoxic, their cytotoxic function is not required for their delivery capability, and indeed non-cytotoxic delivery is superior to cytotoxic delivery when the desired outcome is not death of the target cell. It was first found that a fusion of a lytic granule-secreted protein, such as Granzyme, to a protein of interest can target that protein of interest to lytic granules and cause it to be secreted and even be taken up by target cells and reach the cell cytoplasm. This transfer was demonstrated even for very large proteins such as Cas-9, which is well over 100kDa in size. The cytotoxic properties of granzyme are not required for this transfer, as a mutant granzyme and a pro-granzyme containing an inhibitory di-peptide were both equally capable of facilitating the protein transfer. Even more surprising, it was found that an RNA-protein complex, also of a very large size, when electroporated into lymphocytes or myeloid cells, was also transferred to target cells even without fusion to a lytic granule-secreted protein fusion. The complex was able to reach the interior of the target cells and

indeed was functional there, as the RNA-Protein complex was capable of editing the target cell's genome. This heretofore unknown mechanism allows for lymphocytes to be used as a broad therapeutic delivery system. In particular, it provides for a delivery system for genome editing in target cells.

[0123] It will be understood by a skilled artisan that the leukocytes (lymphocytes and myeloid cells) of the invention are a mode of delivery for bringing the therapeutic agent to its target in a subject. Immune cells are well known in the art to home to disease locations in the body. As such, they are an ideal delivery method for carrying a therapeutic agent to sites of disease. A major stumbling block for current therapeutics is the fact that many known targets are cell internal, while reliable methods of intracellular delivery do not currently exist. As such, most therapeutics are limited to targeting cell surface molecules. This severely limits the pool of available druggable targets. In particular, gene therapy, which is potentially able to treat a wide variety of diseases/conditions, is only feasible if the gene-editing agents can be delivered into the nucleus. Additionally, short circulation half-life and biodistribution problems hamper many promising therapeutics. The cells and compositions of the invention offer a comprehensive solution to all these problems. The therapeutic agent is protected within the leukocyte while it travels through the body, thus limiting degradation and half-life concerns. The leukocyte reduces off-target effects, first by homing to sites of disease, either through its natural homing ability or thanks to targeting moieties on its cell surface. Off-target effects are further limited, as lymphocytes do not secrete lytic vesicles until the cell is activated, a mechanism that can be controlled and targeted directly to disease/target cells. Finally, the lytic vesicles are taken up by the target cells and their cargo is delivered into the target cell's interior, allowing for access to all cellular targets and even for genome editing. The combination of all these aspects of the present technology makes it uniquely suited as a therapeutic delivery system.

[0124] Chimeric molecules

[0125] By a first aspect, there is provided a chimeric molecule comprising a lymphocyte lytic granule-secreted protein, or a variant or fragment thereof, and a molecule of interest.

[0126] It will be understood by a skilled artisan that the lymphocyte lytic granule-secreted protein, or a variant or fragment thereof, is a targeting moiety in the chimeric molecule, and the protein of interest is the cargo. The targeting moiety is not intended to possess any direct

therapeutic function, but rather to facilitate transfer to target cells. Facilitating transfer comprises delivering the cargo to the lytic granule that is secreted by lymphocytes and taken up by target cells. The protein of interest is a therapeutic cargo, that is able to act on its target following delivery by the lymphocyte lytic granule-secreted protein.

[0127] In some embodiments, the chimeric molecule is a chimeric polypeptide. In some embodiments, the molecule of interest is a protein of interest. As used herein, the terms "peptide", "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. In another embodiment, the terms "peptide", "polypeptide" and "protein", as used herein, encompass native peptides, peptidomimetics (typically including non-peptide bonds or other synthetic modifications) and the peptide analogs peptoids and semipeptoids or any combination thereof. In another embodiment, the peptides, polypeptides and proteins described carry modifications rendering them more stable while in the body, or more capable of penetrating into cells. In one embodiment, the terms "peptide", "polypeptide" and "protein" apply to naturally-occurring amino acid polymers. In another embodiment, the terms "peptide", "polypeptide" and "protein" apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analog of a corresponding naturally-occurring amino acid.

[0128] In some embodiments, the chimeric polypeptide is a fusion protein. In some embodiments, the chimeric polypeptide is an artificial polypeptide. In some embodiments, the chimeric polypeptide is a polypeptide not found in nature. As used herein, the term "chimeric polypeptide" refers to a single polypeptide chain comprising at least two distinct protein domains or regions, that are not naturally found in the same protein. The fusion protein may be formed by the joining of two or more peptides through a peptide bond formed between the amino- terminus of one peptide and the carboxyl-terminus of another peptide. The fusion protein may be expressed as a single polypeptide fusion protein from a nucleic acid sequence encoding the single contiguous conjugate. In some embodiments, fusion proteins are created through the joining of two or more genes that originally coded for separate proteins. Recombinant fusion proteins may be created artificially by recombinant DNA technology for use in biological research or therapeutics. "Chimeric" or "chimera" usually designate hybrid proteins made of polypeptides having different functions or physicochemical patterns. For example, a fusion protein can comprise a first part that is a

lymphocyte lytic-granule-secreted protein, and a second part (e.g., genetically fused to the first part) that is a protein of interest (e.g., a protein with a distinct enzymatic activity, i.e., DNA nuclease). Methods of fusion protein generation, recombinant protein generation, recombinant DNA generation, and DNA fusion techniques are well known in the art, and any such method for making the chimeric molecules of the invention may be employed. The chimeric polypeptide may be cleavable, such that upon cleavage it is separated into two or more polypeptides. In one non-limiting example, upon cleavage, a fusion polypeptide comprising a lymphocyte lytic-granule-secreted protein and a protein of interest is split into the lymphocyte lytic-granule-secreted protein and the protein of interest, such that the functionality of the protein of interest is intact.

[0129] As used herein, the term “recombinant protein” refers to a protein which is coded for by a recombinant nucleic acid molecule (DNA or RNA) and is thus not naturally occurring. The term “recombinant DNA or RNA” refers to DNA or RNA molecules formed by laboratory methods of genetic recombination. Generally, this recombinant molecule is in the form of an mRNA, a vector, a plasmid or a virus, used to express the recombinant protein in a cell.

[0130] In some embodiments, the lymphocyte lytic granule-secreted protein is directly conjugated to the protein of interest. In some embodiments, the lymphocyte lytic granule-secreted protein is linked to the protein of interest by a linker. In some embodiments, there are no intervening amino acids between the lymphocyte lytic granule-secreted protein and the protein of interest. In some embodiments, there is an amino acid linker separating the lymphocyte lytic granule-secreted protein and the protein of interest. In some embodiments, the lymphocyte lytic granule-secreted protein is N-terminal to the molecule of interest. In some embodiments, the lymphocyte lytic granule-secreted protein is C-terminal to the molecule of interest.

[0131] As used herein, the term “lymphocyte lytic granule-secreted protein” and “secretory lysosomal protein” are herein used interchangeably and refer to any protein that is secreted by the lymphocyte via lytic granules which are known as secretory lysosomes. In some embodiments, the lymphocyte lytic granule-secreted protein is a protein involved in the granzyme/perforin pathway and is naturally secreted from lytic granules of lymphocytes into target cells during antigen-dependent lymphocyte recognition of target cells. In some

embodiments, antigen-dependent lymphocyte recognition is mediated by an endogenous T Cell Receptor (TCR). In some embodiments, antigen-dependent lymphocyte recognition is mediated by engineered T cell Receptor ligation to an MHC/peptide complex on a target cell. In some embodiments, antigen dependent lymphocyte recognition is mediated by Chimeric Antigen Receptor (CAR) ligation to an antigen on a target cell. In some embodiments, the secretory lysosomal protein is a lytic granule secreted protein. In some embodiments, the lytic granule secreted-protein is a secretory lysosomal protein.

[0132] In some embodiments, the lytic granule is a lytic vesicle. In some embodiments, the lytic granule is a secretory lysosome. The lytic granule is a well-known organelle found in lymphocytes. It is a specialized secretory organelle that, upon activation of cytotoxic lymphocytes such as T cells and NK cells, navigates via microtubules to the apical side of the cell, which is towards the lymphocyte-target cell synapse. The protein content of this granule is disclosed in the art, and can be investigated by isolating these granules by methods known in the art. In some embodiments, the lymphocyte lytic granule- secreted protein is any protein secreted from a lytic granule. In some embodiments, the lymphocyte lytic granule- secreted protein is any protein in a lytic granule. Proteins found in lytic granules are well known in the art and can be found for example in “Supramolecular attack particles are autonomous killing entities released from cytotoxic T cells”, Balint et al., Science, 2020 May 22;368(6493):897-901, herein incorporated by reference in its entirety. In some embodiments, the lymphocyte lytic granule-secreted protein is provided in Table 1. In some embodiments, the lymphocyte lytic granule-secreted protein is selected from the proteins provided in Table 1. In some embodiments, the lymphocyte lytic granule- secreted protein is selected from the group consisting of: granzyme A, granzyme B, granzyme H, granzyme K, granzyme M, Granulysin, Serglycin, and Perforin. In some embodiments, the lymphocyte lytic granule- secreted protein is selected from the group consisting of: granzyme A, granzyme B, granzyme H, granzyme K, granzyme M, and Perforin. In some embodiments, the lymphocyte lytic granule-secreted protein is a granzyme. In some embodiments, a granzyme is selected from granzyme A, granzyme B, granzyme H, granzyme K, and granzyme M. In some embodiments, a granzyme is granzyme B. In some embodiments, the lymphocyte lytic granule-secreted protein is perforin. In some embodiments, the lymphocyte

lytic granule- secreted protein is a human protein. In some embodiments, the lymphocyte
lytic granule- secreted protein is a mammalian protein.

[0133] Table 1: Lytic granule protein list

Gene name	Accession	Gene name	Accession
Keratin, type II cytoskeletal 1	P04264	T-complex protein 1 subunit alpha TCP1	P17987
Keratin, type I cytoskeletal 9	P35527	Skin-specific protein 32 XP32	Q5T750
Serum albumin ALB	P02768	Mucin-like protein 1 MUCL1	Q96DR8
Keratin, type I cytoskeletal 10	P13645; Q7Z3Y7; Q2M2I5; Q7Z3Y8; Q7Z3Z0; Q99456; O76013; Q7Z3Y9	60S ribosomal protein L23a RPL23A	P62750
Keratin, type II cytoskeletal 2 epidermal 2	P35908	Fatty acid synthase FASN	P49327
Complement C3	P01024	Phospholipase B-like 1 PLBD1	Q6P4A8
Hemoglobin subunit alpha HBA1	P69905; P02008	Acid ceramidase ASAH1	Q13510
Desmoplakin DSP	P15924	T-complex protein 1 subunit epsilon CCT5	P48643
Desmoglein-1 DSG1	Q02413	Serine/threonine-protein kinase ULK2 ULK2	Q8IYT8
Keratin, type II cytoskeletal 5	P13647	Isocitrate dehydrogenase [NADP], mitochondrial IDH2	P48735
Alpha-enolase ENO1	P06733; P13929	Lupus La protein SSB	P05455
Apolipoprotein B-100 APOB	P04114	Spectrin alpha chain, non-erythrocytic 1 SPTAN1	Q13813
Keratin, type I cytoskeletal 16	P08779; P35900	Elongation factor Tu, mitochondrial TUFM	P49411
Hornerin HRNR	Q86YZ3	Macrophage migration inhibitory factor MIF	P14174
Vimentin VIM	P08670; P17661; P41219; P07196	Nucleosome assembly protein 1-like 1 NAP1L1	P55209
Pyruvate kinase PKM PKM	P14618; P30613	D-3-phosphoglycerate dehydrogenase PHGDH	O43175

Histone H4 HIST1H4A	P62805	Inorganic pyrophosphatase PPA1	Q15181
Immunoglobulin mu heavy chain	P0DOX6	Carboxypeptidase A4 CPA4	Q9UI42
Glyceraldehyde-3-phosphate dehydrogenase GAPDH	P04406; O14556	Zymogen granule protein 16 homolog B ZG16B	Q96DA0
Junction plakoglobin JUP	P14923; P35222	Protein CREG1 CREG1	O75629
Inter-alpha-trypsin inhibitor heavy chain H2 ITIH2	P19823	Histidine ammonia-lyase HAL	P42357
Alpha-2-macroglobulin A2M	P01023	Loricrin LOR	P23490
Actin, cytoplasmic 1 ACTB	P60709; Q6S8J3; A5A3E0; P0CG38; P0CG39; Q9BYX7	Programmed cell death protein 5 PDCD5	O14737
Keratin, type II cytoskeletal 78	Q8N1N4	Calmodulin-like protein 5 CALML5	Q9NZT1
60 kDa heat shock protein, mitochondrial HSPD1	P10809	60S ribosomal protein L15 RPL15	P61313
Keratin, type I cytoskeletal 14	P02533	Polyadenylate-binding protein 1-like PABPC1L	Q4VXU2
Annexin A2 ANXA2	P07355; A6NMY6	Prohibitin-2 PHB2	Q99623
Peptidyl-prolyl cis-trans isomerase A PPIA	P62937; Q9Y536; A0A075B759	Ezrin EZR	P15311; Q5TZA2
Putative elongation factor 1-alpha-like 3 EEF1A1P5	Q5VTE0; Q05639	Extracellular matrix protein 1 ECM1	Q16610
Phosphoglycerate kinase 1 PGK1	P00558; P07205	Cystatin-A CSTA	P01040
Complement C4-A C4A	P0C0L4; P0C0L5	Protein S100-A16 S100A16	Q96FQ6
Dermcidin DCD	P81605	60S ribosomal protein L31 RPL31	P62899
Immunoglobulin kappa constant IGKC	P01834; P0DOX7	Heterogeneous nuclear ribonucleoprotein U-like protein 2 HNRNPUL2	Q1KMD3
Alpha-2-HS-glycoprotein AHSG	P02765	60S ribosomal protein L6 RPL6	Q02878
Histone H1.4 HIST1H1E	P10412; P16402; Q02539; P22492	Nascent polypeptide-associated complex subunit alpha, muscle-specific form NACA	E9PAV3; Q9BZK3

Inter-alpha-trypsin inhibitor heavy chain H3 ITIH3	Q06033	Proteasome subunit beta type-3 PSMB3	P49720
Keratin, type I cytoskeletal 17	Q04695	Gamma-glutamylcyclotransferase GGCT	O75223
Apolipoprotein A-I APOA1	P02647	Transaldolase TALDO1	P37837
Gelsolin GSN	P06396	Protein POF1B POF1B	Q8WVV4
Profilin-1 PFN1	P07737	Protein S100-A6 S100A6	P06703
Lipoprotein lipase LPL	P06858; Q9Y5X9	Hepatocyte growth factor HGF	P14210
Peroxiredoxin-1 PRDX1	Q06830; Q13162	Plasma protease C1 inhibitor SERPING1	P05155
Plastin-2 LCP1	P13796; P13797	Small nuclear ribonucleoprotein Sm D1 SNRPD1	P62314
Filaggrin-2 FLG2	Q5D862	40S ribosomal protein S24 RPS24	P62847
Prolactin-inducible protein PIP	P12273	C-C motif chemokine 28 CCL28	Q9NRJ3
Fructose-bisphosphate aldolase A ALDOA	P04075; P05062	F-actin-capping protein subunit beta CAPZB	P47756
L-lactate dehydrogenase A chain LDHA	P00338; P07864	Stress-induced-phosphoprotein 1 STIP1	P31948
Desmocollin-1 DSC1	Q08554	Chymotrypsin-like elastase family member 3B CELA3B	P08861
Elongation factor 2 EEF2	P13639	Keratin, type II cytoskeletal 7 7	P08729
Fibrillin-1 FBN1	P35555	Transmembrane protein 109 TMEM109	Q9BVC6
Fibronectin FN1	P02751	Collagen alpha-2(IV) chain COL4A2	P08572
Keratin, type II cytoskeletal 1b	Q7Z794	60S ribosomal protein L7a RPL7A	P62424
Heat shock cognate 71 kDa protein HSPA8	P11142; P54652; P48741	C-1-tetrahydrofolate synthase, cytoplasmic MTHFD1	P11586
Triosephosphate isomerase TPI1	P60174	Cysteine-rich secretory protein LCCL domain-containing 2 CRISPLD2	Q9H0B8
Myosin-9 MYH9	P35579; P35749; P35580; A7E2Y1	Transcription factor BTF3 BTF3	P20290
1-acyl-sn-glycerol-3-phosphate acyltransferase alpha AGPAT1	Q99943	T-complex protein 1 subunit zeta CCT6A	P40227

Protein-glutamine gamma-glutamyltransferase E TGM3	Q08188	40S ribosomal protein S18 RPS18	P62269
Arginase-1 ARG1	P05089	60S ribosomal protein L29 RPL29	P47914
78 kDa glucose-regulated protein HSPA5	P11021	Putative lipocalin 1-like protein 1 LCN1P1	Q5VSP4
Carboxypeptidase A1 CPA1	P15085	Matrix Gla protein MGP	P08493
Protein S100-A9 S100A9	P06702	Elongation factor 1-gamma EEF1G	P26641
Hemoglobin subunit beta HBB	P68871; P02042	Immunoglobulin kappa variable 3D-11 IGKV3D-11	A0A0A0 MRZ8
Tubulin beta chain TUBB	P07437; Q13509; A6NNZ2; Q9H4B7	Histone H2A type 1-B/E HIST1H2AB	P04908
ATP synthase subunit beta, mitochondrial ATP5B	P06576	60S ribosomal protein L30 RPL30	P62888
Keratin, type II cytoskeletal 80	Q6KB66	Serine hydroxymethyltransferase, mitochondrial SHMT2	P34897
Fibrinogen beta chain FGB	P02675	Proteasome subunit alpha type-6 PSMA6	P60900
40S ribosomal protein SA RPSA	P08865	U2 small nuclear ribonucleoprotein B" SNRPB2	P08579
Protein disulfide-isomerase P4HB	P07237	Purine nucleoside phosphorylase PNP	P00491
Hypoxia-inducible factor 1-alpha HIF1A	Q16665	Sex hormone-binding globulin SHBG	P04278
Histone H1.5 HIST1H1B	P16401	High mobility group protein B1 HMGB1	P09429
ATP synthase subunit alpha, mitochondrial ATP5A1	P25705	Histidine-rich glycoprotein HRG	P04196
Histone H3.2 HIST2H3A	Q71DI3; Q6NXT2	RNA binding motif protein, X-linked-like-1 RBMXL1	Q96E39; O75526
Apolipoprotein E APOE	P02649	Ras-related protein Rab-1B RAB1B	Q9H0U4
Keratin, type I cytoskeletal 18	P05783	40S ribosomal protein S3 RPS3	P23396
Fibrinogen gamma chain FGG	P02679	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial ECH1	Q13011
Peroxiredoxin-2 PRDX2	P32119	Glia maturation factor gamma GMFG	O60234
Serpin B3 SERPINB3	P29508; P48594	Rho GDP-dissociation inhibitor 2 ARHGDIB	P52566

Stress-70 protein, mitochondrial HSPA9	P38646	Vitamin K-dependent protein S PROS1	P07225
40S ribosomal protein S19 RPS19	P39019	Complement C1s subcomponent C1S	P09871
Voltage-dependent anion-selective channel protein 1 VDAC1	P21796; Q9Y277	Serine/threonine-protein kinase N3 PKN3	Q6P5Z2
Immunoglobulin lambda constant 2 IGLC2	P0DOY2; A0M8Q6	Collagen alpha-1(I) chain COL1A1	P02452
Inter-alpha-trypsin inhibitor heavy chain H4 ITIH4	Q14624	Protein SETSIP SETSIP	P0DME0
Inter-alpha-trypsin inhibitor heavy chain H1 ITIH1	P19827	Afamin AFM	P43652
Trypsin-1 PRSS1	P07477; Q8NHM4	Anoctamin-9 ANO9	A1A5B4
Caspase-14 CASP14	P31944	Calpain small subunit 1 CAPNS1	P04632; Q96L46
Pigment epithelium-derived factor SERPINF1	P36955	Lamin-B1 LMNB1	P20700
Apolipoprotein C-III APOC3	P02656	Stathmin STMN1	P16949
Apolipoprotein C-II APOC2	P02655	Rab GDP dissociation inhibitor beta GDI2	P50395
Hemopexin HPX	P02790	Parathyroid hormone-related protein PTHLH	P12272
Ubiquitin-40S ribosomal protein S27a RPS27A	P62979	Proteasome subunit alpha type-7 PSMA7	O14818; Q8TAA3
Heat shock protein HSP 90-beta HSP90AB1	P08238; Q58FF7; Q58FF8; Q58FG1; Q12931	Immunoglobulin J chain JCHAIN	P01591
Galectin-1 LGALS1	P09382	Ras-related protein Rab-40C RAB40C	Q96S21
Vitronectin VTN	P04004	Small nuclear ribonucleoprotein Sm D2 SNRPD2	P62316
14-3-3 protein zeta/delta YWHAZ	P63104; P31947	NFX1-type zinc finger-containing protein 1 ZNFX1	Q9P2E3
Cofilin-1 CFL1	P23528; Q9Y281	Calmodulin-1 CALM1	P0DP23
Catalase CAT	P04040	Poly(rC)-binding protein 2 PCBP2	Q15366; P57721
Proliferating cell nuclear antigen PCNA	P12004	14-3-3 protein epsilon YWHAE	P62258
Serpin B12 SERPINB12	Q96P63	Protein disulfide-isomerase A3 PDIA3	P30101

Histone H2B type 1-K HIST1H2BK	O60814	Clathrin heavy chain 1 CLTC	Q00610; P53675
Alpha-1-antitrypsin SERPINA1	P01009	Vitamin D-binding protein GC	P02774
Signal recognition particle 14 kDa protein SRP14	P37108	Proteasome subunit alpha type-5 PSMA5	P28066
Fibrinogen alpha chain FGA	P02671	60S ribosomal protein L7 RPL7	P18124
Collagen alpha-2(VI) chain COL6A2	P12110	Adenylyl cyclase-associated protein 1 CAP1	Q01518; P40123
T-complex protein 1 subunit theta CCT8	P50990	Keratinocyte proline-rich protein KPRP	Q5T749
Endoplasmin HSP90B1	P14625	X-ray repair cross- complementing protein 6 XRCC6	P12956
10 kDa heat shock protein, mitochondrial HSPE1	P61604	C-C motif chemokine 5 CCL5	P13501
Tropomyosin alpha-3 chain TPM3	P06753; P09493	Eukaryotic initiation factor 4A-I EIF4A1	P60842; Q14240; P38919
60S acidic ribosomal protein P0 RPLP0	P05388; Q8NHW5	Eukaryotic translation initiation factor 5B EIF5B	O60841
Nuclease-sensitive element- binding protein 1 YBX1	P67809; P16989	Keratin, type II cytoskeletal 75	O95678
Annexin A6 ANXA6	P08133	Heat shock 70 kDa protein 1A HSPA1A	P0DMV8
Transgelin-2 TAGLN2	P37802	Haptoglobin-related protein HPR	P00739
Immunoglobulin heavy constant alpha 1 IGHA1	P01876; P01877	Tubulin beta-4A chain TUBB4A	P04350
Heterogeneous nuclear ribonucleoproteins A2/B1 HNRNPA2B1	P22626	HLA class I histocompatibility antigen, A-3 alpha chain HLA-A	P04439; P01893
L-lactate dehydrogenase B chain LDHB	P07195	Histone H2B type 1-J HIST1H2BJ	P06899; Q8N257; Q96A08
Antithrombin-III SERPINC1	P01008	Keratin, type I cytoskeletal 19	P08727
Coronin-1A CORO1A	P31146	Gamma-enolase ENO2	P09104
Thrombospondin-1 THBS1	P07996	Keratin, type II cytoskeletal 3	P12035
Alpha-fetoprotein AFP	P02771	Neurofilament heavy polypeptide NEFH	P12036
Protein-glutamine gamma- glutamyltransferase K TGM1	P22735	ADP/ATP translocase 3 SLC25A6	P12236
Corneodesmosin CDSN	Q15517	Heat shock 70 kDa protein 6 HSPA6	P17066

Serum amyloid P-component APCS	P02743	Keratin, type I cytoskeletal 15	P19012
Fatty acid-binding protein, epidermal FABP5	Q01469	Keratin, type II cytoskeletal 4	P19013
Keratin, type II cytoskeletal 6A	P02538	Heat shock 70 kDa protein 1-like HSPA1L	P34931
Small nuclear ribonucleoprotein Sm D3 SNRPD3	P62318	Radixin RDX	P35241
Immunoglobulin kappa variable 2-28 IGKV2-28	A0A075B6P5	Keratin, type II cytoskeletal 6C	P48668
Malate dehydrogenase, mitochondrial MDH2	P40926	Thrombospondin-3 THBS3	P49746
Immunoglobulin kappa variable 3-20 IGKV3-20	P01619; A0A0C4DH25	Tubulin alpha-1B chain TUBA1B	P68363; Q9BQE3; Q9NY65; A6NHL2; Q9H853
Keratin, type II cytoskeletal 6B	P04259	Keratin, type II cytoskeletal 71	Q3SY84
Thioredoxin-dependent peroxide reductase, mitochondrial PRDX3	P30048	Keratin, type II cytoskeletal 79	Q5XKE5
U6 snRNA-associated Sm-like protein LSm4 LSM4	Q9Y4Z0	Putative histone H2B type 2-C HIST2H2BC	Q6DN03
Protein S100-A8 S100A8	P05109	Keratin, type II cytoskeletal 74	Q7RTS7
Nucleophosmin NPM1	P06748	Myosin-14 MYH14	Q7Z406
Alpha-2-antiplasmin SERPINF2	P08697	WD repeat-containing protein 36 WDR36	Q8NI36
Moesin MSN	P26038	Tubulin beta-6 chain TUBB6	Q9BUF5
Immunoglobulin heavy constant gamma 3 IGHG3	P01860	Nascent polypeptide-associated complex subunit alpha-2 NACA2	Q9H009
Collagen alpha-1(VI) chain COL6A1	P12109	Adipocyte plasma membrane-associated protein APMAP	Q9HDC9
Annexin A1 ANXA1	P04083	Keratin, type II cuticular Hb4 84	Q9NSB2; Q43790
Fibulin-1 FBLN1	P23142	Keratin, type II cuticular Hb2 82	Q9NSB4
Ras-related C3 botulinum toxin substrate 2 RAC2	P15153; P60763	Serpin B13 SERPINB13	Q9UIV8
Desmocollin-3 DSC3	Q14574	Keratin, type II cytoskeletal 72	Q14CN4
Keratin, type I cytoskeletal 13	P13646	Putative heat shock protein HSP 90-beta 4 HSP90AB4P	Q58FF6

Plakophilin-1 PKP1	Q13835	Keratin, type II cytoskeletal 73	Q86Y46
40S ribosomal protein S8 RPS8	P62241	Beta-actin-like protein 2 ACTBL2	Q562R1
Tubulin beta-4B chain TUBB4B	P68371	Keratin, type II cytoskeletal 2 oral 76	Q01546
C4b-binding protein alpha chain C4BPA	P04003	Collagen alpha-3(IV) chain COL4A3	Q01955
Protein disulfide-isomerase A6 PDIA6	Q15084	Secretory phospholipase A2 receptor PLA2R1	Q13018
Coactosin-like protein COTL1	Q14019	Tubulin beta-2A chain TUBB2A	Q13885
Trypsin-2 PRSS2	P07478	T-complex protein 1 subunit zeta-2 CCT6B	Q92526
60S ribosomal protein L12 RPL12	P30050	HLA class I histocompatibility antigen, A-2 alpha chain HLA-A	P01892; P01891; P10321
Cell division control protein 42 homolog CDC42	P60953	Histone H2A.Z H2AFZ	P0C0S5
Immunoglobulin gamma-1 heavy chain	P0DOX5	Coagulation factor IX F9	P00740
Serine/arginine-rich splicing factor 2 SRSF2	Q01130; Q9BRL6	Hexokinase-1 HK1	P19367
Pregnancy zone protein PZP	P20742	Bromodomain and WD repeat-containing protein 3 BRWD3	Q6RI45
Lactotransferrin LTF	P02788	40S ribosomal protein S9 RPS9	P46781
Thyroxine-binding globulin SERPINA7	P05543	BPI fold-containing family B member 1 BPIFB1	Q8TDL5
F-actin-capping protein subunit alpha-2 CAPZA2	P47755	Peptidyl-prolyl cis-trans isomerase B PPIB	P23284
40S ribosomal protein S28 RPS28	P62857	Nucleoprotein TPR TPR	P12270
Actin-related protein 2/3 complex subunit 4 ARPC4	P59998	LIM and SH3 domain protein 1 LASP1	Q14847
Immunoglobulin heavy variable 3-74 IGHV3-74	A0A0B4J1X5	Small ubiquitin-related modifier 4 SUMO4	Q6EEV6
40S ribosomal protein S10 RPS10	P46783; Q9NQ39;	Heterogeneous nuclear ribonucleoprotein C-like 2 HNRNPCL2	B2RXH8
T-complex protein 1 subunit beta CCT2	P78371	Protein Z-dependent protease inhibitor SERPINA10	Q9UK55
Annexin A5 ANXA5	P08758	Ubiquitin-conjugating enzyme E2 D3 UBE2D3	P61077
Complement factor H CFH	P08603	Granulysin GNLY	P22749

Immunoglobulin kappa variable 3D-7 IGKV3D-7	A0A0C4DH55	Septin-7 SEPT7	Q16181
Lumican LUM	P51884	Heterogeneous nuclear ribonucleoprotein D-like HNRNPDL	O14979
Immunoglobulin lambda-1 light chain	P0DOX8	Tetranectin CLEC3B	P05452
Calreticulin CALR	P27797	Proline-rich protein 4 PRR4	Q16378
Phosphoglycerate mutase 2 PGAM2	P15259; Q8N0Y7	Annexin A4 ANXA4	P09525
Serotransferrin TF	P02787	Coagulation factor V F5	P12259
14-3-3 protein beta/alpha YWHAB	P31946; P61981	ATP-dependent 6-phosphofructokinase, platelet type PFKP	Q01813
Protein S100-A11 S100A11	P31949	Costars family protein ABRACL ABRACL	Q9P1F3
Beta-2-glycoprotein 1 APOH	P02749	Leukocyte elastase inhibitor SERPINB1	P30740
Heterogeneous nuclear ribonucleoprotein K HNRNPK	P61978	Neutral alpha-glucosidase AB GANAB	Q14697
Collagen alpha-3(VI) chain COL6A3	P12111	Complement C5 C5	P01031
Transketolase TKT	P29401	Actin-related protein 2/3 complex subunit 2 ARPC2	O15144
Collagen alpha-5(IV) chain COL4A5	P29400	Plastin-1 PLS1	Q14651
Immunoglobulin heavy constant gamma 2 IGHG2	P01859; P01861	Putative RNA-binding protein Luc7-like 2 LUC7L2	Q9Y383
GTP-binding nuclear protein Ran RAN	P62826	Inosine-5'-monophosphate dehydrogenase 2 IMPDH2	P12268
Nucleolin NCL	P19338	Core histone macro-H2A.1 H2AFY	O75367
Plasminogen PLG	P00747; Q02325	60S ribosomal protein L3 RPL3	P39023
Cathepsin D CTSD	P07339	Vascular cell adhesion protein 1 VCAM1	P19320
Ras-related protein Rap-1b RAP1B	P61224; A6NIZ1; P62834	PRA1 family protein 3 ARL6IP5	O75915
Alpha-actinin-4 ACTN4	O43707; P12814; P35609; Q9H254	Lymphocyte-specific protein 1 LSP1	P33241
Talin-1 TLN1	Q9Y490; Q9Y4G6	Polypyrimidine tract-binding protein 1 PTBP1	P26599

Serpin A12 SERPINA12	Q8IW75	Glucose-6-phosphate 1-dehydrogenase G6PD	P11413
Tropomyosin beta chain TPM2	P07951	Neuroblast differentiation-associated protein AHNAK AHNAK	Q09666
Prelamin-A/C LMNA	P02545	Apolipoprotein A-IV APOA4	P06727
Filaggrin FLG	P20930	Thymosin beta-4 TMSB4X	P62328
Protein S100-A14 S100A14	Q9HCY8	Apolipoprotein M APOM	O95445
Tryptophan--tRNA ligase, cytoplasmic WARS	P23381	Transitional endoplasmic reticulum ATPase VCP	P55072
DNA replication licensing factor MCM2 MCM2	P49736	Coagulation factor X F10	P00742
4F2 cell-surface antigen heavy chain SLC3A2	P08195	Complement component 1 Q subcomponent-binding protein, mitochondrial C1QBP	Q07021
Carboxypeptidase B CPB1	P15086	Rho-related GTP-binding protein RhoC RHOC	P08134
Heat shock protein beta-1 HSPB1	P04792	Glutathione S-transferase omega-1 GSTO1	P78417
Actin-related protein 2/3 complex subunit 5-like protein ARPC5L	Q9BPX5	Acidic leucine-rich nuclear phosphoprotein 32 family member B ANP32B	Q92688
60S acidic ribosomal protein P2 RPLP2	P05387	3-hydroxyacyl-CoA dehydrogenase type-2 HSD17B10	Q99714
T-complex protein 1 subunit gamma CCT3	P49368	Replication protein A 70 kDa DNA-binding subunit RPA1	P27694
Putative small nuclear ribonucleoprotein G-like protein 15 SNRPGP15	A8MWD9	40S ribosomal protein S7 RPS7	P62081
Multifunctional protein ADE2 PAICS	P22234	Nuclear autoantigenic sperm protein NASP	P49321
Elongation factor 1-delta EEF1D	P29692	Hepatocyte growth factor activator HGFAC	Q04756
Bleomycin hydrolase BLMH	Q13867	60S ribosomal protein L24 RPL24	P83731
Nucleoside diphosphate kinase B NME2	P22392	Mitochondrial import receptor subunit TOM7 homolog TOMM7	Q9P0U1
Actin-related protein 3 ACTR3	P61158	Ubiquitin-like modifier-activating enzyme 1 UBA1	P22314
Voltage-dependent anion-selective channel protein 2 VDAC2	P45880	Complement component C9 C9	P02748

Small nuclear ribonucleoprotein E SNRPE	P62304	Putative 60S ribosomal protein L13a protein RPL13AP3 RPL13AP3	Q6NVV1
40S ribosomal protein S14 RPS14	P62263	60S ribosomal protein L11 RPL11	P62913
Small nuclear ribonucleoprotein-associated proteins B and B' SNRPB	P14678	Cytochrome c oxidase subunit 2 MT-CO2	P00403
Bifunctional purine biosynthesis protein PURHATIC	P31939	Tropomodulin-2 TMOD2	Q9NZR1
Serine/arginine-rich splicing factor 1 SRSF1	Q07955	Fructose-bisphosphate aldolase C ALDOC	P09972
Keratin, type II cytoskeletal 8	P05787; P14136	Tubulin alpha-1A chain TUBA1A	Q71U36; Q13748; Q6PEY2
Signal transducer and activator of transcription 1-alpha/beta STAT1	P42224	ATP synthase subunit f, mitochondrial ATP5J2	P56134
Retroviral-like aspartic protease 1 ASPRV1	Q53RT3	Heterogeneous nuclear ribonucleoprotein A/B HNRNPAB	Q99729
40S ribosomal protein S23 RPS23	P62266	HLA class II histocompatibility antigen, DR alpha chain HLA-DRA	P01903
Heterogeneous nuclear ribonucleoproteins C1/C2 HNRNPC	P07910	Mucin-5B MUC5B	Q9HC84
Histone H2A type 1 HIST1H2AG	P0C0S8; P16104; Q8IUE6; Q96QV6	WD repeat-containing protein 1 WDR1	O75083
Guanine nucleotide-binding protein G(i) subunit alpha-2 GNAI2	P04899; A8MTJ3	Perforin-1 PRF1	P14222
SH3 domain-binding glutamic acid-rich-like protein 3 SH3BGRL3	Q9H299	Granzyme M GZMM	P51124
Collagen alpha-2(I) chain COL1A2	P08123	Activated RNA polymerase II transcriptional coactivator p15 SUB1	P53999
Receptor-type tyrosine-protein phosphatase C PTPRC	P08575	Ras-related protein Rab-11B RAB11B	Q15907
Heterogeneous nuclear ribonucleoprotein U HNRNPU	Q00839	Integrin alpha-L ITGAL	P20701

Poly(rC)-binding protein 1 PCBP1	Q15365	High mobility group protein B2 HMGB2	P26583
Immunoglobulin lambda variable 1-47 IGLV1-47	P01700	HLA class I histocompatibility antigen, B-7 alpha chain HLA-B	P01889; P30486
Copper-transporting ATPase 2 ATP7B	P35670	Heterogeneous nuclear ribonucleoprotein H HNRNPH1	P31943; P55795
40S ribosomal protein S2 RPS2	P15880	Peroxiredoxin-6 PRDX6	P30041
Heterogeneous nuclear ribonucleoprotein A1 HNRNPA1	P09651; Q32P51	60S ribosomal protein L18 RPL18	Q07020
14-3-3 protein theta YWHAQ	P27348	Protein/nucleic acid deglycase DJ-1 PARK7	Q99497
Eukaryotic translation initiation factor 5A-1-like EIF5AL1	Q6IS14	Adenosylhomocysteinase AHCY	P23526
Tubulin alpha-4A chain TUBA4A	P68366	40S ribosomal protein S25 RPS25	P62851
Actin, aortic smooth muscle ACTA2	P62736	Tektin-1 TEK1	Q969V4
Lactadherin MFGE8	Q08431	Putative ubiquitin-conjugating enzyme E2 N-like UBE2NL	Q5JXB2
60S ribosomal protein L14 RPL14	P50914	Acidic leucine-rich nuclear phosphoprotein 32 family member A ANP32A	P39687
Actin-related protein 2/3 complex subunit 5 ARPC5	O15511	Pantetheinase VNN1	O95497
60S ribosomal protein L22 RPL22	P35268	60S ribosomal protein L27 RPL27	P61353
Thioredoxin TXN	P10599	Proteasome activator complex subunit 1 PSME1	Q06323
Immunoglobulin lambda variable 3-9 IGLV3-9	A0A075B6K5	14-3-3 protein eta YWHAH	Q04917
Myosin regulatory light chain 12B MYL12B	O14950; P24844	Rho GDP-dissociation inhibitor 1 ARHGDI1	P52565
Myosin light polypeptide 6 MYL6	P60660; P14649	Heterogeneous nuclear ribonucleoprotein F HNRNPF	P52597
Galectin-7 LGALS7	P47929	T-complex protein 1 subunit eta CCT7	Q99832
ADP/ATP translocase 2 SLC25A5	P05141	Heterogeneous nuclear ribonucleoprotein A3 HNRNPA3	P51991
Haptoglobin HP	P00738	Serine/arginine-rich splicing factor 7 SRSF7	Q16629
Lysozyme C LYZ	P61626	ATP-dependent RNA helicase DDX39A DDX39A	O00148

Macrophage-capping protein CAPG	P40121	Transformer-2 protein homolog beta TRA2B	P62995
Glutathione S-transferase P GSTP1	P09211	Cartilage oligomeric matrix protein COMP	P49747
Glucose-6-phosphate isomerase GPI	P06744	Thrombospondin-4 THBS4	P35443
Chloride intracellular channel protein 1 CLIC1	O00299	Cytokine SCM-1 beta XCL2	Q9UBD3
Heat shock protein HSP 90- alpha HSP90AA1	P07900; Q14568; Q58FG0	Actin-related protein 2/3 complex subunit 1B ARPC1B	O15143
Prothrombin F2	P00734	Granzyme B GZMB	P10144; P20718
RuvB-like 2 RUVBL2	Q9Y230	Interferon gamma IFNG	P01579
Eukaryotic translation initiation factor 2 subunit 1 EIF2S1	P05198	Granzyme A GZMA	P12544
Heterogeneous nuclear ribonucleoprotein R HNRNPR	O43390	Filamin-A FLNA	P21333; O75369

[0134] In some embodiments, human Granzyme B protein comprises or consists of the amino acid sequence provided in accession number NP_004122 or NP_001332940. In some embodiments, Granzyme B is human Granzyme B (GZMB). In some embodiments, Granzyme B comprises the amino acid sequence MQPILLLLAFLLLPRADAGEIIGGHEAKPHSRPYMAYLMIWDQKSLKRCGGFLIRD DFVLTAAHCWGSSINVTLGAHNIKEQEPTQQFIPVKRPIHPAYNPKNFSNDIMLLQ LERKAKRTRAVQPLRLPSNKAQVKPGQTCSVAGWGQTAPLGKHSHTLQEVKMTV QEDRKCESDLRHYYDSTIELCVGDPEIKKTSFKGDSGGPLVCNKVAQGIVSYGRNN GMPPRACTKVSSFVHWIKKTMKRY (SEQ ID NO: 8). In some embodiments, human Granzyme B consists of SEQ ID NO: 8. In some embodiments, mouse Granzyme B protein comprises or consists of the amino acid sequence provided in accession number NP_038570. In some embodiments, mouse Granzyme B comprises the amino acid sequence MKILLLLLTLASRTKAGEIIGGHEVKPHSRPYMALLSIKDQQPEAICGGFLIREDF VLTAAHCEGSIINVTLGAHNIKEQEKTQQVIPMVKCIPHPDYNPKTFSNDIMLLKLK SKAKRTRAVRPLNLPRRNVNVKPGDVCYVAGWGRMAPMGKYSNTLQEVELTVQ KDRECESYFKNRYNKTNQICAGDPKTKRASFRGDSGGPLVCKKVAAGIVSYGYK DGSPPRAF TKVSSFLSWIKKTMKSS (SEQ ID NO: 16). In some embodiments, mouse Granzyme B consists of SEQ ID NO: 16.

[0135] In some embodiments, the lymphocyte lytic granule-secreted protein comprises a signal peptide. In some embodiments, the signal peptide is an endoplasmic reticulum signal peptide (ERSP). In some embodiments, the signal peptide is an endoplasmic reticulum signal sequence (ERSS). In some embodiments, the signal peptide is amino acids 1-18 of SEQ ID NO: 8. In some embodiments, the signal peptide amino acids 1-18 of SEQ ID NO: 16. In some embodiments, the signal peptide comprises amino acids 1-18 of SEQ ID NO: 16. In some embodiments, the signal peptide consists of amino acids 1-18 of SEQ ID NO: 16. In some embodiments, the lymphocyte lytic granule-secreted protein is devoid of a signal peptide. In some embodiments, the granzyme is devoid of a signal peptide. In some embodiments, human Granzyme B is devoid of a signal peptide and comprises or consists of amino acids 19-247 of SEQ ID NO: 8. In some embodiments, a protein devoid of a signal peptide still retains an N-terminal methionine. In some embodiments, human Granzyme B is devoid of a signal peptide and comprises or consists of amino acids 1 and 19-247. In some embodiments, mouse Granzyme B is devoid of the signal peptide and comprises or consists of amino acids 19-247 of SEQ ID NO: 16. In some embodiments, mouse Granzyme B is devoid of the signal peptide and comprises or consists of amino acids 1 and 19-247 of SEQ ID NO: 16. In some embodiments, the granzyme is pro-granzyme. In some embodiments, pro-granzyme comprises an inhibitory dipeptide. In some embodiments, the dipeptide is an N-terminal di-peptide. In some embodiments, the dipeptide is GE. In some embodiments, the dipeptide is amino acids 19-20 of SEQ ID NO: 8. In some embodiments, the dipeptide is amino acids 19-20 of SEQ ID NO: 16. In some embodiments, the Granzyme B is devoid of the inhibitory dipeptide. In some embodiments, human Granzyme B is devoid of the dipeptide and comprises or consists of amino acids 21-247 of SEQ ID NO: 8. In some embodiments, mouse Granzyme B is devoid of the dipeptide and comprises or consists of amino acids 21-247 of SEQ ID NO: 16.

[0136] In some embodiments, the chimeric polypeptide comprises a fragment of the lymphocyte lytic granule-secreted protein. In some embodiments, the fragment is a functional fragment. In some embodiments, the function is entry into the ER. In some embodiments, the function is entry into lytic vesicles. In some embodiments, the function is secretion. In some embodiments, the secretion is secretion into an immune synapse. In some embodiments, the function is entry into lytic granules. In some embodiments, the function is

inclusion of the chimeric molecule or fragments thereof in lytic granules. In some embodiments, the function is inclusion of the molecule of interest in lytic granules. In some embodiments, the function is inclusion of the protein of interest in lytic granules. In some embodiments, the function is directing the chimeric molecule of the invention into lytic granules. In some embodiments, the function is directing the molecule of interest into lytic granules. In some embodiments, the function is secretion in lytic vesicles. In some embodiments, the function is secretion from lytic vesicles. In some embodiments, the function is not cytotoxicity. In some embodiments, the function is delivery into target cells. In some embodiments, the fragment is a fragment capable of delivering the chimeric polypeptide to target cells. In some embodiments, the fragment is a fragment capable of delivering the chimeric polypeptide to lytic granules. In some embodiments, the fragment is a fragment capable of facilitating inclusion of the chimeric polypeptide and/or the protein of interest in lytic granules. In some embodiments, the fragment is a fragment capable of delivering the chimeric polypeptide to the ER. In some embodiments, the fragment is a non-cytotoxic fragment. In some embodiments, the fragment is a fragment lacking lytic activity. In some embodiments, the fragment lacks the enzymatic domain. In some embodiments, the enzymatic activity is protease activity.

[0137] In some embodiments, the fusion protein is naturally trafficked to lytic granules of the modified lymphocytes by the granzyme signaling elements and released upon target recognition to a target cell, through the granzyme pathway. In some embodiments, the fusion protein comprising a lymphocyte lytic granule-targeting moiety/lymphocyte lytic granule-secreted protein/fragment (e.g., granzyme B) and a protein of interest (e.g., CAS nuclease) is naturally trafficked to lytic granules of the modified cells by the granzyme signaling elements, and released upon target recognition to a target cell, through the granzyme pathway. Upon initiation of translation of the fusion protein, comprising, for example, granzyme B and CAS nuclease, the ER signal peptide directs the fusion protein to the ER, where it is co-translationally inserted. As the fusion protein is synthesized in the ER, an N-glycan is added, which targets the translated fusion protein to the Golgi network. In the Golgi, the N-glycan is phosphorylated, and the resultant phosphosugar moiety on the fusion protein then binds to the mannose-6-phosphate receptor, thereby targeting the protein to a lytic granule, where it is sequestered until target cell recognition, and its ensuing release into

the immune synapse. The fusion protein/polypeptide may be cleaved in the lytic granule, thus separating the protein of interest from the targeting moiety, such that upon target cell recognition the stand-alone protein of interest is released into the immune synapse. Alternatively, cleavage may occur in the target cell's cytoplasm or another compartment of the target cell. DNA molecules and/or RNA molecules (e.g., sgRNAs) can also be delivered as a complex with the fusion protein or the protein of interest (e.g., an RNP) to the effector cells or the nucleic acid molecules can be directly delivered to the target cells.

[0138] In some embodiments, the fragment comprises the signal peptide. In some embodiments, the signal peptide is an endoplasmic reticulum signal peptide. In some embodiments, the fragment is an N-terminal fragment. In some embodiments, the lymphocyte lytic granule-secreted protein comprises at least one glycosylation site. In some embodiments, the lymphocyte lytic granule-secreted protein comprises a plurality of glycosylation sites. In some embodiments, the glycosylation is N-linked glycosylation. In some embodiments, the glycosylation is O-linked glycosylation. In some embodiments, a glycosylation site in human Granzyme B is at amino acid 71 of SEQ ID NO: 8. In some embodiments, a glycosylation site in mouse Granzyme B is at amino acid 71 of SEQ ID NO: 16. In some embodiments, a glycosylation site in human Granzyme B is at amino acid 104 of SEQ ID NO: 8. In some embodiments, a glycosylation site in mouse Granzyme B is at amino acid 182 of SEQ ID NO: 16. In some embodiments, the fragment comprises at least one glycosylation site. In some embodiments, the fragment comprises a plurality of glycosylation sites. In some embodiments, the fragment comprises all glycosylation sites in the lymphocyte lytic granule-secreted protein. In some embodiments, the fragment comprises at least the N-terminal 71 amino acids of human Granzyme B. In some embodiments, the fragment comprises at least the N-terminal 71 amino acids of mouse Granzyme B. In some embodiments, the fragment comprises at least the N-terminal 104 amino acids of human Granzyme B. In some embodiments, the fragment comprises at least the N-terminal 182 amino acids of mouse Granzyme B.

[0139] In some embodiments, the fragment comprises at least 10, 20, 25, 30, 40, 50, 60, 70, 75, 80, 90, 100, 110, 120, 125, 130, 140, or 150 amino acids. Each possibility represents a separate embodiment of the invention. In some embodiments, the fragment comprises at least 50 amino acids. In some embodiments, the fragment comprises at least 100 amino acids.

In some embodiments, the fragment comprises at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99 or 100% of the lymphocyte lytic granule-secreted protein. Each possibility represents a separate embodiment of the invention. In some embodiments, the fragment comprises at most 20, 25, 50, 75, 100, 125, 150, 175, 200, 225 or 250 amino acids. Each possibility represents a separate embodiment of the invention. In some embodiments, the fragment comprises at most 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99 or 100% of the lymphocyte lytic granule-secreted protein. Each possibility represents a separate embodiment of the invention.

[0140] In some embodiments, the lymphocyte lytic granule-secreted protein is a variant of the lymphocyte lytic granule-secreted protein. In some embodiments, a variant is a variant of the naturally-occurring protein. In some embodiments, the variant is a variant of Granzyme B. In some embodiments, a variant is a mutant. In some embodiment, a variant is a mutated form of the naturally-occurring protein. In some embodiments, a mutant is a naturally-occurring protein comprising at least one mutation. In some embodiments, a variant is a cleavable variant. In some embodiments, a variant is an uncleavable variant. In some embodiments, the inhibitory dipeptide of the naturally-occurring protein is uncleavable. In some embodiments, the mutation is mutation of the inhibitory dipeptide to render it uncleavable. In some embodiments, the variant is a non-cytotoxic variant. In some embodiments, the mutation reduces cytotoxicity. In some embodiments, the mutation abolishes cytotoxicity. In some embodiments, the mutant is a non-cytotoxic mutant. In some embodiments, the mutant is a non-lytic mutant. In some embodiments, non-cytotoxic is non-lytic. In some embodiments, non-lytic is non-cytotoxic. In some embodiments, the mutation reduces the enzymatic function of the protein. In some embodiments, the mutation abolishes the enzymatic function of the protein. In some embodiments, the enzymatic function is cleavage. In some embodiments, the cleavage is protein cleavage. In some embodiments, the enzymatic function is a protease function. In some embodiments, the enzymatic function induces lysis. In some embodiments, the enzymatic function induces cell death. In some embodiments, the variant is an inactive variant. In some embodiments, the variant is an inert variant. In some embodiments, the variant is a non-cytotoxic variant. In some embodiments, non-cytotoxic variant is not able to induce apoptosis in a target cell. In some embodiments, the variant is a non-lytic variant. In some embodiments, the variant is not able to induce

apoptosis in a target cell. In some embodiments, the variant is a homolog. In some embodiments, the homolog is not cytotoxic in humans. In some embodiments, the homolog has reduced cytotoxicity in humans.

[0141] In some embodiments, the lymphocyte lytic granule-secreted protein is not cytotoxic. In some embodiments, the lymphocyte lytic granule-secreted protein is not lytic. In some embodiments, the lymphocyte lytic granule-secreted protein is not enzymatically active. In some embodiments, the lymphocyte lytic granule-secreted protein is inert. In some embodiments, the lymphocyte lytic granule-secreted protein is inactivated. In some embodiments, inactivity is loss of enzymatic activity. In some embodiments, enzymatic activity is protease activity. In some embodiments, inclusion of the inhibitory dipeptide renders the granzyme inactive. In some embodiments, the inhibitory dipeptide has been mutated. In some embodiments, the mutation renders the inhibitory dipeptide uncleavable resulting in an activatable form of the granzyme. In some embodiments, the inhibitory dipeptide is a mutant uncleavable inhibitory dipeptide. In some embodiments, the mutation is a mutation of amino acid 19 of SEQ ID NO: 8. In some embodiments, the mutation is a mutation of amino acid 19 of SEQ ID NO: 16. In some embodiments, the mutation is a mutation of the first amino acid of the dipeptide. In some embodiments, the mutation is a mutation of amino acid 20 of SEQ ID NO: 8. In some embodiments, the mutation is a mutation of amino acid 20 of SEQ ID NO: 16. In some embodiments, the mutation is a mutation of the second amino acid of the dipeptide. In some embodiments, mutation is a mutation to alanine. In some embodiments, both amino acids of the dipeptide are mutated. In some embodiments, amino acids 19 and 20 of SEQ ID NO: 8 are mutated. In some embodiments, amino acids 19 and 20 of SEQ ID NO: 16 are mutated. In some embodiments, the dipeptide is mutated to AA.

[0142] In some embodiments, the lymphocyte lytic granule-secreted protein and the protein of interest are separated by a linker. In some embodiments, the lymphocyte lytic granule-secreted protein and the protein of interest are linked by a linker. In some embodiments, the lymphocyte lytic granule-secreted protein and the protein of interest are joined by a linker. In some embodiments, the linker is a peptide bond. In some embodiments, the linker is an amino acid linker. In some embodiments, the linker is a chemical linker. In some embodiments, the linker is a flexible linker. In some embodiments, the linker comprises a

plurality of amino acids. In some embodiments, the linker comprises at least, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids. Each possibility represents a separate embodiment of the invention. In some embodiments, the linker comprises at least 5 amino acids. In some embodiments, the linker comprises at most 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 amino acids. Each possibility represents a separate embodiment of the invention. In some embodiments, the linker comprises at most 25 amino acids.

[0143] In some embodiments, the linker is a glycine-serine linker. In some embodiments, the linker comprises or consists of (GS)_n, wherein n is an integer from 1-10. In some embodiments, the linker is GSGSGSGSGS (SEQ ID NO: 9). In some embodiments, the linker comprises or consists of GGGGS (SEQ ID NO: 10). In some embodiments, the linker comprises or consists of (GGGS)_n, wherein n is an integer from 1-5. In some embodiments, the linker is GGGSGGGSGGGSGGGGS (SEQ ID NO: 11). In some embodiments, the linker comprises or consists of GGS (SEQ ID NO: 43). In some embodiments, the linker comprises or consists (GGS)_n, wherein n is an integer from 1-10. In some embodiments, the linker is GSGGSGGSGGS (SEQ ID NO: 44). In some embodiments, the linker is SGFANELGPRLMGK (SEQ ID NO: 72). In some embodiments, the OLLAS linker consists of SEQ ID NO: 72.

[0144] In some embodiments, the linker is a cleavable linker. It will be understood by a skilled artisan that it may be advantageous for the protein of interest to be separated from the lymphocyte lytic granule-secreted protein to better enable the protein to function. However, such separation should only occur after the lymphocytic lytic granule-secreted protein has served its function of facilitating delivery of the protein of interest to the lytic granule and/or the target cell. It will be understood by a skilled artisan that if cleavage occurs in the lytic granule the protein of interest would still be transferred to the target cell. Upon engagement of a lymphocyte to a target cell, all the proteins present in the granule (including the protein of interest even if separate from the lymphocytic lytic granule-secreted protein) passively diffuse to the target cell by the process of lytic granule secretion. This type of protein transfer at the immune synapse is well known in the art. In some embodiments, the linker is a pH-dependent cleavable linker. In some embodiments, the linker is cleavable at acidic pH. It will be understood by a skilled artisan that the lytic granule comprises an acidic pH. A linker that cleaves at acidic pH will ensure that the lymphocyte lytic granule-secreted

protein or fragment thereof remains connected to the protein of interest, at least until they reach the lytic vesicle. Once the lytic granule reaches the target cell, the protein of interest has been released from the lymphocyte lytic-granule-secreted protein and is able to function freely in the target cell. In some embodiments, an acidic pH cleavable linker comprises an aspartic acid-proline (DP) dipeptide. In some embodiments, the linker comprises the amino acid sequence RARDPPVAT (SEQ ID NO: 12). In some embodiments, the linker consists of SEQ ID NO: 12. In some embodiments, the linker comprises the amino acid sequence DXDPHF (SEQ ID NO: 13). In some embodiments, the linker consists of SEQ ID NO: 13. In some embodiments, the linker comprises the amino acid sequence GTGDP (SEQ ID NO: 14). In some embodiments, the linker consists of SEQ ID NO: 14.

[0145] In some embodiments, the linker is a Cathepsin cleavable linker. Non-limiting examples of Cathepsin include, but are not limited to Cathepsin L, Cathepsin B and Cathepsin C. Cathepsins are known to be active in the lytic granules and thus are also useful for targeted cleavage of the linker. In some embodiments, the linker comprises the dipeptide VA. In some embodiments, the linker consists of the dipeptide VA. In some embodiments, the linker comprises the dipeptide GE. In some embodiments, the linker consists of the dipeptide GE.

[0146] In some embodiments, the linker is cleavable in the cytoplasm. In some embodiments, a cytoplasmic cleavable linker is cleavable at cytoplasmic glutathione levels. It will be understood by a skilled artisan that inclusion of an ER signal peptide will trigger co-translational entry of the chimeric polypeptide into the ER, thereby sequestering the linker from the cleavage-inducing environment of the lymphocyte's cytoplasm. Cytoplasmic cleavage would thus be prevented until the fusion protein's arrival in the target cell's cytoplasm, wherein the protein of interest is set free to exert its function. In some embodiments, the cleavable linker comprises the first 11 amino acids of mCherry. In some embodiments, the cleavable linker comprises amino acids 2-11 of mCherry. In some embodiments, mCherry comprises or consists of MVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTK GGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVV TVTQDSSLQDGEFIYKVKL RGTNFP SDGPVMQKKT MGWEASSERMYPEDGALKG EIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQY

ERAEGRHSTGGMDELYK (SEQ ID NO: 1). In some embodiments, mCherry comprises or consists of SEQ ID NO: 1. In some embodiments, the linker comprises VSKGEEDNMA (SEQ ID NO: 2). In some embodiments, the linker consists of SEQ ID NO: 2. In some embodiments, SEQ ID NO: 2 is a cleavable linker. In some embodiments, mCherry is crmCherry. In some embodiments, crmCherry lacks the first 11 amino acids of mCherry. In some embodiments, crmCherry comprises or consists of SEQ ID NO: 3. In some embodiments, crmCherry is encoded by SEQ ID NO: 7.

[0147] In some embodiments, the molecule of interest is a therapeutic molecule. In some embodiments, the molecule is a drug. In some embodiments, the molecule is a biologic. In some embodiments, the molecule is a biologic molecule. In some embodiments, the molecule is a nucleic acid molecule. In some embodiments, the molecule comprises a nucleic acid molecule. In some embodiments, the nucleic acid is DNA. In some embodiments, the nucleic acid is RNA. In some embodiments, the nucleic acid is DNA or RNA. In some embodiments, the molecule is a protein-RNA complex. In some embodiments, the molecule is an RNP. In some embodiments, the molecule is a protein. In some embodiments, the molecule is a protein fragment. In some embodiments, the molecule is a non-natural molecule. In some embodiments, the molecule is a naturally-occurring molecule. In some embodiments, the molecule is a modified form of a naturally-occurring molecule. In some embodiments, the molecule is an enzyme or enzymatically active. In some embodiments, the molecule is a binding molecule. In some embodiments, the molecule of interest is the cargo. In some embodiments, the molecule of interest is a therapeutic agent. In some embodiments, the therapeutic agent is a therapeutic proteinaceous agent.

[0148] In some embodiments, the molecule of interest is a protein of interest. In some embodiments, the protein of interest is a therapeutic agent. In some embodiments, the protein of interest is a fusion protein. In some embodiments, the protein of interest is a cytoplasmic protein. In some embodiments, the protein of interest is active in the cytoplasm. In some embodiments, the protein of interest is a nuclear protein. In some embodiments, the protein of interest is active in the nucleus.

[0149] In some embodiments, the protein of interest is not a membrane protein. In some embodiments, the protein of interest is not a lymphocyte membrane protein. In some embodiments, the protein of interest is not naturally a membrane protein. In some

embodiments, the protein of interest is not naturally membranal in lymphocytes. In some embodiments, the cell does not comprise the molecule of interest within its cellular membrane. In some embodiments, the cell does not comprise the molecule of interest conjugated to its cellular membrane. In some embodiments, the cell does not comprise the therapeutic agent within its cellular membrane. In some embodiments, the cellular membrane is the plasma membrane. In some embodiments, the protein of interest does not comprise a transmembrane domain.

[0150] In some embodiments, the protein of interest is not a naturally secreted protein. In some embodiments, the protein of interest does not comprise a signal peptide. In some embodiments, the protein of interest does not naturally comprise a signal peptide. In some embodiments, the protein of interest is not a receptor ligand. In some embodiments, the protein of interest does not bind a receptor. In some embodiments, the receptor is a surface receptor. In some embodiments, the receptor is a plasma membrane receptor. In some embodiments, the protein of interest is not a targeting protein. In some embodiments, the protein of interest induces an effect in the target cell. In some embodiments, the protein of interest induces a therapeutic effect in the target cell. In some embodiments, the protein of interest binds a surface protein and activates or inhibits that surface protein. In some embodiments, the protein of interest is an antagonist. In some embodiments, the protein of interest is an agonist. In some embodiments, the protein of interest binds a surface protein and induces signaling through that bound receptor. In some embodiments, the protein of interest is a surface receptor ligand. In some embodiments, the protein of interest is a protein that naturally binds its target. In some embodiments, the protein of interest is an antibody or antigen-binding fragment thereof. In some embodiments, the protein of interest is a synthetic binding agent. In some embodiments, the protein of interest is a single chain antibody. In some embodiments, the protein of interest is a single domain antibody. In some embodiments, the protein of interest is a VHH.

[0151] In some embodiments, the protein of interest is not a viral protein. In some embodiments, the protein of interest is not a viral envelope protein. In some embodiments, the protein of interest is not a viral penetration protein. In some embodiments, the protein of interest is not a viral spike protein. In some embodiments, the therapeutic agent is not a full virus. In some embodiments, the therapeutic agent is not a vaccine. In some embodiments,

the therapeutic agent is not an oncolytic virus. In some embodiments, the therapeutic agent is not a viral particle. In some embodiments, the therapeutic agent is not a viral genome. In some embodiments, the therapeutic agent is a viral genome-editing protein. In some embodiments, the protein of interest is an antigen-binding protein or fragment thereof. In some embodiments, the antigen is not a surface antigen. In some embodiments, the antigen is a cytoplasmic antigen. In some embodiments, the antigen is a nuclear antigen. In some embodiments, the antigen is an internal antigen. In some embodiments, the antigen is internal to a target cell. In some embodiments, the molecule of interest is not nanoparticle conjugated. In some embodiments, the molecule of interest is not encapsulated. In some embodiments, encapsulated is nanoparticle encapsulated. In some embodiments, the chimeric molecule is not nanoparticle conjugated. In some embodiments, the chimeric molecule is not encapsulated.

[0152] In some embodiments, the protein of interest is a protein fragment. In some embodiments, the fragment comprises at least one functional domain. In some embodiments, the fragment is a therapeutic fragment. In some embodiments, the fragment comprises at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 75, 80, 90, or 100 amino acids. Each possibility represents a separate embodiment of the invention. In some embodiments, the fragment comprises at least 25 amino acids. In some embodiments, the fragment is not the complete protein. In some embodiments, the fragment lacks a cleavage site. In some embodiments, the fragment lacks a cleavage site that could result in the protein of interest being cleaved from the lymphocytic lytic granule-secreted protein prematurely. In some embodiments, prematurely is before entry to the ER. In some embodiments, prematurely is before entry into the lytic granule. In some embodiments, the protein of interest is not a lymphocytic lytic granule-secreted protein. In some embodiments, the protein of interest is not a targeting moiety.

[0153] In some embodiments, the molecule of interest comprises a molecular weight greater than 25, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 180, 190 or 200 kilodaltons (kDa). Each possibility represents a separate embodiment of the invention. In some embodiments, the molecule of interest comprises a molecular weight greater than 25 kDa. In some embodiments, the molecule of interest comprises a molecular weight greater than 28 kDa.

In some embodiments, the molecule of interest comprises a molecular weight greater than 50 kDa. In some embodiments, the molecule of interest comprises a molecular weight greater than 75 kDa. In some embodiments, the molecule of interest comprises a molecular weight greater than 100 kDa. In some embodiments, the molecule of interest comprises a molecular weight greater than 125 kDa. In some embodiments, the molecule of interest comprises a molecular weight greater than 150 kDa. In some embodiments, the molecule of interest comprises a molecular weight greater than 160 kDa. In some embodiments, the molecule of interest comprises a molecular weight greater than 190 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 25-300, 25-250, 25-200, 25-190, 25-175, 25-165, 28-300, 28-250, 28-200, 28-190, 28-175, 28-165, 30-300, 30-300, 30-200, 30-190, 30-175, 30-165, 50-300, 50-500, 50-200, 50-190, 50-175, 50-165, 75-300, 75-750, 75-200, 75-190, 75-175, 75-165, 100-300, 100-1000, 100-200, 100-190, 100-175, or 100-165 kDa. Each possibility represents a separate embodiment of the invention. In some embodiments, the molecule of interest comprises a molecular weight between 25-300 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 25-200 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 25-190 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 25-165 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 30-300 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 30-200 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 30-190 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 30-165 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 50-300 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 50-200 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 50-190 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 50-165 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 100-300 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 100-200 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 100-190 kDa. In some embodiments, the molecule of interest comprises a molecular weight between 100-165 kDa.

[0154] In some embodiments, the molecule of interest is not cytotoxic. In some embodiments, the chimeric molecule is not cytotoxic. In some embodiments, the lymphocytic lytic granule-secreted protein is not cytotoxic. In some embodiments, the lymphocytic lytic granule-secreted protein is not cytotoxic, and the molecule of interest is cytotoxic. In some embodiments, the lymphocytic lytic granule-secreted protein is cytotoxic, and the molecule of interest is cytotoxic. In some embodiments, the lymphocytic lytic granule-secreted protein is cytotoxic, and the molecule of interest is not cytotoxic. In some embodiments, the lymphocytic lytic granule-secreted protein is not cytotoxic, and the molecule of interest is not cytotoxic. In some embodiments, a cytotoxic protein is a protein that can induce apoptosis in a cell. In some embodiments, the protein of interest is cytotoxic. In some embodiments, the protein of interest is cytotoxic when delivered to the interior of a target cell. In some embodiments, the protein of interest is not cytotoxic when delivered to the surface of a target cell. In some embodiments, the interior is the cytoplasm. In some embodiments, the interior is the nucleus. In some embodiments, the interior is the mitochondria. In some embodiments, cytotoxic is lytic.

[0155] In some embodiments, the molecule of interest is an RNA-protein complex. In some embodiments, an RNA-protein complex is a ribonucleoprotein (RNP). The term “ribonucleotide” or “ribonucleic acid” (RNA), refers to a modified or unmodified nucleotide or polynucleotide comprising at least one ribonucleotide unit. A ribonucleotide unit comprises a hydroxyl group attached to the 2' position of a ribosyl moiety that has a nitrogenous base attached in N-glycosidic linkage at the 1' position of a ribosyl moiety, and a moiety that either allows for linkage to another nucleotide or precludes linkage.

[0156] In some embodiments, the protein is a nuclease. In some embodiments, the molecule of interest is a genome-editing complex. In some embodiments, the protein of interest is a genome-editing protein. In some embodiments, editing is modifying. In some embodiments, a genome-editing protein is selected from the group consisting of a clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) -associated nuclease, a Zinc-finger nuclease (ZFNs), a meganuclease and a transcription activator-like effector nuclease (TALEN). In some embodiments, the genome-editing protein is a meganuclease. In some embodiments, the genome-editing protein is a natural meganuclease. In some embodiments, the genome-editing protein is a modified/engineered meganuclease. In some embodiments,

the meganuclease is specific to a DNA target sequence of a mammalian genome. In some embodiments, the meganuclease is specific to a DNA target sequence of a mammalian gene. In some embodiments, the meganuclease is a PCSK9-specific meganuclease. In some embodiments, the PCSK9-specific meganuclease comprises the amino acid sequence MHMNTKYNKEFLLYLAGFVDGDGSIFARIKPSQRSKFKHKLHLVFAVYQKTQRR WFLDKLVDEIGVG YVLD SGVSFYSLS EIKPLHNFLTQLQPFLKLKQKQANLVLKII EQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLDSLPGSVGGLSPS QASSAASSASSSPGSGISEALRAGAGSGTGYNKEFLLYLAGFVDGDGSIYARIKPVQ RAKFKHELVLGFDVTQKTQRRWFLDKLVDEIGVG YVYDKGSVSA YRLS QIKPLH NFLTQLQPFLKLKQKQANLVLKII EQLPSAKESPDKFLEVCTWVDQIAALNDSKTR KTTSETVRAVLDSLSEKKKSSP (SEQ ID NO: 53). In some embodiments, the PCSK9-specific meganuclease is encoded by the nucleotide sequence provided in SEQ ID NO: 54.

[0157] In some embodiments, the genome-editing protein is a CRISPR-associated protein. In some embodiments, the CRISPR-associated protein is CRISPR-associated protein 9 (Cas9). In some embodiments, the CRISPR-associated protein is Cas9 or a Cas9 ortholog. In some embodiments, the CRISPR-associated protein is Cas9 or a Cas9 variant. In some embodiments, the CRISPR-associated protein is Cas9 or a Cas9 homolog.

[0158] In some embodiments, the CRISPR-associated protein is a CRISPR-associated nuclease. In some embodiments, the CRISPR-associated nuclease is a CPF1 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cas12a nuclease. In some embodiments, the CRISPR-associated nuclease is a Cas13a nuclease. In some embodiments, the CRISPR-associated nuclease is a CasI nuclease. In some embodiments, the CRISPR-associated nuclease is a CasIB nuclease. In some embodiments, the CRISPR-associated nuclease is a Cas2 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cas3 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cas4 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cas5 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cas6 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cas7 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cas8 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cas10O nuclease. In some embodiments, the CRISPR-associated nuclease is a Csy1 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csy2 nuclease.

In some embodiments, the CRISPR-associated nuclease is a Csy3 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cse1 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cse2 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cse1 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cse2 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csa5 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csn2 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csm2 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csm3 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csm4 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csm5 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csm6 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cmr1 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cmr3 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cmr4 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cmr5 nuclease. In some embodiments, the CRISPR-associated nuclease is a Cmr6 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csb1 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csb2 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csb3 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csx17 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csx14 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csx10 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csx16 nuclease. In some embodiments, the CRISPR-associated nuclease is a CsaX nuclease. In some embodiments, the CRISPR-associated nuclease is a Csx3 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csx1 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csx15 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csf1 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csf2 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csf3 nuclease. In some embodiments, the CRISPR-associated nuclease is a Csf4 nuclease. In some embodiments, the CRISPR-associated nuclease is a Prime Editor 1 (PE1) nickase. In some embodiments, PE1 is encoded by SEQ ID NO: 27. In some embodiments, the CRISPR-associated nuclease is a Prime Editor 2 (PE2) nickase. In some embodiments, PE2 is encoded by SEQ ID NO: 28. In some embodiments, the CRISPR-associated nuclease is a Prime Editor 3 (PE3) nickase. In some embodiments,

the CRISPR-associated nuclease is a MAD7 nuclease. In some embodiments, the CRISPR-associated nuclease is a CRISPRi nuclease (CRISPR interference). In some embodiments, the CRISPR-associated nuclease is a CRISPRa nuclease (CRISPR activation). In some embodiments, the CRISPR-associated nuclease is a class 1 CRISPR nuclease. In some embodiments, the genome editing protein is a homing endonuclease. In some embodiments, the genome editing protein is a meganucleases. In some embodiments, meganucleases are available for producing targeted genome perturbations. In some embodiments, one or more of the above endonucleases or homologs thereof, a recombination of the naturally-occurring molecules thereof, codon-optimized version thereof, or modified versions thereof and combination thereof are employed.

[0159] In some embodiments, the Cas9 is *Streptococcus pyogenes* Cas9 (SpCas9). In some embodiments, the Cas9 is *Campylobacter jejuni* Cas9. In some embodiments, *Campylobacter jejuni* Cas9 comprises SEQ ID NO: 19. In some embodiments, *Campylobacter jejuni* Cas9 consists of SEQ ID NO: 19. In some embodiments, the Cas9 is *Streptococcus pyogenes* serotype M1 Cas9. In some embodiments, SpCas9 is Sp serotype M1 Cas9. In some embodiments, *Streptococcus pyogenes* serotype M1 Cas9 comprises SEQ ID NO: 20. In some embodiments, *Streptococcus pyogenes* serotype M1 Cas9 consists of SEQ ID NO: 20. In some embodiments, the Cas9 is *Staphylococcus aureus* Cas9. In some embodiments, *Staphylococcus aureus* Cas9 comprises SEQ ID NO: 21. In some embodiments, *Staphylococcus aureus* Cas9 consists of SEQ ID NO: 21. In some embodiments, the Cas9 is *Neisseria meningitidis* serogroup C (strain 8013) Cas9. In some embodiments, *Neisseria meningitidis* serogroup C (strain 8013) Cas9 comprises SEQ ID NO: 22. In some embodiments, *Neisseria meningitidis* serogroup C (strain 8013) Cas9 consists of SEQ ID NO: 22. In some embodiments, the Cas9 is *Geobacillus stearothermophilus* Cas9. In some embodiments, *Geobacillus stearothermophilus* Cas9 comprises SEQ ID NO: 23. In some embodiments, *Geobacillus stearothermophilus* Cas9 consists of SEQ ID NO: 23. In some embodiments, the CRISPR associated protein is Cas12a. In some embodiments, the Cas12a is CRISPR-associated endonuclease Cas12a *Francisella tularensis* subsp. *novicida* (strain U112). In some embodiments, Cas12a comprises SEQ ID NO: 24. In some embodiments, Cas12a consists of SEQ ID NO: 24. In some embodiments, Cas12a is CRISPR-associated endonuclease Cas12a OS=*Acidaminococcus* sp. (strain

BV3L6). In some embodiments, Cas12a comprises SEQ ID NO: 25. In some embodiments, Cas12a consists of SEQ ID NO: 25. In some embodiments, Cas12a is Cas12a/Cpf1. In some embodiments, Cas12a is type V CRISPR-associated protein Cas12a/Cpf1 of *Lachnospiraceae* bacterium ND2006. In some embodiments, Cas12a comprises SEQ ID NO: 26. In some embodiments, Cas12a consists of SEQ ID NO: 26. In some embodiments, the genome editing protein is prime editor 1 (PE1). In some embodiments, PE1 consists of SEQ ID NO: 73. In some embodiments, the genome editing protein is prime editor 2 (PE2). In some embodiments, PE2 consists of SEQ ID NO: 74. In some embodiments, the protein of interest is selected from SEQ ID Nos: 19-26 and 73,74.

[0160] In some embodiments, the genome editing protein is one of those provided herein above. In some embodiments, the genome editing protein comprises one of the sequences provided hereinabove. In some embodiments, the genome editing protein consists of one of the sequences provided hereinabove. In some embodiments, the genome editing protein corresponds to one of the sequences provided hereinabove. In some embodiments, the genome editing protein is a variant or one of the protein/sequences provided hereinabove. It will be understood by a skilled artisan that the natural protein can be modified, or a variant can be generated, that is optimized for expression in a mammal or specifically a human. Such optimization can include codon optimization, structural optimization, optimization of expression, optimization of genome editing, optimization of specificity, and other optimizations. Any such optimization known in the art may be used, and any variant sequence corresponding or derived from those provided hereinabove may be employed.

[0161] In some embodiments, the RNA in the RNA-protein complex is a guide RNA (gRNA). In some embodiments, the RNA is a gRNA. In some embodiments, the guide RNA is a single guide RNA (sgRNA). In some embodiments the RNA is chemically modified. In some embodiments, the modification increases stability of the RNA. In some embodiments, the modification increases half-life of the RNA. In some embodiments, the modification decreases degradation of the RNA. In some embodiments, the modification decreases cleavage of the RNA. In some embodiments, the modification decreases immunogenicity of the RNA. In some embodiments, the modification decreases off-target effects. Chemical modifications of RNA are well known in the art and include, for example, 2-O-methyl analogs, 2-fluoro analogs, 2-MOE and phosphorothioate, and any such modification may be

employed. In some embodiments, the RNA targets a target protein of interest. In some embodiments, the target protein of interest is a disease-associated protein. In some embodiments, the target protein of interest is a disease-causing protein. In some embodiments, modification of the target protein of interest has a therapeutic benefit. In some embodiments, a disease is characterized by a mutation in the target protein of interest. In some embodiments, a disease is caused by a mutation in the target protein of interest. In some embodiments, a disease is treatable by gene editing of the target protein of interest. In some embodiments, a disease is treatable by altering regulation of the target protein of interest. In some embodiments, regulation is down-regulation. In some embodiments, regulation is up-regulation.

[0162] In some embodiment, the protein of interest further comprises a localization sequence. It will be understood by a skilled artisan that proteins that are intended to act in a particular subcellular location may have enhanced function if they are targeted to that location. In some embodiments, the localization sequence is a nuclear localization sequence (NLS). In some embodiments, the localization sequence is a mitochondrial localization sequence (MLS). In some embodiments, a localization sequence is capable of transporting a protein to which it is linked from the cytoplasm to a target subcellular location. In some embodiments, the location is an organelle. In some embodiments, the organelle comprises a membrane and the sequence is capable of transport across or through the membrane. In some embodiments, the localization sequence is attached to the N-terminus of the protein of interest. In some embodiments, the localization sequence is attached to the C-terminus of the protein of interest. In some embodiments, the localization sequence is internal to the protein of interest. In some embodiments, the localization sequence is directly conjugated to the protein of interest. In some embodiments, the localization sequence is conjugated to the protein of interest by a linker. In some embodiments, the protein of interest is operably linked to the localization sequence. In some embodiments, the localization sequence is operably linked to the protein of interest.

[0163] Nucleic acid molecules

[0164] By another aspect, there is provided a polynucleotide encoding a chimeric molecule of the invention.

[0165] Schematics of exemplary nucleic acid molecules are provided in **Figure 1**.

[0166] In some embodiments, the polynucleotide is a polynucleotide molecule. In some embodiments, the polynucleotide is a vector. In some embodiments, the polynucleotide is an expression vector. In some embodiments, the expression vector is configured for expression in a mammalian cell. In some embodiments, configured for expression is capable of expression. In some embodiments, the expression vector is configured for expression in a human cell. In some embodiments, the expression vector is configured for expression in a lymphocyte.

[0167] The term "expression" as used herein refers to the biosynthesis of a gene product, including the transcription and/or translation of said gene product. Thus, expression of a nucleic acid molecule may refer to transcription of the nucleic acid fragment (e.g., transcription resulting in mRNA or other functional RNA) and/or translation of RNA into a precursor or mature protein (polypeptide).

[0168] Expressing of a gene within a cell is well known to one skilled in the art. It can be carried out by, among many methods, transfection, electroporation, viral infection, or direct alteration of the cell's genome. In some embodiments, the gene is an open reading frame. In some embodiments, the open reading frame encodes the chimeric polypeptide of the invention. In some embodiments, the open reading frame is in an expression vector such as plasmid or viral vector. Expression vectors are well known in the art and are available commercially from companies such as Addgene, Sigma Aldrich, Genscript and many others.

[0169] A vector nucleic acid sequence generally contains at least an origin of replication for propagation in a cell and optionally additional elements, such as a heterologous polynucleotide sequence, expression control element (e.g., a promoter, enhancer), selectable marker (e.g., antibiotic resistance), poly-Adenine sequence.

[0170] In some embodiments, the vector is a viral vector. The vector may be a DNA plasmid delivered via non-viral methods or via viral methods. The viral vector may be a retroviral vector, a herpesviral vector, an adenoviral vector, an adeno-associated viral vector or a poxviral vector. The promoter may be active in mammalian cells. The promoters may be active in a human cell. The promoter may be active in a lymphocyte.

[0171] In some embodiments, the open reading frame is operably linked to at least one regulatory element. In some embodiments, the regulatory element is a promoter. The term

“operably linked” is intended to mean that the nucleotide sequence of interest is linked to the regulatory element or elements in a manner that allows for expression of the nucleotide sequence (e.g., in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell).

[0172] In some embodiments, the vector is introduced into the cell by standard methods including electroporation (e.g., as described in From et al., Proc. Natl. Acad. Sci. USA 82, 5824 (1985)), Heat shock, infection by viral vectors, high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface (Klein et al., Nature 327. 70-73 (1987)), and/or the like.

[0173] The term "promoter" as used herein refers to a group of transcriptional control modules that are clustered around the initiation site for an RNA polymerase i.e., RNA polymerase II. Promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

[0174] In some embodiments, nucleic acid sequences are transcribed by RNA polymerase II (RNAP II and Pol II). RNAP II is an enzyme found in eukaryotic cells. It catalyzes the transcription of DNA to synthesize precursors of mRNA and most snRNA and microRNA.

[0175] In some embodiments, mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1 ($\pm$), pGL3, pZeoSV2($\pm$), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

[0176] In some embodiments, expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses are used by the present invention. SV40 vectors include pSVT7 and pMT2. In some embodiments, vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein Bar virus include pHEBO, and p2O5. Other exemplary vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter,

murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0177] In some embodiments, recombinant viral vectors, which offer advantages such as lateral infection and targeting specificity, are used for in vivo expression. In one embodiment, lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. In one embodiment, the result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. In one embodiment, viral vectors are produced that are unable to spread laterally. In one embodiment, this characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

[0178] Various methods can be used to introduce the expression vector of the present invention into cells. Such methods are generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, Mich. (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor Mich. (1995), *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, Butterworths, Boston Mass. (1988) and Gilboa et al. [*Biotechniques* 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

[0179] It will be appreciated that other than containing the necessary elements for the transcription and translation of the inserted coding sequence (encoding the polypeptide), the expression construct of the present invention can also include sequences engineered to optimize stability, production, purification, yield or activity of the expressed polypeptide.

[0180] In some embodiments, the nucleic acid sequence encoding human Granzyme B is provided by accession number NM_004131, NM_001346011, or NR_144343. In some embodiments, the nucleic acid sequence encoding human Granzyme B is provided by accession number NM_004131 or NM_001346011. In some embodiments, the nucleic acid sequence encoding human Granzyme B is provided by accession number NM_004131. In

some embodiments, human Granzyme B is encoded by the nucleotide sequence
agctccaaccagggcagccttctgagaagatgcaaccaatctgcttctgctggccttctctgctgcccagggcagatgcagg
ggagatcatcgggggacatgaggccaagccccactcccgcccctacatggcttatcttatgatctgggatcagaagtctctgaaga
ggtgcggtggcttctgatacgagacgacttcgtgctgacagctgtcactgttggggaagctccataaatgtcaccttgggggcc
cacaatatcaaagaacaggagccgacccagcagtttatccctgtgaaaagacccatccccatccagcctataatcctaagaactt
ctccaacgacatcatgctactgcagctggagagaaaggccaagcggaccagagctgtgcagccccctcaggctacctagcaaca
aggccccaggtgaagccagggcagacatgcagtgtggccggctgggggcagacggccccctgggaaaacactcacacacac
tacaagaggtgaagatgacagtgcaggaagatcgaaagtgcgaatctgacttacgccattattacgacagtaccattgagttgtgc
gtgggggacccagagattaaaaagacttcccttaagggggactctggaggccctcttgtgtgtaacaaggtggcccagggcattgt
ctcctatggacgaaacaatggcatgcctccacgagcctgcaccaaagtctcaagctttgtactggataaagaaaacatgaaac
gctactaactacaggaagcaaactaagcccccgctgtaatgaaacaccttctctggagccaagtccagatttacactgggagaggt
gccagcaactgaataaatacctcttagctgagtgga (SEQ ID NO: 15). In some embodiments, SEQ ID
NO: 15 encodes SEQ ID NO: 8. It will be understood that modification of SEQ ID NO: 15
can be made in order to modify SEQ ID NO: 8. For example, to remove the first 18 amino
acids of SEQ ID NO: 8 one can remove the first 54 nucleotides of SEQ ID NO: 15. If an
ATG is removed it can be added back to the beginning of the open reading frame.

[0181] In some embodiments, the nucleic acid sequence encoding mouse Granzyme B is
provided by accession number NM_013542. In some embodiments, mouse Granzyme B is
encoded by the nucleotide sequence
ATGAAGATCCTCCTGCTACTGCTGACCTTGTCTCTGGCCTCCAGGACAAAGGCA
GGGGAGATCATCGGGGGACATGAAGTCAAGCCCCACTCTCGACCCTACATGGC
CTTACTTTCGATCAAGGATCAGCAGCCTGAGGCGATATGTGGGGGCTTCCTTAT
TCGAGAGGACTTTGTGCTGACTGCTGCTCACTGTGAAGGAAGTATAATAAATG
TCACTTTGGGGGCCCACAACATCAAAGAACAGGAGAAGACCCAGCAAGTCAT
CCCTATGGTAAAATGCATTCCCCACCCAGACTATAATCCTAAGACATTCTCCAA
TGACATCATGCTGCTAAAGCTGAAGAGTAAGGCCAAGAGGACTAGAGCTGTG
AGGCCCCTCAACCTGCCCAGGCGCAATGTCAATGTGAAGCCAGGAGATGTGTG
CTATGTGGCTGGTTGGGGAAGGATGGCCCCAATGGGCAAATACTCAAACACGC
TACAAGAGGTTGAGCTGACAGTACAGAAGGATCGGGAGTGTGAGTCCTACTTT
AAAAATCGTTACAACAAAACCAATCAGATATGTGCGGGGGACCCAAAGACCA
AACGTGCTTCCTTTCGGGGGGATTCTGGAGGCCCGCTTGTGTGTAAAAAAGTG

GCTGCAGGCATAGTTTCCTATGGATATAAGGATGGTTCACCTCCACGTGCTTTC
ACCAAAGTCTCGAGTTTCTTATCCTGGATAAAGAAAACAATGAAAAGCAGC

(SEQ ID NO: 17). In some embodiments, SEQ ID NO: 17 encodes SEQ ID NO: 16. It will be understood that modification of SEQ ID NO: 17 can be made in order to modify SEQ ID NO: 16. For example, to remove the first 18 amino acids of SEQ ID NO: 17 one can remove the first 54 nucleotides of SEQ ID NO: 16. If an ATG is removed it can be added back to the beginning of the open reading frame.

[0182] In some embodiments, the nucleotide sequence encoding mutant, non-activatable human Granzyme B is encoded by the sequence
atgcaaccaatcctgcttctgctggccttctctgctgcccagggcagatgcagcagcaatcatcgggggacatgaggccaagc
cccactcccggccctacatggcttatcttatgatctgggatcagaagtctctgaagaggtgcggtggcttcctgatacagagcactt
cgtgctgacagctgctcactgttggggaagctccataaatgtcaccttggggggccacaatatcaaagaacaggagccgaccag
cagtttatccctgtgaaaagaccatccccatccagcctataatcctaagaacttctccaacgacatcatgctactgcagctggaga
gaaaggccaagcggaccagagctgtgcagccctcaggctacctagcaacaaggcccaggtgaagccagggcagacatgca
gtgtggccggctgggggcagacggccccctgggaaaacactcacacacactacaagaggtgaagatgacagtgcaggaaga
tcgaaagtgcgaatctgacttacgccattattacgacagtaccattgagttgtgcgtgggggaccagagattaaaaagacttccttt
aagggggactctggaggccctcttgtgtgtaacaaggtggcccagggcattgtctcctatggacgaaacaatggcatgcctccac
gagcctgcaccaaagtctcaagctttgtacactggataaagaaaaccatgaaacgcta (SEQ ID NO: 18). In some
embodiments, the nucleotide sequence encoding mutant, non-activatable mouse Granzyme
B is encoded by the sequence
atgaagatcctcctgctactgctgaccttgtctctggcctccaggacaaaggcagcagcaatcatcgggggacatgaagtcaagcc
ccactctcgaccctacatggccttactttcgatcaaggatcagcagcctgaggcgatatgtgggggcttccttattcgagaggacttt
gtgctgactgctgctcactgtgaaggaagtataataaatgtcactttggggggccacaacatcaaagaacaggagaagaccagc
aagtcatccctatggtaaaatgcattccccaccagactataatcctaagacattctccaatgacatcatgctgctaaagctgaagagt
aaggccaagaggactagagctgtgaggccctcaacctgccagggcgcaatgtcaatgtgaagccaggagatgtgtgctatgtg
gctggttggggaaggatggccccaatgggcaaatactcaaacacgctacaagaggttgagctgacagtacagaaggatcgggga
gtgtgagtcctactttaaaaatcgttacaacaaaaccaatcagatatgtgcgggggacccaaagaccaaacgtgcttcctttcgggg
ggattctggaggcccgttgtgtgtaaaaaagtggtgcaggcatagtttctatggatataaggatggttcacctccacgtgctttca
ccaaagtctcgagtttcttctcctggataaagaaaacaatgaaaagcag (SEQ ID NO: 41).

[0183] In some embodiments, the nucleotide sequence encoding Streptococcus pyogenes
Cas9 (SpCas9) is

atggacaagaagtatagcatcggcctggatatcggcacaactccgtgggctgggccgtgatcaccgacgagtacaaggtgcc
agcaagaagttaaggtgctgggcaacaccgatagacactccatcaagaagaatctgatcggcgccctgctgttcgactctggcg
agacagccgaggccacacggctgaagagaaccgcccggagaaggtatacacgccggaagaataggatctgctacctgcagga
gatcttcagcaacgagatggccaaggtggacgattctttcttcaccgcctggaggagagcttcctggtggaggaggataagaagc
acgagcggcaccctatctttggcaacatcgtggacgaggtggcctatcacgagaagtacccaacaatctatcacctgaggaagaa
gctggtggactccaccgataaggccgacctgcgcctgatctatctggccctggcccacatgatcaagttccggggccactttctgat
cgagggcgatctgaaccagacaatagcgatgtggacaagctgttcatccagctggtgcagacctacaatcagctgtttgaggag
aaccctcatcaatgcctctggagtggacgcaaaggcaatcctgagcgccagactgtccaagtctagaaggctggagaacctgatc
ggccagctgccaggcgagaagaagaacggcctgtttggcaatctgatcgcctgtccctgggcctgacaccaacttcaagtcta
atcttgatctggccgaggacgccaagctgcagctgtccaaggacacctatgacgatgacctggataacctgctggcccagatcgg
cgatcagtacgccgacctgttctggccgccaagaatctgtctgacgccatcctgctgagcgatatcctgcgcgtgaacaccgaga
tcacaaaggccccctgagcgccctccatgatcaagagatatgacgagcaccaccaggatctgacctgctgaaggccctggtga
ggcagcagctgcctgagaagtacaaggagatcttctttgatcagagcaagaatggatacgcaggatatacgcaggaggagcatc
ccaggaggagtctacaagtttatcaagcctatcctggagaagatggacggcacagaggagctgctggtgaagctgaatcgga
ggacctgctgaggaagcagcgccactttgataacggcagcatccctcaccagatccacctgggagagctgcacgcaatcctgcg
ccggcaggaggacttctacccatttctgaaggataaccgggagaagatcgagaagatcctgacattcagaatccctactatgtgg
gacctctggcccggggcaatagcagatttgctggatgacctgcaagtcaggagagacaatcacacctggaacttcgaggagg
tggtggataaggcgccctctgccagagcttcatcgagcggatgaccaatttgacaagaacctgcctaataagaaggtgctgcc
aagcactctctgctgtacgagtatttcaccgtgtataacgagctgacaaaggtgaagtacgtgaccgagggcatgagaaagcctgc
cttctgagcggcgagcagaagaaggccatcgtggacctgctgtttaagaccaataggaaggtgacagtgaagcagctgaagga
ggactatttcaagaagatcgagtgtttgattctgtggagatcagcggcgtggaggacaggtttaacgcctccctgggcacctacca
cgatctgctgaagatcatcaaggataaggacttctggacaacgaggagaatgaggatatcctggaggacatcgtgctgacctg
acactgtttgaggataggagatgatcgaggagcgcctgaagacatatgccacctgttcgatgacaaagtgatgaagcagctga
agagaaggcgctacaccggatggggccggctgagcagaaagctgatcaatggcatccgcgacaagcagtctggcaagacaat
cctggactttctgaagagcgtggcttcgccaaccggaacttcatgcagctgatccacgatgactccctgaccttcaaggaggatat
ccagaaggcacaggtgtctggacaggcgacagcctgcacgagcacatcgccaacctggccggctctcctgccatcaagaagg
gcactctgcagacctgaaggtggtggacgagctggtgaaagtgatgggcaggcacaagccagagaacatcgtgatcgagatg
gcccgcgagaatcagaccacacagaagggccagaagaactcccgggagagaatgaagagaatcgaggagggcatcaagga
gctgggctctcagatcctgaaggagcaccctgggagaacacacagctgcagaatgagaagctgtatctgtactatctgcagaat
ggccgggatatgtacgtggaccaggagctggatatcaacagactgtctgattatgacgtggatcacatcgtgccacagtccttctg
aaggatgactctatcgacaataaggtgctgaccaggagcgacaagaaccgcggaagtccgataatgtgccctctgaggaggtg
gtgaagaagatgaagaactactggaggcagctgctgaatgccaagctgatcacacagaggaagtttgataacctgaccaaggca

gagaggggaggactgtccgagctggacaaggccggcttcatcaagcggcagctggtggagacaagacagatcacaagcacg
 tggcccagatcctggattctagaatgaacacaaagtacgatgagaatgacaagctgatcaggagggtgaaagtgatcacctgaa
 gtccaagctggtgtctgacttttaggaaggatttccagttttataaggtgcgcgagatcaacaattatcaccacgcccacgacgcctac
 ctgaacgccgtggtgggcacagccctgatcaagaagtaccctaagctggagtccgagttcgtgtacggcgactataaggtgtacg
 atgtgcgcaagatgatcgccaagtctgagcaggagatcggcaaggccaccgccaagtatttcttttacagcaacatcatgaatttctt
 taagaccgagatcacactggccaatggcgagatcaggaagcgcccactgatcgagacaaacggcgagacaggcgagatcgtg
 tgggacaagggcagggttttggcacctgtgcgcaaggtgctgagcatgccccaaagtgaatatcgtgaagaagaccgagggtgca
 gacaggcggcttctccaaggagtctatcctgcctaagcggaactccgataagctgatcgccagaaagaaggactgggaccccaa
 gaagtatggcggcttcgacagccctacagtggcctactccgtgctggtggtggccaaggtggagaagggaagagcaagaagc
 tgaagtccgtgaaggagctgctgggcatcaccatcatggagcgcagctccttcgagaagaatcctatcgactttctggaggccaag
 ggctataaggaggtgaagaaggacctgatcatcaagctgccaaagtactctctgtttgagctggagaacggaaggaagagaatgc
 tggcaagcggcgagagctgcagaagggaatgagctggccctgccctccaagtacgtgaacttctgtatctggcctcccacta
 cgagaagctgaagggtctcctgaggataacgagcagaagcagctgtttgtggagcagcacaagcactatctggacgagatcat
 cgagcagatcagcgagttctccaagagagtgatcctggccgacgccaatctggataaggtgctgtccgcctacaacaagcacccg
 ggataagccaatcagagagcaggccgagaatatcatccacctgtttaccctgacaaacctgggagcaccagcagccttcaagtatt
 ttgacaccacaatcgacaggaagcggtacaccagcacaaggaggtgctggacgccacactgatccaccagtccatcaccggc
 ctgtacgagacacggatcgacctgtctcagctgggaggcgat (SEQ ID NO: 42).

[0184] In some embodiments, a plasmid encoding human Granzyme B comprising mutation of the GE dipeptide to AA and linked to crmCherry is provided in SEQ ID NO: 29. A schematic of the coding region of this plasmid is presented as the first row (labeled 1_2) of **Figure 1**. In some embodiments, a plasmid encoding human Granzyme B linked to crmCherry is provided in SEQ ID NO: 30. A schematic of the coding region of this plasmid is presented as the second row (labeled 1_3) of **Figure 1**. In some embodiments, a plasmid encoding human Granzyme B comprising mutation of the GE dipeptide to AA and linked to full-length Cas9 with an N-terminal NLS followed by P2A peptide-crmCherry is provided in SEQ ID NO: 31. A schematic of the coding region of this plasmid is presented as the third row (labeled 1_4) of **Figure 1**. In some embodiments, a plasmid encoding human Granzyme B comprising mutation of the GE dipeptide to AA but lacking the ER signal peptide and linked to full-length Cas9 with an N-terminal NLS followed by P2A peptide-crmCherry is provided in SEQ ID NO: 66. A schematic of the coding region of this plasmid is presented as the seventh row (labeled 1_16) of **Figure 1**. In some embodiments, a plasmid encoding

human Granzyme B and linked to full-length Cas9 with an N-terminal NLS followed by a P2A-crmCherry is provided in SEQ ID NO: 32. A schematic of the coding region of this plasmid is presented as the fourth row (labeled 1_5) of **Figure 1**. In some embodiments, a plasmid encoding human Granzyme B lacking the ER signal peptide and linked to full-length Cas9 with an N-terminal NLS followed by a P2A-crmCherry is provided in SEQ ID NO: 65. A schematic of the coding region of this plasmid is presented as the eighth row (labeled 1_15) of **Figure 1**. In some embodiments, a plasmid encoding human Granzyme B comprising mutation of the GE dipeptide to AA and linked to full-length Cas9 with an N-terminal NLS by the cleavable linker denoted by SEQ ID NO: 2 and followed by P2A-crmCherry is provided in SEQ ID NO: 33. A schematic of the coding region of this plasmid is presented as the fifth row (labeled 1_6) of **Figure 1**. In some embodiments, a plasmid encoding human Granzyme B comprising mutation of the GE dipeptide to AA but lacking the ER signal peptide and linked to full-length Cas9 with an N-terminal NLS by the cleavable linker denoted by SEQ ID NO: 2 and followed by P2A-crmCherry is provided in SEQ ID NO: 67. A schematic of the coding region of this plasmid is presented as the ninth row (labeled 1_17) of **Figure 1**. In some embodiments, a plasmid encoding human wild-type Granzyme B linked to full-length Cas9 with an N-terminal NLS by the cleavable linker denoted by SEQ ID NO: 2 and then followed by a P2A-crmCherry is provided in SEQ ID NO: 34. A schematic of the coding region of this plasmid is presented as the sixth row (labeled 1_7) of **Figure 1**. In some embodiments, a plasmid encoding human wild-type Granzyme B lacking the ER signal peptide linked to full-length Cas9 with an N-terminal NLS by the cleavable linker denoted by SEQ ID NO: 2 and then followed by a P2A-crmCherry is provided in SEQ ID NO: 68. A schematic of the coding region of this plasmid is presented as the tenth row (labeled 1_18) of **Figure 1**. In some embodiments, a plasmid encoding mouse wild-type Granzyme B linked to crmCherry is provided in SEQ ID NO: 35. In some embodiments, a plasmid encoding mouse Granzyme B comprising mutation of the GE dipeptide to AA and linked to crmCherry is provided in SEQ ID NO: 36. In some embodiments, a plasmid encoding mouse wild-type Granzyme B and linked to full-length Cas9 with an N-terminal NLS followed by a P2A peptide and then crmCherry is provided in SEQ ID NO: 37. In some embodiments, a plasmid encoding mouse Granzyme B comprising mutation of the GE dipeptide to AA and linked to full-length Cas9 with an N-terminal NLS and then followed by a P2A peptide and then crmCherry is provided in SEQ ID NO: 38. In

some embodiments, a plasmid encoding mouse wild-type Granzyme B and linked to full-length Cas9 with an N-terminal NLS by a cleavable linker and followed by a P2A peptide and then crmCherry is provided in SEQ ID NO: 39. In some embodiments, a plasmid encoding mouse Granzyme B comprising mutation of the GE dipeptide to AA and linked to full-length Cas9 with an N-terminal NLS by a cleavable linker and then followed by a P2A peptide and then crmCherry is provided in SEQ ID NO: 40.

[0185] In some embodiments, a plasmid encoding human Granzyme B comprising mutation of the GE dipeptide to AA and linked to a meganuclease with an N-terminal NLS is provided in SEQ ID NO: 69. A schematic of the coding region of this plasmid is presented as the eleventh row (labeled 2_4) of **Figure 1**. In some embodiments, a plasmid encoding human Granzyme B comprising mutation of the GE dipeptide to AA but lacking the ER signal peptide and linked to a meganuclease with an N-terminal NLS is provided in SEQ ID NO: 70. A schematic of the coding region of this plasmid is presented as the twelfth row (labeled 2_5) of **Figure 1**. In some embodiments, a plasmid encoding human Granzyme B comprising mutation of the GE dipeptide to AA and linked to a meganuclease with an N-terminal NLS followed by GFP is provided in SEQ ID NO: 71. A schematic of the coding region of this plasmid is presented as the thirteenth row (labeled 2_6) of **Figure 1**. In some embodiments, a plasmid encoding a meganuclease with an N-terminal NLS is provided in SEQ ID NO: 5. In some embodiments, a plasmid encoding a meganuclease with an N-terminal NLS and a C-terminal NLS is provided in SEQ ID NO: 6.

[0186] Modified cells

[0187] By another aspect, there is provided a modified cell comprising a therapeutic agent.

[0188] In some embodiments, the cell is a cell that expresses Granzyme. In some embodiments, the cell naturally expresses granzyme. In some embodiments, the cell expresses granzyme before modification. In some embodiments, the Granzyme is Granzyme B. In some embodiments, the cell is a cell that expresses Perforin. In some embodiments, the cell expresses perforin before the modification. In some embodiments, the cell is a cell that expresses Granzyme and Perforin. In some embodiments, expression is secretion. Cells that express/secrete the combination of Granzyme and Perforin are well known in the art and any such cells may be used. In some embodiments, the cell is an immune cell. In some embodiments the cell is a white blood cell. In some embodiments, the cell is a leukocyte. In

some embodiments, a leukocyte is a lymphocyte or a myeloid cell. In some embodiments, the cell is a CD45+ cell. In some embodiments, the white blood cell (CD45+ cell) expresses Granzyme and/or Perforin. In some embodiments, the cell is capable of forming an immune synapse. In some embodiments, the cell is a cell that produces immune synapses. In some embodiments, the cell is characterized by the ability to form an immune synapse with a target cell. In some embodiments, the cell is a lymphocyte. In some embodiments, the cell is a myeloid cell. In some embodiments, the cell is selected from a lymphocyte and a myeloid cell. In some embodiments, the myeloid cell is a macrophage. In some embodiments, myeloid cells comprise macrophages. In some embodiments, the myeloid cell is a dendritic cell. In some embodiments, myeloid cells comprise dendritic cells. In some embodiments, the lymphocyte is a T cell. In some embodiments, the lymphocyte is an NK cell. In some embodiments, the lymphocyte is selected from a T cell and an NK cell. In some embodiments, the cell is selected from a T cell, an NK cell and a myeloid cell. In some embodiments, the cell is selected from a T cell, an NK cell and a macrophage. In some embodiments, a cell that can form an immune synapse is selected from a T cell, an NK cell, a B cell, a mast cell, a neutrophil and a macrophage. In some embodiments, a cell that can form an immune synapse is selected from a T cell, an NK cell, a B cell, a mast cell, a neutrophil and a myeloid cell. In some embodiments, a cell that can form an immune synapse is selected from a T cell, an NK cell, a B cell, a mast cell and a macrophage. a cell that can form an immune synapse is selected from a T cell, an NK cell, a B cell, and a macrophage. In some embodiments, a cell that can form an immune synapse is selected from a T cell, an NK cell, a B cell, a mast cell and a macrophage. a cell that can form an immune synapse is selected from a T cell, an NK cell, a B cell, and a myeloid cell. In some embodiments, a cell that can form an immune synapse is selected from a T cell, an NK cell, and a macrophage. In some embodiments, a cell that can form an immune synapse is selected from a T cell, an NK cell, and a myeloid cell. In some embodiments, the cell is selected from a lymphocyte and a cell that is capable of producing a phagocytic synapse. In some embodiments, an immune synapse is a phagocytic synapse. In some embodiments, the cell is selected from a lymphocyte and a macrophage. In some embodiments, the cell is selected from a lymphocyte and a myeloid cell.

[0189] As used herein, the term “immune synapse” and “immunological synapse” are used interchangeably to refer to a physical interface between an immune cell and a target cell, which is often an antigen-providing cell. In some embodiments, an immune synapse is a supramolecular activation cluster. In some embodiments, the synapse comprises three concentric rings of protein clusters that mediate transfer of proteins between the immune cell and its target. In some embodiments, the synapse comprises adhesion molecules in the periphery. It will be understood by a skilled artisan that the adhesion molecules of the immunological synapse result in increased avidity and sustained contact between effector and target cell. This increased avidity/contact enables more potent transport of molecules. In this way synapse formation produces a solution to the problem of successful and efficient transfer of therapeutic molecules. In some embodiments, the synapse comprises a high density of T cell receptors and co-stimulatory molecules. In some embodiments, the high density is in the center of the immunological synapse. In some embodiments, immune synapse formation triggers immune cell activation. In some embodiments, immune synapse formation triggers lymphocyte activation. In some embodiments, immune synapse formation triggers T cell activation. In some embodiments, activation is activation above a minimum threshold needed for execution of effector function. In some embodiments, activation is activation above or equal to artificial activation by an anti-CD3 antibody. In some embodiments, activation is activation above or equal to artificial activation by IL-2. In some embodiments, activation is activation above artificial activation by an anti-CD3 antibody. In some embodiments, activation is activation above artificial activation by IL-2.

[0190] In some embodiments, the cell is a cell of adoptive cell transfer. In some embodiments, the cell is a therapeutic cell. In some embodiments, the cell is a tumor infiltrating lymphocyte (TIL). In some embodiments, the cell is an adoptive T cell. In some embodiments, the cell is an adoptive NK cell. In some embodiments, the cell is a CAR cell. In some embodiments, the CAR cell is a CAR T cell. In some embodiments, the CAR cell is a CAR NK cell. In some embodiments, the cell is a cell in culture. In some embodiments, the cell has been expanded. In some embodiments, the cell is in vivo.

[0191] In some embodiments, the cell is modified to express the therapeutic agent and/or molecule and/or protein. In some embodiment, the modification is performed in-vitro or ex-vivo. In some embodiments, the modification is performed by introduction of a protein of

interest or a ribonucleoprotein of interest or a nucleotide sequence of interest or other molecule of interest. In vitro introduction of molecule of interest to cells is well known in the art and may be performed for a non-limiting example by electroporation, transfection, infection, by plasmid, virus and liposomes. In some embodiment the modification is performed by introducing a nucleotide sequence and/or molecule (e.g., DNA and RNA) to be expressed in the lymphocyte.

[0192] In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a human cell. In some embodiments, the cell is a primary cell. In some embodiments, the cell is a peripheral blood cell. In some embodiments, the lymphocyte is a peripheral blood lymphocyte. In some embodiments, the peripheral blood cell is a peripheral blood mononuclear cell (PBMC). In some embodiments, the cell is a bone marrow cell. In some embodiments, the lymphocyte is a bone marrow lymphocyte. In some embodiments, the lymphocytes are tissue resident lymphocytes. In some embodiments, the cell is a cell in culture. In some embodiments, the cell is an ex vivo cell. In some embodiments, the cell is from a subject. In some embodiments, the cell is in a subject. In some embodiments, the cell is from a cell line. In some embodiments, the cell line is a granzyme knockdown cell line.

[0193] In some embodiments, the cell is a naturally-occurring cell. In some embodiments, the cell is a differentiated from another cell in culture. In some embodiments, the differentiation is trans-differentiation. In some embodiments, the differentiation is a naturally-occurring differentiation. Methods of producing cells in culture by differentiation are well known in the art and any such method may be used to produce the cells used to produce the modified cells of the invention. In some embodiments, stem cells are differentiated to produce the cells. In some embodiments, the stem cells are hematopoietic stem cells (HSCs). In some embodiments, the stem cells are embryonic stem cells (ESCs). In some embodiments, the stem cells are not ESCs. In some embodiments, the cells are not derived from embryonic cells. In some embodiments, the stem cells are multipotent stem cells. In some embodiments, the stem cells are pluripotent stem cells. In some embodiments, the stem cells are induced pluripotent stem cells (iPSCs). In some embodiments, the stem cells are mesenchymal stromal cells or mesenchymal stem cells (MSCs). In some embodiments, the cells are not derived or trans-differentiated from MSCs.

[0194] In some embodiments, the cell comprises secretory lysosomes. In some embodiments, the cell comprises lytic granules. In some embodiments, the cell is a lytic cell. In some embodiments, the lymphocyte comprises lytic granules. In some embodiments, the lymphocyte expresses lytic granules in response to activation. In some embodiments, the lytic granules become polarized in response to activation. In some embodiments, the lymphocyte comprises polarized lytic granules. In some embodiments, the cell expresses at least one lymphocyte lytic granule-secreted protein. In some embodiments, the lymphocyte expresses at least one lymphocyte lytic granule-secreted protein. In some embodiments, the expression is in response to activation. In some embodiments, the lymphocyte is a T cell. In some embodiments, the lymphocyte is a B cell. In some embodiments, the lymphocyte is an NK cell. In some embodiments, the lymphocyte is a naïve lymphocyte. In some embodiments, the lymphocyte is an activated lymphocyte. Activation of lymphocytes is well known in the art and can be done by any known method including those disclosed hereinbelow. Such methods include anti-CD3 stimulation and/or IL-2 stimulation. In some embodiments, the T cell is a CD8 positive T cell. In some embodiments, the T cell is a cytotoxic T lymphocyte. In some embodiments, the T cell has been modified to inhibit its cytotoxicity. In some embodiments, the T cell has been modified to abolish its cytotoxicity. In some embodiments, the T cell is a helper T cell. In some embodiments, the T cell is a CD4 positive T cell. In some embodiments, the T cell is an $\alpha\beta$ T-cell. In some embodiments, the T cell is a $\gamma\delta$ T-cell. In some embodiments, the T cell is a regulatory T cell. In some embodiments, the lymphocyte is an NK cell. In some embodiments, the lymphocyte is selected from a T cell and an NK cell. In some embodiments, the NK cell is a natural killer T (NKT) cell.

[0195] In some embodiments, the cell is a non-cytotoxic cell. In some embodiments, the cell is a non-lytic cell. In some embodiments, the cell does not induce lysis in a target cell. In some embodiments, the cell does not induce apoptosis in a target cell. In some embodiments, the lymphocyte is a non-cytotoxic lymphocyte. In some embodiments, the cell is non-cytotoxic before modification. In some embodiments, the lymphocyte is non-cytotoxic before modification. In some embodiments, the cell is naturally cytotoxic and is modified to reduce cytotoxicity. In some embodiments, the cell is naturally cytotoxic and is modified to inhibit cytotoxicity. In some embodiments, the cell is naturally cytotoxic and is modified to

abolish cytotoxicity. In some embodiments, the modification is mutation of the TCR. In some embodiments, the modification is removal of the TCR. In some embodiments, the modification is down-regulation of the TCR. In some embodiments, the modification is inhibition of expression of the TCR. In some embodiments, the TCR is the endogenous TCR. In some embodiments, the modification is mutation of a granzyme. In some embodiments, the cell is a cell of a granzyme knockdown cell line. In some embodiments, the knockdown is CRISPR removal. In some embodiments, the modification is mutation of a proinflammatory cytokine. In some embodiments, the cell is a cell of a proinflammatory cytokine knockdown cell line. In some embodiments, cytotoxicity is endogenous cytotoxicity. In some embodiments, the cell is not cytotoxic, and the chimeric molecule is cytotoxic. In some embodiments, the cell is not cytotoxic, and the molecule of interest is cytotoxic. In some embodiments, the cell comprises mutation or knockout of a cytotoxic protein. In some embodiments, the cytotoxic protein is a cytotoxic protein other than a lytic granule-secreted protein. In some embodiments, the knockout is by genetic ablation of the locus encoding the cytotoxic protein. In some embodiments, the genetic ablation is by a genome-editing protein. In some embodiments, the genetic ablation is by CRISPR.

[0196] In some embodiments, the modified cells are further modified/engineered to prevent lytic effect on target cells upon/following activation. In some embodiments, activation is activation of the granzyme-perforin pathway. In some embodiments, the modified lymphocytes are engineered to knockdown endogenous expression of a lymphocytes lytic-granule-secreted protein. In some embodiments, knockdown is knockout. In some embodiments, the modified cells are engineered to knockdown a gene encoding for cell-mediating killing elements. Example of such killing elements include, but are not limited to endogenous granzyme, FasL and Trail. In some embodiments, endogenous expression of FasL is knocked down. In some embodiments, endogenous expression of a Trail receptor is knocked down. In some embodiments, endogenous expression of a proinflammatory cytokine is knocked down. In some embodiments, endogenous expression of a proinflammatory cytokine is inhibited. Proinflammatory cytokines are well known in the art and include, for non-limiting example, TNFa and INFg. In some embodiments, knocked down is knocked out. Genetic knockdown/knockout can be accomplished by any convenient method known in the art, such as genome-editing, for example with CRISPR/Cas9 or

introduction of sequences encoding a specific siRNA, shRNA, miRNA or similar inhibitory nucleic acid molecules. In some embodiments, the further modification/engineering is performed in vitro. In some embodiments, the further modification/engineering is performed ex vivo.

[0197] In some embodiments, the cell comprises a therapeutic agent and a targeting moiety. In some embodiments, the targeting moiety is a separate molecule from the therapeutic agent. In some embodiments, the therapeutic agent is not a targeting molecule. In some embodiments, the targeting moiety is an engineered molecule. In some embodiments, the targeting moiety is non-naturally occurring. In some embodiments, the targeting moiety is an engineered TCR. In some embodiments, the targeting moiety is a chimeric antigen receptor (CAR). In some embodiments, the targeting moiety activates the lymphocyte upon binding its target. In some embodiments, the targeting moiety initiates an activation cascade upon binding its target. In some embodiments, the targeting moiety comprises a target-binding domain. In some embodiments, the targeting moiety comprises an activation domain. In some embodiments, the targeting moiety is a transmembrane protein. In some embodiments, the targeting moiety comprises a co-activation domain. In some embodiments, the lymphocyte is a CAR-T cell. In some embodiments, the lymphocyte is a CAR-NK cell. In some embodiments, the lymphocyte is a TIL.

[0198] As used herein, the terms "CAR-T cell" and "CAR-NK cell" refer to an engineered receptor which has specificity for at least one target protein of interest and is grafted onto an immune cell (a lymphocyte). In some embodiments, the CAR-T cell has the specificity of a monoclonal antibody grafted onto a T-cell. In some embodiments, the CAR-NK cell has the specificity of a monoclonal antibody grafted onto a NK-cell. In some embodiments, the T cell is selected from a cytotoxic T lymphocyte and a regulatory T cell. It will be understood by a skilled artisan that if the cell of the invention has been rendered non-cytotoxic or is naturally non-cytotoxic, activation will lead to secretion of vesicles containing the therapeutic agent or molecule of interest but will still not lead to killing of the target cell. In some embodiments, the target cell is an engaged cell. In some embodiments, engaged is engaged by the CAR.

[0199] CAR-T and CAR-NK cells and their vectors are well known in the art. Such cells target and engage the protein for which the receptor binds. In some embodiments, a CAR-T

or CAR-NK cell targets at least one viral protein. In some embodiments, a CAR-T or CAR-NK cell targets at least one cancer protein. In some embodiments, a CAR-T or CAR-NK cell targets at least one surface protein. In some embodiments, the surface protein is a target protein. In some embodiments, the surface protein is on the surface of a target cell.

[0200] Construction of CAR-T cells is well known in the art. In one non-limiting example, a monoclonal antibody to a viral protein can be made and then a vector coding for the antibody will be constructed. The vector will also comprise a costimulatory signal region. In some embodiments, the costimulatory signal region comprises the intracellular domain of a known T cell or NK cell stimulatory molecule. In some embodiments, the intracellular domain is selected from at least one of the following: CD3Z, CD27, CD28, 4-1BB, OX40, CD30, CD40, PD- 1, ICOS, lymphocyte function-associated antigen- 1 (LFA- 1), CD2, CD 7, LIGHT, NKG2C, B7- H3, and a ligand that specifically binds with CD83. In some embodiments, the vector also comprises a CD3Z signaling domain. This vector is then transfected, for example by lentiviral infection, or electroporated into a lymphocyte.

[0201] In some embodiments, the cell is from a subject. In some embodiments, the cell is autologous to the subject. In some embodiments, the cell is allogeneic to the subject. In some embodiments, the cell is a universal allogeneic lymphocyte. In some embodiments, the cell is a non-immunogenic lymphocyte. In some embodiments, the cell is an off-the-shelf lymphocyte. In some embodiments, the cell is syngeneic to the subject. In some embodiments, the cells share a matched HLA type to the subject. Autologous cells refer to cells derived from the same subject to which they are re-introduced following modification (e.g., ex vivo/in vitro modification. The cells may be extracted from the patient's blood by Leukapheresis. Methods of cell/lymphocyte extraction are well known in the art and any such method may be employed. In some embodiments, the cells are for adoptive cell transfer. In some embodiments, the lymphocytes are for adoptive cell transplant.

[0202] In some embodiments, the therapeutic agent is the molecule of interest. In some embodiments, the therapeutic agent is the protein of interest. In some embodiments, the therapeutic agent is the chimeric molecule. In some embodiments, the therapeutic agent is the chimeric polypeptide. In some embodiments, the therapeutic agent is a protein. In some embodiments, the therapeutic agent is a proteinaceous agent. In some embodiments, the therapeutic agent is exogenous to the leukocyte. In some embodiments, the therapeutic agent

is not naturally occurring. In some embodiments, the therapeutic agent is not cytotoxic. In some embodiments, the therapeutic agent is not lytic. In some embodiments, the therapeutic agent is a chimeric molecule of the invention. In some embodiments, the therapeutic agent is a chimeric polypeptide of the invention.

[0203] In some embodiments, the therapeutic agent acts in the cytoplasm. In some embodiments, the therapeutic agent acts in the nucleus. In some embodiments, the therapeutic agent acts within a cell. In some embodiments, the therapeutic agent's target is an internal target. In some embodiments, the therapeutic agent's target is a nuclear target. In some embodiments, the therapeutic agent's target is a cytoplasmic target. In some embodiments, the therapeutic agent's target is not a cell surface target. In some embodiments, the therapeutic agent's target is not a receptor.

[0204] In some embodiments, the therapeutic agent treats a disease, disorder, or condition. In some embodiments, the therapeutic agent treats a disease. Examples of such disease include, but are not limited to a genetic disease, an autoimmune disease, a bacterial disease, a viral disease, an inflammatory disease, a proliferative disease, a cardiovascular disease, a degenerative disease, a brain disease, a digestive disease, a liver disease, a neurological disease and an energy homeostasis disease. In some embodiments, a proliferative disease is cancer. In some embodiments, the disease is not cancer. In some embodiments, the therapeutic agent is not an anti-cancer therapeutic. In some embodiments, the leukocyte comprises a targeting moiety that targets a cell of the disease. It will be understood by a skilled artisan that the cells at the route of a disease can be discerned and in turn treated by lymphocytes of the invention. For example, a neuronal disease can be treated with lymphocytes with a neuron targeting moiety, an energy homeostasis disease can be treated with lymphocytes with a pancreatic targeting moiety, etc. In some embodiments, the therapeutic agent treats a condition. Examples of conditions include, but are not limited to inflammatory conditions, aging-related conditions, degenerative conditions and many others.

[0205] As used herein, the terms "treatment" or "treating" of a disease, disorder, or condition encompasses alleviation of at least one symptom thereof, a reduction in the severity thereof, or inhibition of the progression thereof. Treatment need not mean that the disease, disorder, or condition is totally cured. To be an effective treatment, a useful composition or method

herein needs only to reduce the severity of a disease, disorder, or condition, reduce the severity of symptoms associated therewith, or provide improvement to a patient or subject's quality of life.

[0206] In some embodiments, the disease is a genetic disease. In some embodiments, the condition is a genetic condition. In some embodiments, the disorder is a genetic disorder. In some embodiments, a genetic disease, condition or disorder is one that is caused by a genetic mutation. In some embodiments, a disease is a disease condition or disorder. In some embodiments, the disease is not cancer. In some embodiments, the genetic mutation is a somatic mutation. In some embodiments, the genetic mutation is a germ-line mutation. In some embodiments, the genetic disease, disorder or condition is treatable by gene therapy. In some embodiments, the therapeutic agent is a gene editing agent. In some embodiments, the therapeutic agent is a gene editing protein. In some embodiments, the therapeutic agent is a gene editing complex. In some embodiments, the complex is an RNA-protein complex. In some embodiments, gene editing is genome-editing. In some embodiments, the therapeutic agent comprises Cas9 or a homolog, ortholog or variant thereof.

[0207] Genetic disease and disorders are well known in the art and treatment of any such disease/disorder is envisioned. Examples of genetic disease and disorders include, but are not limited to: Angelmen syndrome, ankylosing spondylitis, Apert syndrome, congenital adrenal hyperplasia, cystic fibrosis, Down syndrome, fragile X syndrome, haemochromatosis, hemophilia, Huntington's disease, Klinefelter syndrome, Marfan syndrome, muscular dystrophy, neurofibromatosis, Noonan syndrome, Prader-Willi syndrome, Rett syndrome, Tay-Sachs disease, thalassemia, Tourette syndrome, Turner syndrome, Von Willebrand disease and Williams syndrome.

[0208] In some embodiments, the therapeutic agent is in a lytic granule of the cell. In some embodiments, the therapeutic agent is in a lytic granule of the lymphocyte. In some embodiments, the therapeutic agent is in a secretory granule of the cell. In some embodiments, the therapeutic agent is in a secretory granule of the lymphocyte. In some embodiments, the therapeutic agent is in a secretory lysosome of the cell. In some embodiments, the therapeutic agent is in a secretory lysosome of the lymphocyte. In some embodiments, the therapeutic agent is not in an exosome of the cell. In some embodiments, a lytic granule of the lymphocyte comprises the therapeutic agent. In some embodiments, a

secretory granule of the cell comprises the therapeutic agent. In some embodiments, a secretory granule of the lymphocyte comprises the therapeutic agent. In some embodiments, at least 50% of the therapeutic agent/protein of interest is within the granule/lysosome. In some embodiments, at least 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 97, 99 or 100% of the therapeutic agent/protein of interest is within the granule/lysosome. Each possibility represents a separate embodiment of the invention. In some embodiments, at least 75% of the therapeutic agent/protein of interest is within the granule/lysosome. It will be understood by a skilled artisan that having the therapeutic agent in the lytic/secretory granule and not in exosomes (or not solely in exosomes) is advantageous. Exosomes are produced indiscriminately, and the therapeutic agent would be continuously released if it were in exosomes. Further, under standard conditions exosomes are not highly produced. In contrast, the lytic/secretory vesicle is only secreted upon cellular activation. Thus, as the lymphocyte travels through the subject before engaging its target the therapeutic agent in the lytic vesicle is not released, though exosomal content may be. Upon engagement of the target cell, the lymphocyte becomes activated and large numbers of lytic vesicles are released. Thus, employing the lytic pathway and not the exosome pathway ensures target delivery and robust delivery at the same time. Advantageously, delivery of therapeutic agents via leukocytes (lymphocytes and myeloids), which are activated by recognition of target cells, will result in increased specificity to target cells or cells within a target tissue compared to other known methods of in vivo delivery.

[0209] Pharmaceutical compositions

[0210] By another aspect there is provided a composition comprising a modified cell of the invention.

[0211] In some embodiments, the composition is a pharmaceutical composition. In some embodiments, the composition is a therapeutic composition. In some embodiments, the composition is a composition for use in treatment. In some embodiments, the treatment is treatment of a subject in need thereof. In some embodiments, treatment is treatment of a disease, condition or disorder that is treatable by the therapeutic agent.

[0212] In some embodiments, the composition comprises a population of modified cells. In some embodiments, the composition comprises a plurality of modified cells. In some embodiments, the composition comprises at least 1, 2, 3, 4, 5, 6, 7, 9, 10, 20, 30, 40, 50, 60,

70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900 million or 1 billion modified cells. Each possibility represents a separate embodiment of the invention.

[0213] In some embodiments, the composition is formulated for systemic administration. In some embodiments, the composition is formulated for administration to a subject. In some embodiments, the composition is formulated for intravenous administration. In some embodiments, the composition is formulated for local administration. In some embodiments, the composition is formulated for administration to a subject. In some embodiments, formulated for administration to a subject is formulated without unknown chemical content. In some embodiments, a composition formulated for administration to a subject is chemically defined. In some embodiments, a composition formulated for administration does not comprise animal serum. In some embodiments, animal serum is non-human animal serum. In some embodiments, the subject is a human. In some embodiments, the subject is afflicted with a disease.

[0214] As used herein, the terms “administering,” “administration,” and like terms refer to any method which, in sound medical practice, delivers a composition containing an active agent to a subject in such a manner as to provide a therapeutic effect. One aspect of the present subject matter provides for intravenous administration of a therapeutically effective amount of a composition of the present subject matter to a patient in need thereof. Other suitable routes of administration can include parenteral, oral, subcutaneous, intrathecal, intramuscular, or intraperitoneal.

[0215] The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

[0216] In some embodiments, the composition comprises a pharmaceutically acceptable excipient, carrier or adjuvant. As used herein, the term “carrier,” “excipient,” or “adjuvant” refers to any component of a pharmaceutical composition that is not the active agent. As used herein, the term “pharmaceutically acceptable carrier” refers to non-toxic, inert solid, semi-solid liquid filler, diluent, encapsulating material, formulation auxiliary of any type, or simply a sterile aqueous medium, such as saline. Some examples of the materials that can serve as pharmaceutically acceptable carriers are sugars, such as lactose, glucose and sucrose, starches such as corn starch and potato starch, cellulose and its derivatives such as

sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt, gelatin, talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol, polyols such as glycerin, sorbitol, mannitol and polyethylene glycol; esters such as ethyl oleate and ethyl laurate, agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline, Ringer's solution; ethyl alcohol and phosphate buffer solutions, as well as other non-toxic compatible substances used in pharmaceutical formulations. Some non-limiting examples of substances which can serve as a carrier herein include sugar, starch, cellulose and its derivatives, powdered tragacanth, malt, gelatin, talc, stearic acid, magnesium stearate, calcium sulfate, vegetable oils, polyols, alginic acid, pyrogen-free water, isotonic saline, phosphate buffer solutions, cocoa butter (suppository base), emulsifier as well as other non-toxic pharmaceutically compatible substances used in other pharmaceutical formulations. Wetting agents and lubricants such as sodium lauryl sulfate, as well as coloring agents, flavoring agents, excipients, stabilizers, antioxidants, and preservatives may also be present. Any non-toxic, inert, and effective carrier may be used to formulate the compositions contemplated herein. Suitable pharmaceutically acceptable carriers, excipients, and diluents in this regard are well known to those of skill in the art, such as those described in The Merck Index, Thirteenth Edition, Budavari et al., Eds., Merck & Co., Inc., Rahway, N.J. (2001); the CTFA (Cosmetic, Toiletry, and Fragrance Association) International Cosmetic Ingredient Dictionary and Handbook, Tenth Edition (2004); and the "Inactive Ingredient Guide," U.S. Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER) Office of Management, the contents of all of which are hereby incorporated by reference in their entirety. Examples of pharmaceutically acceptable excipients, carriers and diluents useful in the present compositions include distilled water, physiological saline, Ringer's solution, dextrose solution, Hank's solution, and DMSO. These additional inactive components, as well as effective formulations and administration procedures, are well known in the art and are described in standard textbooks, such as Goodman and Gillman's: The Pharmacological Bases of Therapeutics, 8th Ed., Gilman et al. Eds. Pergamon Press (1990); Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, Pa. (1990); and Remington: The Science and Practice of Pharmacy, 21st Ed., Lippincott Williams & Wilkins, Philadelphia, Pa., (2005), each of which is incorporated by reference

herein in its entirety. The presently described composition may also be contained in artificially created structures such as liposomes, ISCOMS, slow-releasing particles, and other vehicles which increase the half-life of the peptides or polypeptides in serum. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. Liposomes for use with the presently described peptides are formed from standard vesicle-forming lipids which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally determined by considerations such as liposome size and stability in the blood. A variety of methods are available for preparing liposomes as reviewed, for example, by Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York, and see also U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

[0217] The carrier may comprise, in total, from about 0.1% to about 99.99999% by weight of the pharmaceutical compositions presented herein.

[0218] In some embodiments, the composition is a carrier suitable for administration to a human. In some embodiments, the composition does not comprise non-human proteins. In some embodiments, the composition is devoid of non-human proteins. In some embodiments, the carrier contains media for the cell. In some embodiments, the media is lymphocyte media. In some embodiments, the media is myeloid media. In some embodiments, the media is macrophage media. In some embodiments, the media is chemically defined media. In some embodiments, the media is devoid of animal protein. In some embodiments, the media is devoid of animal serum. In some embodiments, the composition is devoid of animal serum. In some embodiments, the composition is non-immunogenic. In some embodiments, non-immunogenic in non-immunogenic to the subject.

[0219] Methods of use

[0220] By one aspect, there is provided a method of delivering a therapeutic agent to a target cell, the method comprising contacting the target cell with a modified cell of the invention, thereby delivering a therapeutic agent to a target cell.

[0221] By another aspect, there is provided a method of delivering a therapeutic agent to a target cell, the method comprising contacting the target cell with a composition of the invention, thereby delivering a therapeutic agent to a target cell.

[0222] By another aspect, there is provided a method of delivering a therapeutic agent to a target cell, the method comprising contacting the target cell with a modified cell expressing a chimeric molecule of the invention, thereby delivering a therapeutic agent to a target cell.

[0223] By another aspect, there is provided a method of delivering a therapeutic agent to a target cell, the method comprising contacting the target cell with a modified cell expressing a polynucleotide of the invention, thereby delivering a therapeutic agent to a target cell.

[0224] In some embodiments, the method is an in vivo method. In some embodiments, the method is an in vitro method. In some embodiments, the method is an ex vivo method. In some embodiments, the method is performed ex vivo. In some embodiments, the target cell is in a subject. In some embodiments, the subject is in need of treatment. In some embodiments, treatment is by the therapeutic agent. In some embodiments, treatment is with the therapeutic agent. In some embodiments, the subject is amenable to treatment by the therapeutic agent. In some embodiments, the method is a method of genome-editing. In some embodiments, the method is a method of gene therapy. In some embodiments, the method is a method of editing a genomic locus. In some embodiments, the subject suffers from a disease. In some embodiments, the subject suffers from a disease or condition. In some embodiments, the disease or condition are treatable by the therapeutic agent.

[0225] In some embodiments, there is provided a method of genome editing within a target cell, the method comprising contacting the target cell with a modified cell of the invention, thereby delivering a gene editing agent to a target cell, wherein the gene editing agent modifies/edits a gene within the nucleus of the target cell. In some embodiments, genome editing comprises modifying a gene within a nucleus of the target cell. In some embodiments, the gene editing agent is a gene editing protein. In some embodiments, the gene editing agent modifies a gene within a nucleus of the target cell.

[0226] In some embodiments, the method of genome editing is a method of treating a genetic disease. In some embodiments, the method of genome editing further comprises providing a genome editing targeting nucleic acid molecule. In some embodiments, the nucleic acid is an RNA. In some embodiments, the RNA is a guide RNA. In some embodiments, the guide RNA is an sgRNA. In some embodiments, the targeting nucleic acid molecule is compatible with the genome editing protein. Targeting nucleic acid molecules for use with genome editing proteins (such as CAS9) are well known in the art. Methods of designing these

sequences are also well known, as are methods for selecting the target sequence for the genome editing. Any such method may be employed. In some embodiments, the nucleic acid molecule is chemically modified. In some embodiments, the nucleic acid molecule comprises a chemically modified backbone.

[0227] In some embodiments, the targeting nucleic acid molecule is provided with the genome editing protein. In some embodiments, the genome editing protein and the targeting nucleic acid molecule are provided together. In some embodiments, the genome editing protein and the targeting nucleic acid molecule are provided in an RNP. In some embodiments, the genome editing protein and the targeting nucleic acid molecule are provided separately. In some embodiments, the genome editing protein and the targeting nucleic acid molecule are provided separately to the target cell. In some embodiments, the genome editing protein and the targeting nucleic acid molecule are provided separately to subject.

[0228] Methods of nucleic acid molecule delivery are well known in the art and are described herein. Any such method of delivery may be employed. Methods of delivery in vitro such as nucleofection, lipofection, transfection, and viral delivery may be employed. Methods of in vivo delivery such as lentivirus, nanoparticle delivery, microparticle delivery, lipid delivery, ligand conjugated delivery and many more are envisioned. Any method known in the art for nucleic acid delivery may be employed to deliver the targeting nucleic acid to the target cells.

[0229] By another aspect, there is provided a method of treating a subject in need thereof, the method comprising administering to the subject a modified cell of the invention, thereby treating a subject.

[0230] By another aspect, there is provided a method of treating a subject in need thereof, the method comprising administering to the subject a composition of the invention, thereby treating a subject.

[0231] By another aspect, there is provided a method of treating a subject in need thereof, the method comprising administering to the subject a modified cell expressing a chimeric molecule of the invention, thereby treating a subject.

[0232] By another aspect, there is provided a method of treating a subject in need thereof, the method comprising administering to the subject a modified cell expressing a polynucleotide of the invention, thereby treating a subject.

[0233] By another aspect, there is provided a method of producing a modified cell of the invention, the method comprising providing the cell, and introducing a therapeutic agent into the cell.

[0234] By another aspect, there is provided a method of producing a modified cell of the invention, the method comprising providing the cell, and introducing a chimeric polypeptide of the invention into the cell.

[0235] In some embodiments, the target cell is a cell of the disease. In some embodiments, the target cell is a cell to which the cell naturally homes. In some embodiments, the target cell is a cell to which lymphocytes naturally home. In some embodiments, the target cell is a cell expressing a surface protein that is a target of a targeting moiety on the cell. In some embodiments, the cell is autologous to the subject. In some embodiments, the cell is allogeneic to the subject. In some embodiments, the cell is syngeneic to the subject. In some embodiments, the cell is an in vitro cell. In some embodiments, the cell is an ex vivo cell. In some embodiments, the cell is in culture. In some embodiments, the cell is in a subject. In some embodiments, the cell is a population of cells expanded in culture.

[0236] In some embodiments, the method further comprises obtaining cells. In some embodiments, obtaining is extracting the cells from a subject. In some embodiments, the method further comprises extracting cells from the subject. In some embodiments, the method further comprises obtaining lymphocytes. In some embodiments, obtaining is extracting the lymphocytes from a subject. In some embodiments, the method further comprises extracting lymphocytes from the subject. In some embodiments, the method further comprises expanding the extracted cells. In some embodiments, the method further comprises activating the extracted cells. In some embodiments, the method further comprises activating the extracted lymphocytes. In some embodiments, the method further comprises modifying the extracted cells. In some embodiments, modifying is modifying with a targeting moiety. In some embodiments, modifying is with the therapeutic agent. In some embodiments, modifying is expressing in the cell. In some embodiments, expressing in the cell is modifying. In some embodiments, modifying is with the chimeric molecule of the

invention. In some embodiments, modifying is expressing within the cells. In some embodiments, modifying produces modified cells. In some embodiments, modified cells are modified cells of the invention. In some embodiments, the method comprises administering the cells to the subject. In some embodiments, the administering is returning the cells to the subject.

[0237] In some embodiments, the modifying or expressing is done after the cells are activated. In some embodiments, the modifying or expressing is done immediately after the activating. In some embodiments, the cells are administered while activated. In some embodiments, the modifying or expressing is done while the cells are active. In some embodiments, the cells are not allowed to return to a stable mode before modifying or expressing. In some embodiments, the modifying or expressing is done in close proximity to the administering. It will be understood by a skilled artisan that for cargo that is transiently loaded it is advantageous not to have the modified cells proliferating for an extended time before administration as this would dilute the concentration of cargo in the effector cells. In some embodiments, the cells are modified not more than 3, 6, 12, 24, 36, 48, 60, 72, 84, 96, 108, or 120 hours before administering. Each possibility represents a separate embodiment of the invention. In some embodiments, the cells are modified not more than 24 hours before administering. In some embodiments, the cells are modified not more than 48 hours before administering. In some embodiments, the cells are modified not more than 72 hours before administering. In some embodiments, the cells are modified not more than 96 hours before administering. In some embodiments, the cells are modified not more than 120 hours before administering. In some embodiments, the modifying or expressing the therapeutic agent is done after the cells are activated. In some embodiments, modifying or expressing a targeting moiety is done before activating. It will be understood by a skilled artisan that in current methods known in the art for expressing cytotoxic proteins/CARs/cytokines and similar molecules in effector cells the effector cells are not activated first. Rather the expression is done first and then the cells are activated. In the instant method the effector cells are first activated. This activation induces the cytoplasm to be full of lytic granules ensuring that the expressed therapeutic agent/chimeric polypeptide will go primarily into the granules and thus facilitate transfer.

[0238] In some embodiments, the modified cells are modified to comprise the cargo of interest within granules (secretory lysosomes, such as lytic granules) thereof. In some embodiments, modification after activation results in loading of cargo into granules. In some embodiments, the cargo of interest comprises a fusion protein disclosed hereinabove. In some embodiments, the obtained cells or the modified cells are further engineered to prevent lytic effect on target cells upon/following activation of the granzyme-perforin pathway. In some embodiments, the modified cells are engineered to knock out endogenous expression of the lymphocytes lytic-granule-secreted protein. In some embodiments, the modified cells are engineered to knock out genes encoding for cell-mediating killing elements like endogenous granzyme and/or FasL and/or Trail. In some embodiments, endogenous expression of FasL and Trail receptors, which are typically expressed on T-cell membranes and initiate apoptosis upon binding to their ligands on target cells, may be knocked out. Genetic knockout can be accomplished by any convenient method as known in the art, e.g., editing with CRISPR/Cas9; introduction of sequences encoding a specific siRNA, shRNA, and the like. In some embodiments, the cell is modified to express a targeting nucleic acid molecule.

[0239] In some embodiments, delivery is delivery to the cytoplasm of the target cell. In some embodiments, delivery is delivery to the nucleus of the target cell. In some embodiments, delivery is delivery to the cytoplasm or nucleus of a target cell. In some embodiments, delivery is through a perforin pore. In some embodiments, the delivery does not comprise endocytosis by the target cell. In some embodiments, delivery is not by exosomes. In some embodiments, delivery is not by extracellular vesicles. In some embodiments, the extracellular vesicles are not lytic extracellular vesicles. In some embodiments, delivery is not via endosomes in the target cell. In some embodiments, delivery is not via phagocytosis. In some embodiments, delivery is delivery to an immune synapse. In some embodiments, delivery comprises editing a genome of the target cell. In some embodiments, delivery comprises editing a genomic locus of the target cell. In some embodiments, delivery comprises gene therapy. In some embodiments, the method further comprises delivering a targeting nucleic acid molecule to the target cell. In some embodiments, the method further comprises delivering a targeting nucleic acid molecule to the subject.

[0240] By another aspect, there is provided a kit comprising a chimeric polypeptide of the invention.

[0241] By another aspect, there is provided a kit comprising a polynucleotide of the invention.

[0242] By another aspect, there is provided a kit comprising a modified cell of the invention.

[0243] By another aspect, there is provided a kit comprising a composition of the invention.

[0244] In some embodiments, the kit further comprises a cell. In some embodiments, the kit further comprises means for expressing the polypeptide in a cell. In some embodiments, the method further comprises means for expressing the protein of interest in the cell. In some embodiments, the kit further comprises means for expressing the polynucleotide in a cell. In some embodiments, the kit further comprises instructions for performing a method of the invention.

[0245] By another aspect, there is provided a modified cell of the invention, a composition of the invention, a modified cell expressing a chimeric molecule of the invention or a modified cell expressing a polynucleotide of the invention for use in a method of delivering a therapeutic agent to a target cell.

[0246] By another aspect, there is provided a modified cell of the invention, a composition of the invention, a modified cell expressing a chimeric molecule of the invention or a modified cell expressing a polynucleotide of the invention for use in a method of treating a subject in need thereof.

[0247] In some embodiments, the use comprises a method of delivery/treating comprising administering the modified cell or composition to the target cell or the subject. In some embodiment, the use comprises a method of the invention. In some embodiments, the use comprises a method provided hereinabove. In some embodiments, the use comprises providing the modified cell. In some embodiments, the use comprises extracting the cell from the subject. In some embodiments, the use comprises modifying the cell. In some embodiments, the use comprises returning the cell to the subject. In some embodiments, the use is use in gene therapy.

[0248] As used herein, the term "about" when combined with a value refers to plus and minus 10% of the reference value. For example, a length of about 1000 nanometers (nm) refers to a length of 1000 nm $\pm$ 100 nm.

[0249] It is noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polynucleotide" includes a plurality of such polynucleotides and reference to "the polypeptide" includes reference to one or more polypeptides and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[0250] In those instances where a convention analogous to "at least one of A, B, and C, etc." is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (e.g., "a system having at least one of A, B, and C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description, claims, or drawings, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase "A or B" will be understood to include the possibilities of "A" or "B" or "A and B."

[0251] It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments pertaining to the invention are specifically embraced by the present invention and are disclosed herein just as if each and every combination was individually and explicitly disclosed. In addition, all sub-combinations of the various embodiments and elements thereof are also specifically embraced by the present invention and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

[0252] Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

[0253] Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

[0254] Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference. Other general references are provided throughout this document.

[0255] **Materials and methods**

[0256] CD8 T cell production and maintenance: Peripheral Blood Mononuclear Cells (PBMC) were isolated from Buffy coats by density gradient centrifugation using Ficoll-Paque. PBMC cells were collected and placed into pre-warmed RPMI 1640 medium (BI, cat# 01-100-1A) and then counted and frozen. 20 million cells were frozen per cryopreservation vial.

[0257] 5 PBMC vials (100 million cells) were defrosted and seeded at a density of 2 million cells per 1ml of complete medium supplemented with 50ng/ml of anti-CD3 antibody and 300U/ml of IL2. Cells were maintained in culture for 5 days. 100U/ml of IL2 was added in each medium supplement during culture.

[0258] CD8 positive cells were isolated by negative selection using CD8 isolation kit (Milteny #130-096-495). The CD8 cells were seeded in cRPMI medium with 100U/ml of IL2 at a density of 1 million cells/ml. Cells at day 6-10 in culture were used for experiments.

[0259] Cell line culture: YTS, P815, and K562 cell lines were maintained in RPMI medium supplemented with 10% Fetal Calf Serum, 1mM Sodium Pyruvate, 4mM L-Glutamine, 1% Penicillin-Streptomycin, and 0.1mM MEM Eagle Non-essential Amino Acids.

[0260] MCF-7 cells were maintained in MEM-alpha medium supplemented with 10% Fetal Calf Serum, 1mM Sodium Pyruvate, 2mM L-Glutamine, 1% Penicillin-Streptomycin, 0.1mM MEM Eagle Non-essential Amino Acids, 1.5 g/L Sodium Bicarbonate, and 0.01 mg/ml insulin. Mel A2- and Mel A2+ were grown in DMEM medium supplemented with 10% Fetal Calf Serum, 1mM Sodium Pyruvate, 2mM L-Glutamine, 1% Penicillin-Streptomycin, 0.1mM MEM Eagle Non-essential Amino Acids, 1.5 g/L.

[0261] Electroporation (EP): For each electroporation $1-8 \times 10^6$ cells were harvested, and medium was replaced by washing three times in Optimem (Thermofisher). Cells were placed in Eppendorf tubes at a concentration of 10×10^6 cells per ml. 3-10ug of insert (DNA plasmid, mRNA, SiRNA or CAS9+sgRNA complex (RNP) were added per 10^6 cells. For mock control the cells were electroporated without insert. The electroporation was done using either Amaxa Nucleofector (Lonza) or Nepa21 gene electroporator (Nepa Gene). Before electroporation, the cells were resuspended in either specific electroporation buffer for the Amaxa (Lonza) or in Optimem (Thermofisher) for the Nepa21. Cells and insert were placed into a 2mm electroporation cuvette for Nepa21 and cell project cuvette (Lonza) for

Amaza. Electroporation with the Nepa21 (NepaGene Unit) was performed using 225-250V pulse length of 2.5ms. Electroporation with Amaza was performed using cell project specific conditions defined by the device.

[0262] After run completion, 400ul of pre-warmed RPMI medium was added to the cuvette and the cells were left in the cuvette for a 20-30 min recovery. The cells were transferred from the cuvette to 24 or 6-well plates at 10^6 cells per ml.

[0263] Cells were maintained in culture from 2 hours to 3 days before being used for experiments.

[0264] Transfection of cells was conducted according to manufacturer's instructions. Briefly, 300K cells were plated in 6-well plates the day prior to the day of transfection. The following day, lipid nanoparticle mixes were prepared with 2ug of DNA together with the Lipofectamin 3000 reagent (ThermoFischer cat#L3000015) and applied on cells in a gentle dropwise manner.

[0265] Transfer assay: $0.5-1 \times 10^5$ K562, P815 or Mel A2+/- target cells were either labeled with Tag-it at final concentrations of 1.6uM according to manufacturer's instruction (Biolegend cat# 425101) or transfected with GFP or gene targeting sgRNA or PNA reporter system and seeded to 96 well U shape plates. $1-6 \times 10^5$ T, YTS, or K562 effector cells were loaded with Cas9-sgRNA RNP complexes or were made to express Granzyme-Cherry, Granzyme-Cas9, Granzyme-meganuclease or Granzyme-meganuclease-GFP fusion constructs and were co-cultured with the target cells. Cells were co-incubated for 2-16 hours at 37°C. Transfer of fusion protein or RNPs was monitored by flow cytometry, fluorescence microscopy, or subsequent genomic sequence analysis in gene of interest for specific gene editing events that occurred as a result of introducing the gene editing proteins to the target cells. For re-directed transfer assay, P815 target cells were pre-coated for 1 hour, in PBS with 2ug/ml of OKT-3 anti-CD3 Ab (Biolegend cat# BLG-317326) at room temperature, washed in PBS, and then co-cultured with target cells. Prior to co-culture with effector cells, target cells were treated with a pan-Caspase inhibitor Z-VAD-FMK (R&D Systems cat# FMK001) for 2 hours at 50uM and its concentration was maintained following the addition of the effector cells and following the cell sorting procedure.

[0266] Cell staining for FACS analysis: Following co-culture, cells were stained for cells viability using the Fixable Viability Dye eFluor™ blue 450 (ThermoFisher cat# 65-0863-18) or near-IR 633/635 (ThermoFisher cat# L10119). Blue and near-IR viability dye, Fixable Viability Dye eFluor™ 450 and 633/635 staining was carried out at room temperature in 100 microliters in U shape 96 well plates. Sample acquisition was done in the following devices: 5-laser Fortessa cell analyzer (BioRad), MacsQuant (Milteny), or BD FACSAria III cell sorter. In addition to measuring the viability dye, cells were also monitored for GFP, PNA RFP and mCherry signal. Prior to fluorescence analysis, forward side scatter gates were initially positioned in accordance with each cell type specific scatter patterns as determined for each cell type by itself. For mCherry signal analysis, target cells were then selected based on GFP expression or Tag-it labeling. For analyzing cells expressing the PNA reporter system, GFP expression was determined on cells gated for RFP expression. Intracellular staining using anti-Crispr/Cas9 Alexa Fluor 488 (Abcam cat#ab191468) and anti-Granzyme B Alexa Fluor 647 (Biolegend cat#515406) was performed with the intracellular Cytofix (Biolegend cat#420801) and Perm Wash (Biolegend cat# 421002) buffers according to the manufacturer's instructions. Analysis was performed using the Flowjo (Becton Dickinson) or FCS Express (DeNovo) software. For cell sorting, the target cell population was isolated based on a two-log difference in Tag-it or RFP signal. Subsequently, target cells sorted after co-cultured with T or YTS cells were supplemented with Pan-caspase inhibitor Z-VAD-FMK (R&D Systems cat#FMK001) in a final concentration of 50uM and an increased concentration of 2% penicillin-streptomycin. Fluorescence microscopy: Images of GFP, RFP, or mCherry expressing cells were captured with the Observer Z1 (Zeiss) fluorescent microscope and Axiovision software (Zeiss) using a range of 2-200msec exposure.

[0267] Granzyme Knock-down: PBMCs were isolated as before. Activated T cells were transduced with retrovirus carrying an engineered antigen-specific TCR against melanoma cells on an HLA-2 background. After 2 days the transduced T cells were collected and plated in triplicate in 6-well plates.

[0268] The cells were then electroporated with GZMB-CAS9 fusion protein as well as an siRNA against the endogenous Granzyme B. The Granzyme B siRNA was designed to target the 3' UTR of endogenously expressed Granzyme B. The siRNA does not recognize the Granzyme B-Cas9 fusion mRNA which contains only the open reading frame of Granzyme

B. Two siRNAs were designed and synthesized, siGzmB-1 and siGzmB-2 (AxoLab, Germany). In order to evaluate the siRNA activity, T cells were electroporated with 300pmol of either siGzmB-1 or siGzmB-2 and tested for Granzyme B protein levels using Granzyme B antibodies and FACS analysis. siGzmB-1 Sense strand: 5'g*g*AgCcAaGuCcAgAuUuA-3' (SEQ ID NO: 45). Antisense strand 5'-U*A*aAuCuGgAcUuGgCuCc*u*u-3' (SEQ ID NO: 46) siGzmB-2 Sense strand 5'-c*g*CuGuAaUgAaAcAcCuU-3' (SEQ ID NO: 47) Antisense strand 5'-A*A*gGuGuUuCaUuAcAgCg*u*u-3' (SEQ ID NO: 48) Lower case nucleotides denote 2'-O-methyl residues. Upper case nucleotides denote 2'-Fluoro residues. An "*" denotes a phosphorothioate backbone modification. Cells were either mock prepared with an empty vector, electroporated with only hGZMB_crmCherry or double electroporated with the expression plasmid and the siRNA.

[0269] The next day target cells were prepared. MART1 expressing melanoma cell lines either expressing HLA-A2 or not were used. Both cell lines were stained with CFSE to distinguish them from the effector T cells. Each well of lymphocyte cells was split in two to produce replicate plates and 2.5×10^4 target cells were added to each well. The cells were allowed to co-culture for 3 hours and then were analyzed by flow cytometry to determine crmCherry transfer.

[0270] RNA-protein complex generation and nucleofection: Cas9 protein (Alt-R S.p. Cas9 Nuclease V3, IDT) or Cas9-GFP (CAS9GFPPRO, Sigma) were mixed with synthetic modified sgRNA. EMX1_1, Rosa26_1, GzmB_1 and GzmB_2, were ordered from Synthego, with 2'-O-methyl and 3' phosphorothioate inter-nucleotide linkages at the first three 5' and 3' terminal RNA residues. For sgRNA sequences and target genes see Table 2. HBB1 sgRNA was ordered from AxoLab with multiple modification along the entire oligo c*u*u*GCC*C*Cf*Af*Cf*AfGGfGfCAGfUfAAgUUUUAGagcuagaaauagcaaGUUaAaA uAaggcuGUccGUUAucAAc*u*u*g*a*a*a*a*a*gugG*ca*c*c*g*a*g*u*cg*g*u*g*c*u *u*u*u*u Capital letters indicate unmodified RNA. Lower case letters indicate 2'-O-methyl residues. "f" indicates 2'-Fluoro residues. "*" indicates phosphorothioate backbone modification. For RNA-protein preparation, 400-573 pmol of Cas9 or Cas9-GFP were mixed with 480-687 pmol sgRNA (keeping ratio of 1.2-1.3 of sgRNA to Cas9 protein) and incubated for 10-20min in RT. The RNA-Protein complex and 400-571 pmol Electroporation Enhancer (IDT) was added to the cells just before electroporation.

[0271] Table 2: sgRNA sequences

sgRNA name	Spacer sequence	SEQ ID NO:	Target gene
EMX1_1	GUCACCUCCAAUGACUAGGG	49	EMX1
Rosa26_1	ACUCCAGUCUUUCUAGAAGA	50	Rosa26
GzmB_1	CUGACAGCUGCUCACUGUUG	51	Granzyme B
GzmB_2	AAGUCUCUGAAGAGGUGCGG	52	Granzyme B
Luc_1	UUGCACGAGAUCGCCAGCGGCGG	75	Luciferase

[0272] RNA-Protein complex transfer assay: 10^5 Tag-it labeled targets cells or Ab coated target cells (for activation) were seeded to 96well U shape plates. After a 2-hour incubation, the target cells were washed and 6×10^5 effector cells (T, K562 or YTS cells) post nucleofection were added to the target cells. 10 μ l Caspase-8 inhibitor was again added for a final concentration of 50 μ M. The cells were co-cultured at 37°C for 4 hours.

[0273] Preparation of samples for SDS Page electroporation: Cells were lysed in RIPA buffer (x20 of cell pellet volume). RIPA buffer was 50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% SDS. Cells were lysed for 30 minutes at 4°C, followed by centrifugation for 10 minutes at 14000RPM. The supernatant was transferred to new Eppendorf tubes and protein concentration was measured by Bradford reagent. To interpolate protein concentration, OD values were plotted to a reference graph of BSA amount vs OD.

[0274] Samples were mixed 1:4 in 4X Laemmli Sample Buffer (BioRad, cat# 1610747) and then heated at 70°C for 10 minutes. Samples were run on Criterion™ TGX Stain-Free Precast Midi Gels. Running conditions were 50 minutes, 200V in Tris/Glycine/SDS running buffer (Biorad cat# 161-0772). Gels were transferred using Trans-Blot TURBO (BioRad Cat# 1704159) at 2.5A and 25V for 10 min.

[0275] Immunoblotting: Blocking was done with 3% BSA in TBST (Cell Signaling Cat#9997S) (0.1% Tween20) for 1 hour at room temperature (RT). The primary antibody used was Rabbit anti-Cas9 (Clontech Cat# 632607). It was diluted 1:2500 in blocking solution and incubation was for 1.5 hours at RT. The secondary antibody was Goat anti Rabbit HRP (Jackson Cat# 115-035-062). It was diluted at 1:10000 in blocking solution and

incubation was for 1 hour at RT. Blots were stained with ponceau-red to confirm loading uniformity.

Example 1: Primary T cells and the NK cell line YTS efficiently transfer Granzyme-Cherry protein to target cells

[0276] In order to test the ability of a Granzyme B fusion protein to transfer a protein of interest to a target cell, a Granzyme B-mCherry fusion protein was created. mCherry was used (SEQ ID NO: 1). The first 11 amino acids, MVSKGEEDNMA (SEQ ID NO: 4), were removed due to possible cleavage sites. This produced the still fluorescent truncated protein crmCherry (SEQ ID NO: 3). Inactive human Granzyme B (G19A/E20A) was placed N-terminal to the crmCherry with a glycine-serine linker (SEQ ID NO: 11) between them. The nucleic acid sequence encoding this fusion protein was inserted into the pMAX expression vector to produce a pMAX-hGZMB-crmCherry vector (SEQ ID NO: 29) (**Fig. 1**).

[0277] In order to assess the subcellular localization profile of the Granzyme B_crmCherry fusion protein, the pMAX-hGZMB-crmCherry vector was expressed in primary human CD8 positive T cells isolated from healthy donors. Cherry fluorescence was confirmed in the electroporated cells.

[0278] Human K562 chronic myelogenous leukemia cells (human erythroleukemic cell line) were used as the target cell. The cells were electroporated with pMaxGFP vector and GFP fluorescence was confirmed in the target cells. The primary T cells were also confirmed to be GFP negative and the K562 cells were confirmed to be Cherry negative.

[0279] Electroporated primary T cells were co-cultured with electroporated K562 cells at an effector to target ratio of 6:1 for 4 hours. Following the co-culture, the cells were analyzed by flow cytometry. The K562 cells could be separated by gating on the target cell population using only forward- and side-scatter (**Fig. 2A**). In order to further ensure that only K562 cells are being analyzed, GFP positive cells were selected within this population (**Fig. 2B**). When this GFP positive population was analyzed for Cherry expression, it was found to be highly Cherry positive (**Fig. 2C**). Indeed, greater than 90% of the GFP positive K562 cells (94.8%) were found to be also Cherry positive. When control cells electroporated with an empty vector were used for the co-culture, the K562 cells were 100% Cherry negative as would be expected (**Fig. 2C**).

[0280] These experiments were repeated with cells of the YTS NK cell line in place of the primary T cells. After co-culture, the K562 cells were again gated based first on forward- and side-scatter and then on GFP expression. The target cancer cells were once again found to be highly Cherry positive (**Fig. 2D**) with 88.1% of the cells found to express Cherry. These results, taken together, show the high efficiency by which T cells and NK cells can transfer Granzyme fusion proteins to target cells. Importantly, when a mRNA IVT product of the coding region of a crmCherry expressing plasmid alone (without fusion to Granzyme) was expressed in GZMB-KO YTS cells, no transfer of Cherry to Tag-it positive K562 target cells was observed (**Fig. 2E**).

Example 2: Granzyme B knock-down and/or knockout potentiates transfer of Granzyme B-crmCherry to target cells

[0281] In order to test the effect of endogenous Granzyme knock-down on the transfer of exogenous Granzyme B fusion proteins, an siRNA molecule against only endogenous Granzyme B was utilized (see Materials and Methods). Primary human T cells were isolated and transduced with a retroviral vector bearing an antigen-specific engineered TCR which is reactive to MART1 and restricted to HLA-A2. These cells were also electroporated with a mRNA IVT product encoding hGZMB_crmCherry (plasmid 1_3 in **Figure 1**) with or without an siRNA knock-down of endogenous Granzyme B.

[0282] Target melanoma cells employed in this assay express HLA-A2 and the surface melanoma antigen MART1, or only MART1 but not HLA-A2. These target cells were stained with CFSE which allows them to be easily distinguished from the T cells. The T cells were co-cultured with the target melanoma cells for 4 hours and then Cherry expression in the CFSE positive cells was assessed. In the HLA-A2 expressing melanoma cells, the T cells with knockdown of endogenous Granzyme B produced a 25% increase in mCherry fluorescent cells as compared to co-culture with non- knockdown cells.

[0283] Further, cell lines were generated in which the endogenous Granzyme B is knocked out. YTS cells were electroporated by Nepa gene electroporator (200 volt, 5 ms), with RNA-Protein complex for CRISPR knockout. Alt-R® S.p. Cas9 Nuclease V3 (IDT) was incubated with either GzmB_1 (SEQ ID NO:51) or GzmB_2 (SEQ ID NO:52) synthetic single guide RNA (sgRNA, Synthego) against Granzyme B to produce stable RNA-protein complex. The YTS cells were electroporated with either RNA-protein complex containing only sgRNA

GzmB_1, only GzmB_2 or with a mixture of both complexes in a 1:1 ratio. These complexes were electroporated into the YTS cells and the cells were allowed to recover in culture for 3 days. Granzyme B expression in the YTS cells was measured after 10 days in culture. As shown in **Figure 3A**, YTS cells electroporated with RNA-Protein GzmB_1 showed nearly 95% reduction in Granzyme B expression relative to parental YTS cells. YTS cells electroporated with RNA-Protein GzmB_2 or mixture of GzmB_1 and GzmB_2 showed significantly lower percentage of Granzyme B reduction (data not shown).

[0284] In order to confirm that the knockout cells indeed have reduced cytotoxic effect, GZMB-KO YTS cells were co-cultured with K562 target cells for either 24 or 48 hours. At 24 hours of co-culture, the percentage of K562 cells gated within the forward scatter typical for untreated cells was significantly higher in K562 cells co-culture with the GZMB KO cells as compared to those co-cultured with non-KO YTS cells. This can be seen by the higher average FCS in the cells co-cultured with the non-knockout cells (**Fig. 3B**). The significant increase in size in K562 co-cultured with parental YTS expressing Granzyme B is typical of osmotic instability induced as a result of perforin-granzyme cell death pathway and is absent in the K562 co-cultured with GZMB KO YTS cells which display a similar size and granularity as naïve non-treated K562 cells.

[0285] To further validate the reduced cytotoxic effect of the GZMB-KO YTS cells, viability of K562 cells was tested after 48 hours of co-culture with parental or KO YTS NK cells. Cells were stained with viability dye (ThermoFisher) as described herein above. As seen in figure **3D**, K562 cells cultured with GZMB-KO YTS cells exhibit reduced dead cell staining (36%) relative to K562 cultured with parental YTS cells (60%). Thus, the GZMB-KO YTS cell line exhibited reduced cytotoxic/lytic activity in comparison to the parental YTS cell line.

[0286] This same co-culture system was used to check mCherry transfer. The GZMB-KO cells and parental YTS cells were transfected with the plasmid expressing granzyme fused to crmCherry (plasmid 1_3 in **Figure 1**). They were then co-cultured with K562 cells for 4 hours as was done previously (Example 1) and the mCherry expression was measured in the target cells. As can be seen in **Figure 3C**, not only did the KO cells effectively transfer mCherry, but the transfer efficiency it actually increased (4.6X MFI ratio increase over

negative cells for KO vs. 2.8X for WT Granzyme), again attesting to the superiority of using non-cytotoxic cells, and particularly GZMB-KO cells, for transfer.

[0287] Taken together these results point-out for effector cells with reduced cytotoxicity as potent delivery vehicles.

Example 3: Granzyme B fused to Cas9 transfers to target cells

[0288] Having established that inactive Granzyme B can successfully transfer mCherry to target cells it was next tested if significantly larger proteins could be transferred in this manner and retain enzymatic activity within the target cells. mCherry has a molecular weight of only about 27 kDa, and the crmCherry is even smaller with a weight of only about 25.5 kDa. In contrast, Cas9, a genome-editing protein, has a molecular weight of over 163 kDa. A second genome-editing protein, meganuclease which is specific to PCSK9, was also investigated. The meganuclease has a molecular weight of about 37 kDa.

[0289] The full Cas9 coding sequence was placed after Granzyme B with a linker (GS linker) between them in the pMax expression plasmid (see **Fig. 1**). The Cas9 also contained an N-terminal HA tag. In order to easily track cells that received the plasmid, crmCherry was inserted downstream of Cas9. A P2A self-cleaving peptide was placed between the Cas9 and the crmCherry. This should result in Cherry expression in the cells that are effectively electroporated, but since the Cherry would be uncoupled from the Granzyme it would not be expected to reach granulocytic vesicles or be transferred. Indeed, when the pMAX-hGZMB_HA-Cas9_P2A_crmCherry vector was expressed in HEK293 cells, the Cherry had a diffuse cytoplasmic staining similar to what was observed when crmCherry was expressed alone. In contrast the Cas9 showed punctate staining indicating entrance into vesicles similar to that observed for Granzyme_crmCherry (data not shown). Meganuclease was also linked to Granzyme B, but with an OLLAS flexible linker. As meganuclease is much smaller than Cas9, GFP could be directly conjugated to the meganuclease itself without the need for a P2A peptide (see **Fig. 1**).

[0290] In order to confirm that the new fusion proteins retained their DNA editing activity, a reporter system (PNA Bio) was employed. Granzyme B is a sufficiently large protein that there is a concern, especially when the linker is not cleavable, that it could impair the function of the editing protein. The PNA reporter system carries genes encoding two fluorescent proteins (RFP and GFP) linked by a specific editing targeting sequence. The RFP

and GFP coding sequences are designed to express the red but not the green fluorescent protein as it is placed out of frame. The system contains two GFP encoding sequences, one placed -1 frameshift from the RFP and one in -2 frameshift from the RFP. Upon expression of an editing nuclease, a double strand break is induced at the editing target site between the RFP and GFP expressing sequences, leading to frameshift mutations and expression of GFP. Following GFP expression, normalized by RFP in cells, demonstrates if the editing nuclease is functionally cutting and to what extent. For evaluating the editing efficiencies of the fused Cas9, K562 cells were electroporated by Nepa gene electroporator (250 volts 2.5 seconds) with 5-8 μ g of EMX1_1-PNA reporter system plasmids (PNA Bio), and 2 μ g of a plasmid expressing EMX1_1 guide (SEQ ID NO: 49). A GZMB-CAS9 fusion protein which lacked the ER signal peptide (ERSP) was expressed (2 μ g) and as a positive control, Cas9 expressing plasmid was also expressed (2 μ g of Cas9 fused to blue fluorescent protein). Removal of the ERSP was necessary to ensure cytoplasmic expression and nuclear localization of the fusion protein within the K562 cells. The same experiment was performed using expression of the meganuclease fusion protein also lacking the ERSP (2 μ g) and a PCSK9-PNA reporter system plasmid (5 μ g). Meganuclease expressing plasmid was used as a positive control (2 μ g). Following 3 days in culture, the editing efficiencies were evaluated by following the GFP signal normalized to the RFP signal by FACS analysis. The results are presented in Table 3. The fusion proteins containing CAS9 or meganuclease both retained their functionality.

[0291] Table 3: Editing quantification with PNA editing system

Plasmid	Description	Percent GFP positive within RFP positive
PNA only	Negative control	3.65%
PCSK9-PNA and meganuclease	Positive control	73.34%
PCSK9-PNA and SP free GZMB-meganuclease (construct 2_6 in Fig. 1)	Fusion with flexible linker	26.25%
EMX-PNA, gRNA and CAS9-BFP	Positive control	93.90%
EMX-PNA, gRNA and SP free GZMB-CAS9 (construct 1_4 in Fig. 1)	Fusion with flexible linker	21.62%
EMX-PNA, gRNA and SP free GZMB-CAS9 (construct 1_6 in Fig. 1)	Fusion with cleavable linker	30.22%

[0292] Next, the ability of the fusion proteins to edit the endogenous genome was tested. In the PNA system, many copies of the target plasmid may be present in a given cell, making editing more likely. However, in an endogenous diploid genome there are only two copies of the target sequence. To test the ability of the fusion proteins to cut endogenous targets, a similar experiment was performed only without the PNA plasmid. The control plasmids and the test plasmids were expressed in K562 cells. The sgRNA was expressed along with the CAS9 plasmids. Following 3 days in culture, genomic DNA was extracted using QuickExtract™ DNA Extraction Solution (Lucigen). The region flanking the editing target was amplified by PCR (Q5 DNA Polymerase, NEB) and the products were sent for next-generation sequencing. Primers used are provided in Table 5. The number of editing events was calculated over the total number of reads and editing efficiencies were normalized to fluorescent cells (indicating insert positive events) as determined by FACS analysis. The results of the genome-editing are summarized in Table 4. The fusion proteins containing CAS9 or meganuclease both retained their genomic editing functionality.

[0293] Table 4: Editing quantification of genomic loci

Plasmid	Description	Editing in genome, %
PCSK9 Meganuclease (SEQ ID NO: 5)	Positive control	76%
ERSP free GZMB Meganuclease (construct 2_5 in Fig. 1 ; SEQ ID NO: 70)	Fusion with flexible linker	15%
CAS9-BFP	Positive control	97%
GZMB-CAS9 lacking ERSP (construct 1_16 in Fig. 1 ; SEQ ID NO: 66)	Fusion with flexible linker	30%
GZMB-CAS9 lacking ERSP (construct 1_17 in Fig. 1 ; SEQ ID NO: 67)	Fusion with cleavable linker	19.5%
GZMB-CAS9 lacking ERSP (construct 1_18 in Fig. 1 ; SEQ ID NO: 68)	Fusion with cleavable linker	14%

[0294] Table 5: Primer list

Target	Name	Sequence	SEQ ID NO:

hEMX1	oETX54_hEMX1gPCRf	ACACTGACGACATGGTTCTACAGTTC CAGAACCGGAGGACAAAGTAC	55
	oETX55_hEMX1gPCRr	TACGGTAGCAGAGACTTGGTCTTCCA GGCCTCCCCAAAGC	56
mRosa	oETX46_mROSAgPCRf	ACACTGACGACATGGTTCTACAGAG GCGGATCACAAGCAATA	57
	oETX47_mROSAgPCRr	TACGGTAGCAGAGACTTGGTCTGGG AGGGGAGTGTTGCAA	58
hPCSK9	oETX48_hPCSK9gPCRf	ACACTGACGACATGGTTCTACACCTG GCTATGGTAGGGACAG	59
	oETX49_hPCSK9gPCRr	TACGGTAGCAGAGACTTGGTCTGCAG TGGGGTGGTGACTTAC	60
hHbb	oETX50_hHBBgPCRf	ACACTGACGACATGGTTCTACACCAA CTCCTAAGCCAGTGC	61
	oETX51_hHBBgPCRr	TACGGTAGCAGAGACTTGGTCTAGTG CCTATCAGAAACCCAAG	62
PNA	oETX67_PNAngsF	ACACTGACGACATGGTTCTACACCAC AACGAGGACTACACCA	63
	oETX68_PNAngsR	TACGGTAGCAGAGACTTGGTCTTGGT GCAGATGAACTTCAGG	64

[0295] As it is now confirmed that the fusion proteins are still functionally able to induce editing, next, the expression profile of the fusion proteins in Granzyme B and perforin expressing cells was evaluated. pMAX-hGZMB_HA-Cas9_P2A_crmCherry vector was electroporated into primary CD8 T cells. 48 hours after electroporation the cells were stained with an anti-Granzyme B antibody as well as an anti-Cas9 and were imaged along with the crmCherry. As can be seen in **Figure 4A**, which shows 4 representative cells, Cas9 and

Granzyme B colocalized within vesicles while mCherry showed a diffuse cytosolic pattern. This indicates that even with its large size Cas9 can enter lytic granules within lymphocytes.

[0296] As it is now confirmed that the fusion proteins are expressed in effector cells expressing Granzyme B and perforin in an expression profile characteristic of lytic granules, the transfer of CAS9 and meganuclease fused to Granzyme B from effector to target cells was evaluated next. 1×10^6 YTS cells were electroporated with 10ug of mRNA IVT product encoding Granzyme B-CAS9 using the Nepa gene unit at 225V with a pulse length of 2.5ms. Fusion protein expressing cells were co-cultured with target cells (K562) in a 1:6 effector to target cell ratio for 4 hours and the transfer of Cas9 was monitored. The target cells were Tag-it labeled and therefore could be easily separated from the NK cells by their Tag-it fluorescence (**Fig. 4B**). Following separation of the Tag-it positive cells using a FACS Aria III FACS sorter, the sorted cells were lysed and run on a western blot. Expression of Cas9 protein in these cells was determined by blot hybridization with an anti-Cas9 antibody. As can be seen in **Figure 4C**, CAS9 protein was detectable in the K562 cells indicating that even such a large protein could be effectively transferred by the lytic granule. Notably, in the lymphocytes themselves two bands are detected. These are the Granzyme-CAS9 fusion protein before cleavage (expected size 190 kDa) and the already cleaved CAS9 (expected size 160 kDa). As cleavage is ongoing in the lytic vesicle, both proteins are detected. In the target cell, only the lower band is detected as by this point the CAS9 is fully separated from the Granzyme.

[0297] Next, YTS Knockout effector cells (generated as described above) were used to evaluate transfer of Granzyme-CAS9 fusion protein to K562 target cells. Since no endogenous Granzyme B is produced in the knockout effector cells, transfer of the fusion protein to target cells was evaluated by FACS detection of Granzyme B, using intracellular staining with Alexa Fluor 647 labeled anti-Granzyme B antibodies, in Tag-it positive K562 target cells. As seen in **Fig. 4D**, Granzyme B was clearly present in K562 cells cultured with GZMB-CAS9-expressing GZMB-KO YTS but was absent from K562 cells cultured with mock transfected GZMB-KO YTS cells. This clearly demonstrates that GZMB-KO YTS effector cells can transfer GZMB-CAS9 fusion protein.

Example 4: Gene-editing after Granzyme mediated transfer

[0298] The ability of CAS9 and meganuclease transferred by Granzyme fusion to carry out genome-editing in the target cells was assessed. CD8 cells were isolated and activated as before. Electroporation of Granzyme-Cas9 mRNA into the CD8 cells and into YTS cells was carried out as before. K562 and melanoma target cells were co-cultured as before and electroporated with the EMX_1 sgRNA encoding plasmid and the PNA-reporter plasmid with an EMX sequence in the cleavage target area. The target cells were electroporated with a NepaGene unit at 250V with a pulse length of 2.5 ms. Cells were allowed to recover and 10^5 cells were seeded in U shaped plates. To this, 6×10^5 T cells or YTS cells with Granzyme-Cas9 or Granzyme-meganuclease were added. T cells or YTS cells electroporated without an insert were used as control. The cells were co-cultured for 4 hours. RFP positive target cells were separated from RFP negative effector cells by a FACS Aria III FACS sorter, and GFP within the RFP positive population was monitored as evidence for gene-editing events. As can be seen in **Figure 5A-B**, cleavage in the target cells occurred as evidenced by the expression of GFP in RFP-positive cells. Thus, it is clear that not only can the CAS9 be transferred from the effector to the target cell by fusion with Granzyme B, but the CAS9 is fully functional and capable of inducing editing in the target cells. Similar results were obtained using T cells (**Fig. 5B**) and YTS cells (**Fig. 5A**) and both in target K562 and melanoma cells.

[0299] The editing efficiency of Granzyme-Cas9 transferred by YTS cells was also evaluated using a GZMB-CAS9 fusion protein with a linker sequence (SEQ ID NO: 2) between the Granzyme and the CAS9 that is predicted to be cleaved in acidic pH (plasmid 1_6, SEQ ID NO: 33). mRNA IVT product of the coding region of this construct was electroporated into YTS cells as before. The editing activity of CAS9 within K562 target cells expressing the PNA-reporter plasmid with an EMX sequence in the cleavage target area and an EMX_1 sgRNA encoding plasmid was tested. Effector cells expressing GZMB-CAS9 were co-cultured with the above mentioned K562 target cells. After 4 hours of co-culture, target cells were sorted based the RFP signal as described herein above and grown for 3 days. The editing efficiencies were evaluated by following the GFP signal normalized to the RFP signal by FACS analysis. As shown in **Figure Fig. 5C**, a substantial three-fold shift in GFP fluorescence is observed in K562 target cells cultured with GZMB-CAS9 expressing YTS effector cells as compared to K562 target cells cultured with mock

transfected effector YTS cells. This indicates that CAS9 transferred by YTS NK cells to K562 target cells retains its editing function even after separation from GZMB in the acidic pH of the lytic granules.

[0300] The editing efficiency of Granzyme-Meganuclease transferred by YTS cells was evaluated using a GZMB-Meganuclease fusion protein (plasmid 2_4; SEQ ID NO: 69). mRNA IVT product of the coding region of this construct was electroporated into YTS cells as before. The editing capability of the PCSK9-Meganuclease within K562 target cells, expressing the PNA-reporter plasmid with a PCSK9 sequence in the cleavage target area was tested. Co-culture between effector and target cells and sorting of RFP positive target cells was performed as described herein above. The editing efficiencies were evaluated after 3 days by following the GFP signal normalized to the RFP signal by FACS analysis. As shown in figure **Fig. 5D**, a substantial shift in GFP fluorescence is observed in K562 target cells cultured with GZMB-Meganuclease expressing YTS effector cells relative to K562 target cells cultured with mock transfected effector YTS cells. This indicates that the GZMB-Meganuclease fusion protein is being transferred by YTS NK cells to K562 target cells and retains its editing function.

[0301] The editing efficiency of Granzyme-Cas9 transferred by YTS cells was also evaluated using a GZMB-CAS9 fusion protein with a linker sequence (SEQ ID NO: 2) between the Granzyme and the CAS9 that is predicted to be cleaved in acidic pH (plasmid 1_6, SEQ ID NO: 33). mRNA IVT product of the coding region of this construct was electroporated into YTS cells as before. The editing activity of CAS9 within K562 target cells expressing a plasmid encoding the PNA-reporter plasmid with a Luciferase sequence in the cleavage target area and a Luciferase_sgRNA was tested (SEQ ID NO: 75). Effector cells expressing the above mentioned Granzyme B-CAS9 were co-cultured with the above mentioned K562 target cells. Cells were grown for 48 hours in co-culture after which editing efficiencies were evaluated by DNA extraction and PCR amplification of the flanking region of the recognition site of the Luciferase sgRNA. YTS mediated transfer of Granzyme B-CAS9 resulted in 15.7% of editing in K562 cells. This indicates that CAS9 transferred by YTS NK cells to K562 target cells retains its editing function even after separation from GZMB in the acidic pH of the lytic granules.

[0302] Having shown editing of the exogenous PNA plasmid, editing of the endogenous genome was now tested. Genome-editing mediated by transfer of the Granzyme B-CAS9 fusion protein in co-culture was performed as described hereinabove. Target cells were electroporated with a plasmid encoding EMX-1 sgRNA fused to mCherry. After 4 hours of co-culture, target cells were sorted based on mCherry signal and incubated for 3 days, after which genomic DNA was extracted. Edited reads were quantified by next generation sequencing. T cell transfer Granzyme B-CAS9 fusion protein produced an editing of 2% in melanoma cells, 1.25% in K562 cell and YTS cell transfer into K562 cells produced 0.71% editing, normalized to viable cells and transfer efficiency. This demonstrates that endogenous editing is possible in target cells that received the genome-editing protein by Granzyme mediated transfer.

[0303] The ability of PCSK9-specific meganuclease to carry out gene editing in the genome of the target cell, following Granzyme B mediated delivery, was assessed. Transfection of mRNA IVT product of the coding region of Granzyme-meganuclease from plasmid 2_4 into YTS cells was carried out as before. K562 target cells were labeled with Tag-it as described herein above. Effector and target cells were co-cultured for 4 hours after which target cells were sorted by a FACS Aria III FACS sorter based on Tag-it signal. Sorted target cells were cultured for 3 additional days and genomic DNA was extracted. Edited reads were quantified by next generation sequencing. YTS cell transfer of GZMB-meganuclease into K562 cells produced 1.4% genome editing after normalizing for the viable cells and transfer efficiency. This demonstrates that endogenous editing is possible in target cells that received the genome-editing protein by Granzyme mediated.

Example 5: Myeloid cells are capable of transferring genome-editing proteins

[0304] Myeloid cells are known to also express perforin and granzyme similar to lymphocytes, but they do not produce lytic granules. K562, Chronic Myeloid Leukemia cell line was used as an experimental model to test the ability of myeloid cells to successfully transfer cargo. To investigate whether K562 can be used as a potent delivery vehicle, first the transfer of crmCherry fused to Granzyme B from K562 cells to melanoma cells was tested. K562 cells were electroporated with mRNA IVT product of the coding region of plasmid 1_3 as described hereinabove. K562 cells and Tag-it labeled melanoma cells were co-cultured for 4 hours and mCherry signal within the Tag-it positive cells was monitored.

As can be seen in **Figure 6A**, ~30% of the Tag-it positive target melanoma cells were detected as being mCherry positive. This clearly demonstrates that myeloid cells are also capable of Granzyme mediated protein transfer.

[0305] The transfer of meganuclease by K562 as effector cells was tested next. K562 cells were electroporated with 5 ug of plasmid 2_6 (shown in **Figure 1**) encoding a human mutant Granzyme B linked to meganuclease and GFP. The K562 cells were incubated with target melanoma cells stained with Tag-it and co-cultured for 4 hours. GFP was detected in the Tag-it positive cells (~30% of Tag-it positive cells) indicating that the meganuclease was successfully transferred (**Fig. 6B**). Thus, surprisingly, myeloid cells were also competent to transfer genome-editing proteins.

[0306] The ability of CAS9 and meganuclease transferred by Granzyme fusion to carry out editing in target cells was assessed. K562 cells were electroporated with Granzyme-PCSK9-specific meganuclease mRNA IVT products coding sequence of plasmid 2_4 as described herein above. Manipulated K562 cells were co-cultured with the melanoma target cells transfected with PNA-reporter plasmid with an PCSK9 sequence in the cleavage target area (for meganuclease) using lipofectamine 3000 as described hereinabove. 5×10^4 transfected melanoma target cells were seeded in U shaped 96 well plates. To this, 2×10^5 K562 cells with Granzyme-PCSK9 meganuclease were added. K562 cells electroporated without an insert were also used as a control. The cells were co-cultured for 4 hours. RFP positive target cells were separated from RFP negative effector cells by a FACS Aria III FACS sorter. Sorted cells were kept in culture for 3 days after which GFP within the RFP positive population was monitored as evidence for gene-editing events. As can be seen in **Figure 6C**, cleavage in the target cells occurred as evidenced by the expression of GFP in RFP positive melanoma target cells. Thus, it is clear that not only can the editing nuclease be transferred from myeloid effector cells to target cells by fusion with Granzyme B, but the nucleases are also fully functional and capable of inducing editing in the target cells.

[0307] The ability of PCSK9-specific meganuclease to carry out gene editing in the genome of melanoma target cells, following Granzyme B mediated delivery by K562 as effector cells, was assessed. Electroporation of mRNA IVT product of the coding region of Granzyme-meganuclease (plasmid 2_4 described in **Figure 1**) into YTS cells was carried out as before. K562 target cells were labeled with Tag-it as described herein above. Effector

and target cells were co-cultured for 4 hours after which target cells were sorted by a FACS Aria III FACS sorter based on Tag-it signal. Sorted target cells were cultured for 3 additional days and genomic DNA was extracted. Edited reads were quantified by next generation sequencing. K562 cell transfer of GZMB-meganuclease into melanoma cells produced 2% genome editing after normalizing for the viable cells and transfer efficiency. This demonstrates that endogenous editing is possible in target cells that received the genome-editing protein by Granzyme mediated.

Example 6: T cells and NK cells mediate transfer of CAS9 + gRNA complex to target cell

[0308] In addition to Granzyme B mediated transfer, direct transfer of protein-RNA complex by lymphocytes was also tested. Primary CD8 positive T cells were isolated and activated as before. Cas9-GFP was incubated with a synthetic single guide RNA (sgRNA) against EMX1 to produce stable RNA-protein complex. This complex was electroporated into the primary T cells with the Nepa21 (NepaGene Unit) at 225V and pulse length of 2.5ms. Following a short recovery culture GFP expression in the T cells was confirmed (>90% GFP positive) (**Fig. 7A**).

[0309] Two different target cells were labeled with CFSE as before: human K562 cells and mouse p815. The electroporated T cells and labeled target cells were co-cultured for 4 hours as before. After co-culture the cells were stained with a viability dye (Near infrared APC-Cy7 LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, cat# L10119) and with anti-CD8 conjugated to Alexa-Fluor 421 (Biolegend). The cells were analyzed both by flow-cytometry and by microscopy with a ImageStream®X Mk II Imaging Flow Cytometer (Luminex).

[0310] The effector cells and the target cells were distinguished by CD8 staining, as the p815 cells and K562 cells are negative for CD8 (**Fig. 7B**). When the CD8 negative cells were examined a large proportion of them were found to be GFP positive (**Fig. 7C**) indicating that the Cas9-GFP fusion protein was successfully transferred to the target cells. Cell imaging confirmed the presence of CD8-/GFP+ cells (**Fig. 7D**). The GFP signal in the target cells was disperse, indicating that protein reaches the cytoplasm. Similar results were found for transfer to p815 cells and K562 cells. The results are summarized in Table 6.

[0311] The same experiments were performed with YTS cells effector cells and K562 or human MCF7 breast cancer cells as target cells. The YTS cells also expressed high levels of GFP (>50% GFP positive) (**Fig. 8A**). The target cells were stained with Tag-it cell viability dye and could therefore be distinguished from the YTS cells due to their being Tag-it positive (**Fig. 8B and 8D**). The Tag-it positive cells were then assessed for GFP expression. Both K562 (**Fig. 8C**) and MCF7 cells (**Fig. 8E**) were found to be highly GFP positive indicating that even the very large RNA-Protein complex was able to transfer and even without fusion to Granzyme B. The results of transfer are summarized in Table 6.

[0312] Table 6: Results of RNA-Protein complex transfer

Effector Cells	Target Cells	Transfer results	Target cell viability
T cells	Mouse p815 (replicate 1)	40%	50%
T cells	Mouse p815 (replicate 2)	25%	92%
T cells	Human K562	65%	80%
YTS cells	Human K562	56%	65%
YTS cells	Human K562	37%	80%
YTS cells	Human MCF7	77%	47%

[0313] Even though the RNP did not contain Granzyme protein, it was hypothesized that the transfer was still mediated by the lytic granule and synapse formation. This would mean that exosomes or other extracellular vesicles could not simply be isolated from the cells as direct contact and recognition of the target cells would be necessary for transfer. To test this hypothesis YTS cells were transduced with CAS9-GFP and cultured for 14-16 hours after which media from the cells was collected. The conditioned media was added to target K562 cells and melanoma cells and incubated overnight. Neither the K562 (**Fig. 8F**) nor the melanoma cells (**Fig. 8G**) were found to become GFP positive. When the effector cells were directly co-cultured with the target K562 cells (**Fig. 8H**) and melanoma cells (**Fig. 8I**) the target cells became highly GFP positive indicating protein transfer. This indicates that secreted vesicles, like exosomes, are not responsible for this non-Granzyme mediated transfer, but rather direct cellular contact is required.

[0314] As it is now confirmed that CAS9-sgRNA complexes are transferred from effector to target cells, the ability of the RNA-Protein complex to carry out gene-editing in the target

cells was also assessed. YTS and T cells were electroporated with RNA-Protein complexes as described hereinabove. The target K562 and melanoma cells were electroporated or transfected (respectively) with a PNA-reporter plasmid with an EMX1 sequence in the cleavage target area. Effector and target cells were co-cultured for 4 hours followed by sorting of target cells based on the RFP signal of the reporter plasmid. Sorted target cells were cultured for 3 days and editing was measured as before based on the presence of GFP signal in RFP gated cells. Even though the RNA-Protein complex did not comprise Granzyme, the CAS9 complex transferred, retained its sgRNA and was able to induce editing in the target cell (**Fig. 9A**). Similar results were observed for YTS cells and T cells used as the effector cells and for melanoma and K562 cells used as the target cells. Importantly, YTS cells that had endogenous Granzyme B knocked out and thus had reduced cytotoxicity, were also competent for transfer, sgRNA retention and editing in the target cell (**Fig. 9B**).

[0315] Next, it was examined if myeloid cells could also transfer RNA-protein complex that was capable of performing gene-editing in target cells. K562 cells were transduced with the CAS9-sgRNA complex as was done with the T cells and YTS cells. They were then co-cultured with melanoma cells expressing the editing reporter plasmid and it was observed that editing still occurred in the target cells (**Fig. 9C**). This indicates that myeloid cells, and indeed other perforin/granzyme expressing cells, are capable of transferring cargo and indeed are specifically capable of delivering functional genome-editing proteins/complexes.

[0316] Having shown editing of the exogenous PNA plasmid, editing of the endogenous genome by RNP delivery was tested. T cells, YTS cells, Granzyme B KO YTS cells and K562 cells were all used as effector cells to test RNP delivery and genome-editing. Melanoma cells, K562 cells and mouse P815 cells were all used as target cells. Editing events were quantified as described hereinabove by next-generation sequencing of the target locus. The results of the endogenous gene editing are summarized in Table 7. These results confirm that not only is the RNP transferred, but the guide RNA stays associated with the CAS9 protein during transfer and the complex is able to carry out genome-editing in the nucleus of the target cell.

[0317] Table 7: Results of endogenous gene editing after RNP transfer normalized to viable cell result.

Effector cell	Target cell	Editing
T Cell	MEL	8.3%
YTS GZMB KO	K562	2.87%
YTS	MEL	5%
YTS GZMB KO	MEL	2.8%
K562	MEL	5.6%

[0318] Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

CLAIMS:

1. A method of delivering a non-lytic therapeutic protein of interest into a target cell, the method comprising contacting said target cell with a modified leukocyte wherein said modified leukocyte comprises reduced cytotoxicity as compared to a non-modified leukocyte and comprises said non-lytic therapeutic protein of interest, thereby delivering a non-lytic therapeutic protein of interest into a target cell.
2. A method of delivering a genome editing protein into a target cell, the method comprising contacting said target cell with a modified leukocyte wherein said modified leukocyte comprises said genome editing protein, thereby delivering a non-lytic therapeutic protein of interest into a target cell.
3. The method of claim 1 or 2, wherein the non-lytic therapeutic protein of interest or genome editing protein is delivered to the cytoplasm or nucleus of the target cell.
4. The method of any one of claims 1 to 3, wherein said modified leukocyte is capable of forming an immune synapse with said target cell.
5. The method of claims 1 to 4, wherein said modified leukocyte comprises said non-lytic therapeutic protein of interest or genome editing protein within a secretory lysosome.
6. The method of any one of claims 1 to 5, wherein said modified leukocyte does not comprise said non-lytic therapeutic protein of interest or genome editing protein within or conjugated to said modified cell's cellular membrane.
7. The method of any one of claims 1 to 6, wherein said modified leukocyte is selected from a modified T cell, modified natural killer (NK) cell and a modified myeloid cell.
8. The method of any one of claims 1 to 7, wherein said modified leukocyte is a modified non-cytotoxic leukocyte, or wherein said modified leukocyte has been further modified to reduce cytotoxicity.

9. The method of any one of claims 1 to 8, wherein said modified leukocyte comprises a knockout or knockdown or at least one endogenous cytotoxic protein, optionally wherein said endogenous cytotoxic protein is Granzyme B.
10. The method of any one of claims 1 to 9, wherein said modified leukocyte comprises a mutation in at least one endogenous cytotoxic protein and wherein said mutation decreases cytotoxicity of said endogenous cytotoxic protein.
11. The method of any one of claims 1 to 9, wherein said modified leukocyte comprises an anti-sense mediated reduction of at least one endogenous cytotoxic protein and wherein said anti-sense mediated decreases cytotoxicity of said endogenous cytotoxic protein.
12. The method of any one of claims 1 and 3 to 11, wherein said non-lytic therapeutic protein of interest does not comprise any of:
 - a. a naturally secreted protein;
 - b. a membranal protein expressed in the membrane of the modified leukocyte;
 - c. a surface receptor-binding protein;
 - d. a viral penetration or envelope protein; and
 - e. a nanoparticle conjugated or encapsulated protein.
13. The method of any one of claims 1 to 12, wherein said non-lytic therapeutic protein of interest or genome editing protein is a cytoplasmic or nuclear protein.
14. The method of any one of claims 1 to 13, wherein said non-lytic therapeutic protein of interest or genome editing protein does not comprise a signal peptide.
15. The method of any one of claims 1 to 14, wherein said non-lytic therapeutic protein of interest or genome editing protein is a ribonuclear-protein (RNP) complex.
16. The method of any one of claims 1 and 3 to 15, wherein said non-lytic therapeutic protein of interest comprises a genome editing protein.

17. The method of any one of claims 2 to 16, wherein said genome editing protein modifies a gene within a nucleus of the target cell.
18. The method of any one of claims 2 to 17, wherein said genome editing protein is a meganuclease.
19. The method of any one of claims 1 to 18, wherein said non-lytic therapeutic protein of interest or genome editing protein comprises a molecule weight of at least 50 kDa.
20. The method of any one of claims 2 to 19, wherein said genome editing protein is CRISPR associated protein 9 (Cas9).
21. The method of any one of claims 1 to 20, wherein said non-lytic therapeutic protein of interest is a chimeric protein comprising a lymphocyte lytic granule-secreted protein or a functional fragment or variant thereof and a therapeutic polypeptide or wherein said genome editing protein is a chimeric protein comprising a lymphocyte lytic granule-secreted protein or a functional fragment or variant thereof and said genome editing protein.
22. The method of claim 21, wherein said lymphocyte lytic granule-secreted protein or a functional fragment or variant thereof comprises a signal peptide, optionally wherein said signal peptide is an N-terminal signal peptide.
23. The method of claim 20 or 22, wherein said lymphocyte lytic granule-secreted protein is directly conjugated to said therapeutic polypeptide or genome editing protein by a peptide bond or is indirectly conjugated by a protein linker.
24. The method of claim 23, wherein said linker is a cleavable linker, optionally wherein said linker is cleaved in a secretory granule or at acidic pH.
25. The method of any one of claims 20 to 24, wherein said therapeutic polypeptide or genome editing protein comprises a nuclear localization sequence (NLS).

26. The method of any one of claims 20 to 25, wherein said lytic granule-secreted protein is a lytic protein comprising at least one inactivating mutation, wherein said inactivating mutation inhibits the lytic function of said lytic protein.
27. The method of any one of claims 20 to 26, wherein said lymphocyte lytic granule secreted protein is selected from the group consisting of: granzyme A, granzyme B, granzyme H, granzyme K, granzyme M, Granulysin, Serglycin, and Perforin.
28. The method of claim 27, wherein said lymphocyte lytic granule secreted protein is Granzyme B.
29. The method of any one of claims 1 to 28, wherein said transfer is not mediated by exosomes.
30. The method of any one of claims 1 to 29, wherein delivering to a cytoplasm does not comprise entering an endosome.
31. The method of any one of claims 1 to 30, further comprising providing said leukocyte, activating said leukocyte and expressing said non-lytic therapeutic protein of interest or genome editing protein in said leukocyte after said activating to produce said modified leukocyte.
32. The method of claim 31, wherein said expression is done not more than 5 days before said contacting.
33. The method of any one of claims 1 to 32, wherein said target cell is in a subject in need of treatment with said non-lytic therapeutic protein of interest or genome editing protein, and said method comprises administering a pharmaceutical composition comprising said modified leukocyte.
34. The method of claim 33, wherein said subject is in need to gene therapy and said modified leukocyte comprises a genome editing protein.
35. The method of claim 33 or 34, wherein said modified leukocyte is autologous or allogeneic to said subject.

36. The method of any one of claims 33 to 35, comprising extracting leukocytes from said subject, activating said leukocytes, expressing said non-cytotoxic therapeutic protein of interest or genome editing protein in said leukocytes after said activating to produce said modified leukocytes and returning said modified leukocytes to said subject.
37. The method of claim 36, wherein said expressing is done not more than 5 days before said returning.
38. The method of any one of claims 33 to 37, wherein said treatment does not comprise killing said target cell.
39. The method of any one of claims 33 to 38, wherein said subject does not suffer from cancer.
40. A non-lytic chimeric polypeptide comprising a lymphocyte lytic granule-secreted protein or a functional fragment or variant thereof and a protein of interest.
41. The chimeric polypeptide of claim 40, wherein said protein of interest does not bind a cell surface receptor.
42. The chimeric polypeptide of claim 40 or 41, wherein said lymphocyte lytic granule-secreted protein is directly conjugated to said protein of interest by a peptide bond or is indirectly conjugated by a protein linker.
43. The chimeric polypeptide of claim 42, wherein said linker is a cleavable linker, optionally wherein cleavable linker is cleaved in a secretory granule or at acidic pH.
44. The chimeric polypeptide of any one of claims 40 to 43, wherein said protein of interest comprises at least one NLS.
45. The chimeric polypeptide of any one of claims 40 to 44, wherein said lymphocyte lytic granule-secreted protein is selected from the group consisting of: granzyme A, granzyme B, granzyme H, granzyme K, granzyme M, and Perforin.
46. The chimeric polypeptide of claim 45, wherein said lymphocyte lytic granule-secreted protein is granzyme B.

47. The chimeric polypeptide of any one of claims 40 to 46, wherein said protein of interest is a genome-editing protein.
48. The chimeric polypeptide of claim 47, wherein said genome-editing protein is CRISPR associated protein 9 (Cas9).
49. The chimeric polypeptide of claim 47, wherein said genome-editing protein is a meganuclease.
50. The chimeric polypeptide of any one of claims 40 to 49, wherein said protein of interest comprises a molecular weight of at least 50kDa.
51. A polynucleotide encoding a chimeric polypeptide of any one of claims 40 to 50.
52. The polynucleotide of claim 51, wherein said polynucleotide is an expression vector capable of expressing said chimeric polypeptide in a lymphocyte or myeloid cell.
53. A modified leukocyte with reduced cytotoxicity as compared to a non-modified leukocyte, comprising at least one of:
- a. a non-cytotoxic chimeric polypeptide of any one of claims 40 to 50;
 - b. a polynucleotide of claim 51 or 52; and
 - c. a secretory granule comprising a non-cytotoxic therapeutic protein of interest.
54. The modified leukocyte of claim 53, wherein said leukocyte is capable of forming an immune synapse with a target cell.
55. The modified leukocyte of claims 53 or 54, wherein said leukocyte is selected from a T cell, a natural killer (NK) cell, and a myeloid cell.
56. The modified leukocyte of any one of claims 53 to 55, wherein said modified leukocyte does not comprise said non-cytotoxic therapeutic protein of interest within or conjugated to said modified leukocyte's cellular membrane.

57. The modified leukocyte of any one of claims 53 to 56, wherein said modified leukocyte is a modified non-cytotoxic leukocyte, or wherein said modified leukocyte comprises a mutation of at least one endogenous cytotoxic protein wherein said mutation decreases the cytotoxicity of said endogenous cytotoxic protein.
58. The modified leukocyte of any one of claims 53 to 57, wherein said modified cell comprises a knockout or knockdown or at least one endogenous cytotoxic protein, optionally wherein said endogenous cytotoxic protein is Granzyme B.
59. A therapeutic composition comprising a modified leukocyte of any one of claims 53 to 58.
60. The therapeutic composition of claim 59, formulated for administration to a subject, and comprising a pharmaceutically acceptable carrier, excipient, or adjuvant or both.
61. A kit comprising at least one of:
- a. a non-cytotoxic chimeric polypeptide of any one of claims 40 to 50;
 - b. a polynucleotide of claim 51 or 52;
 - c. a modified leukocyte of any one of claims 53 to 58; and
 - d. a therapeutic composition of claim 59 or 60.

IN Granzyme B inhibitory dipeptide
GZB – mature Granzyme B
GSL – glycine serine linker
2A – bicistronic sequence
CL - cleavage linker
No ERSP- NO ER signal peptide
OLLAS - linker

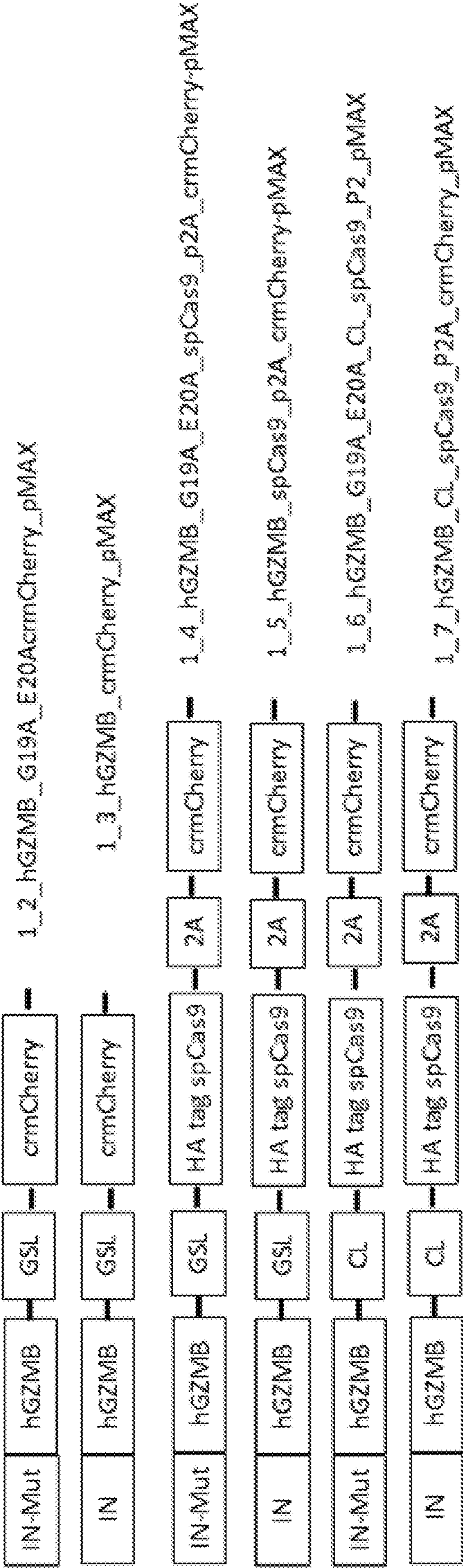


Figure 1

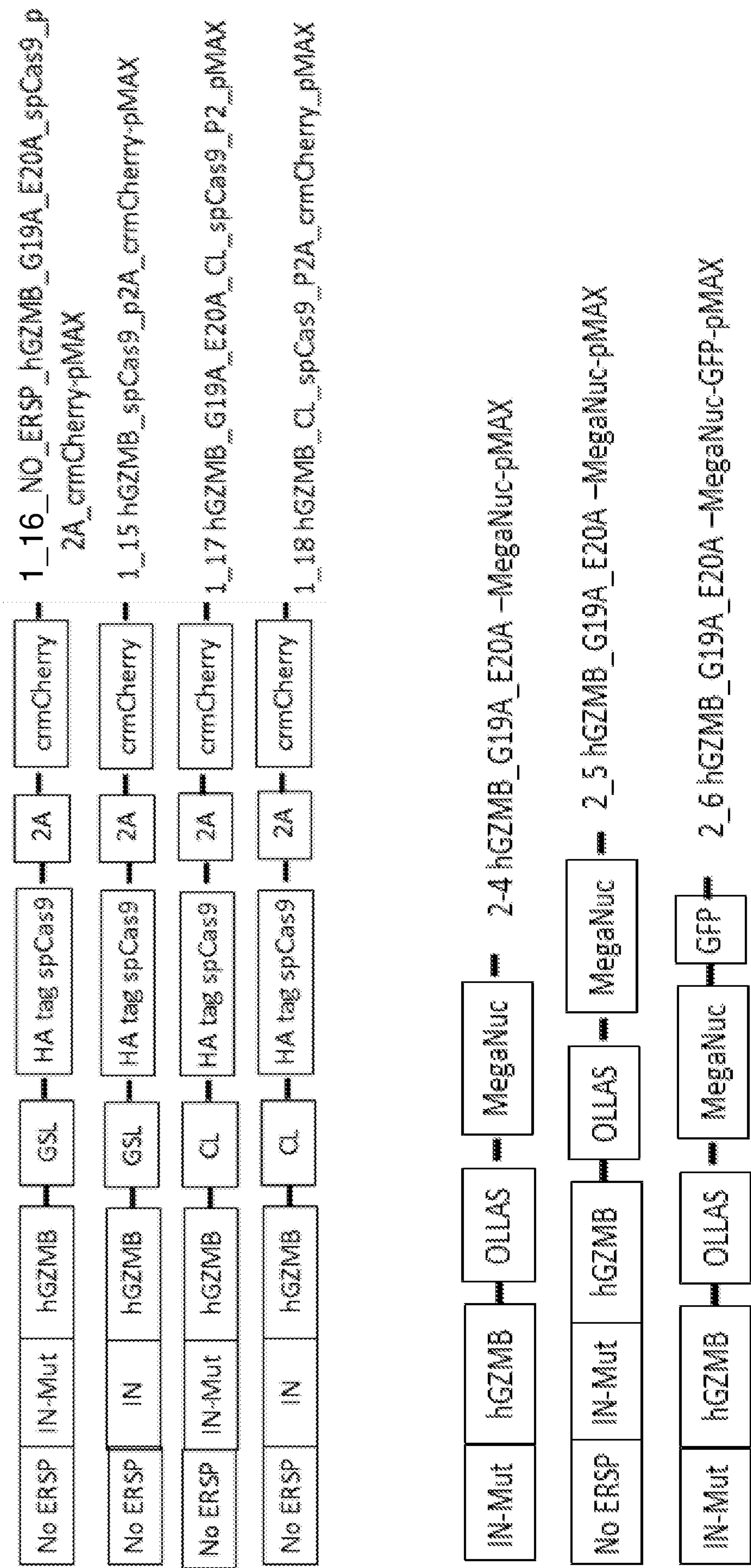


Figure 1 continued

Figure 2A

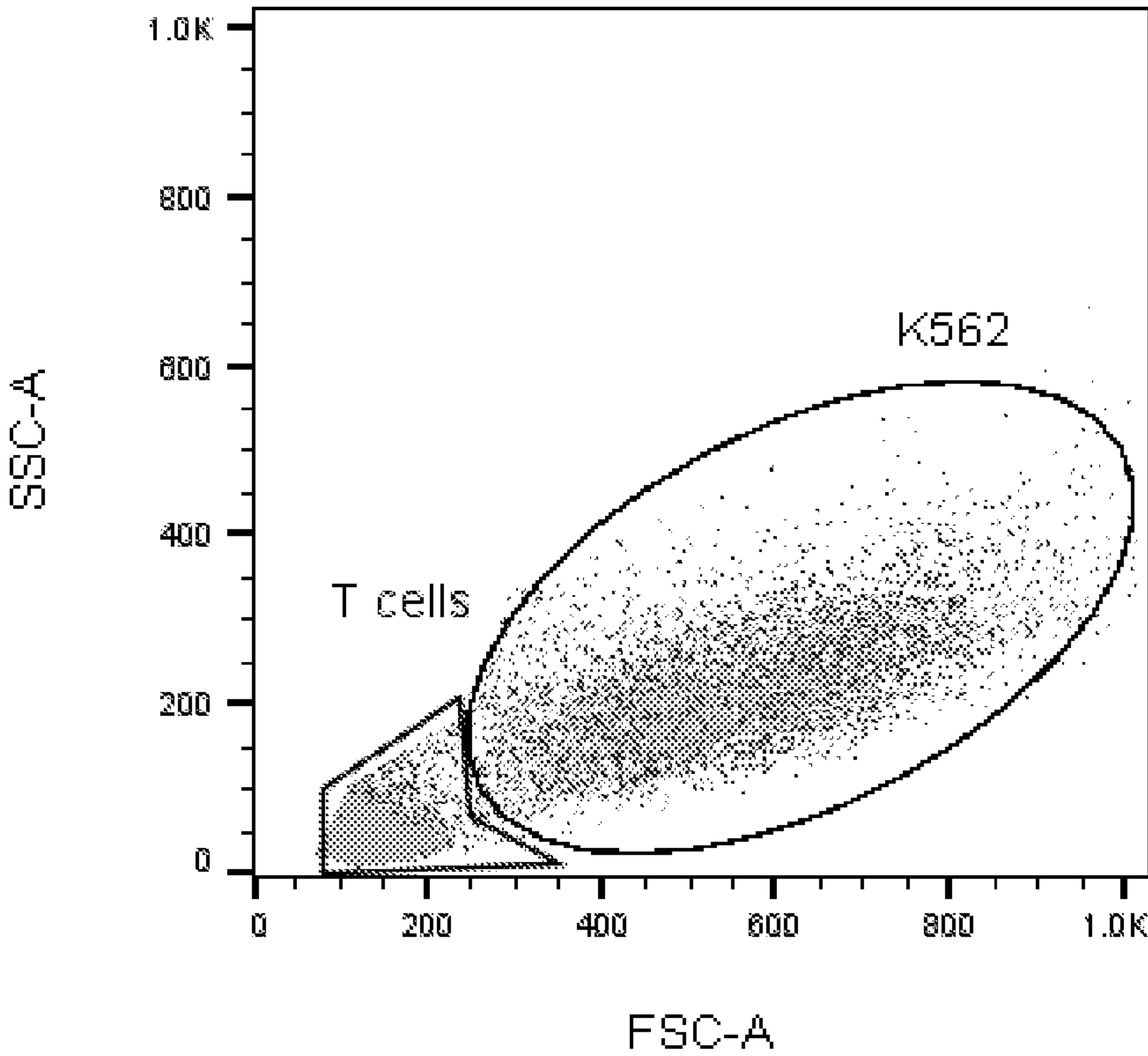


Figure 2B

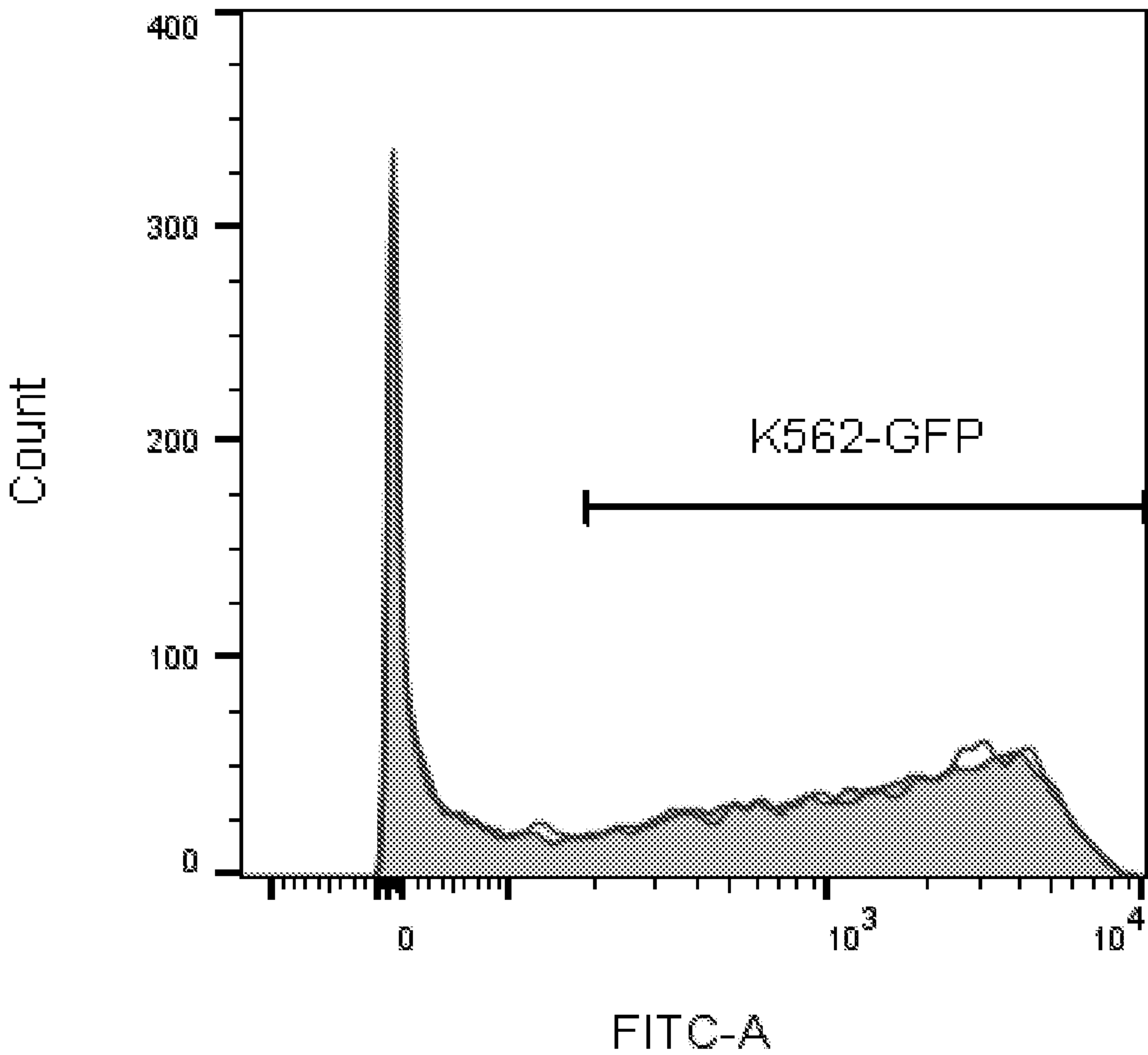


Figure 2C

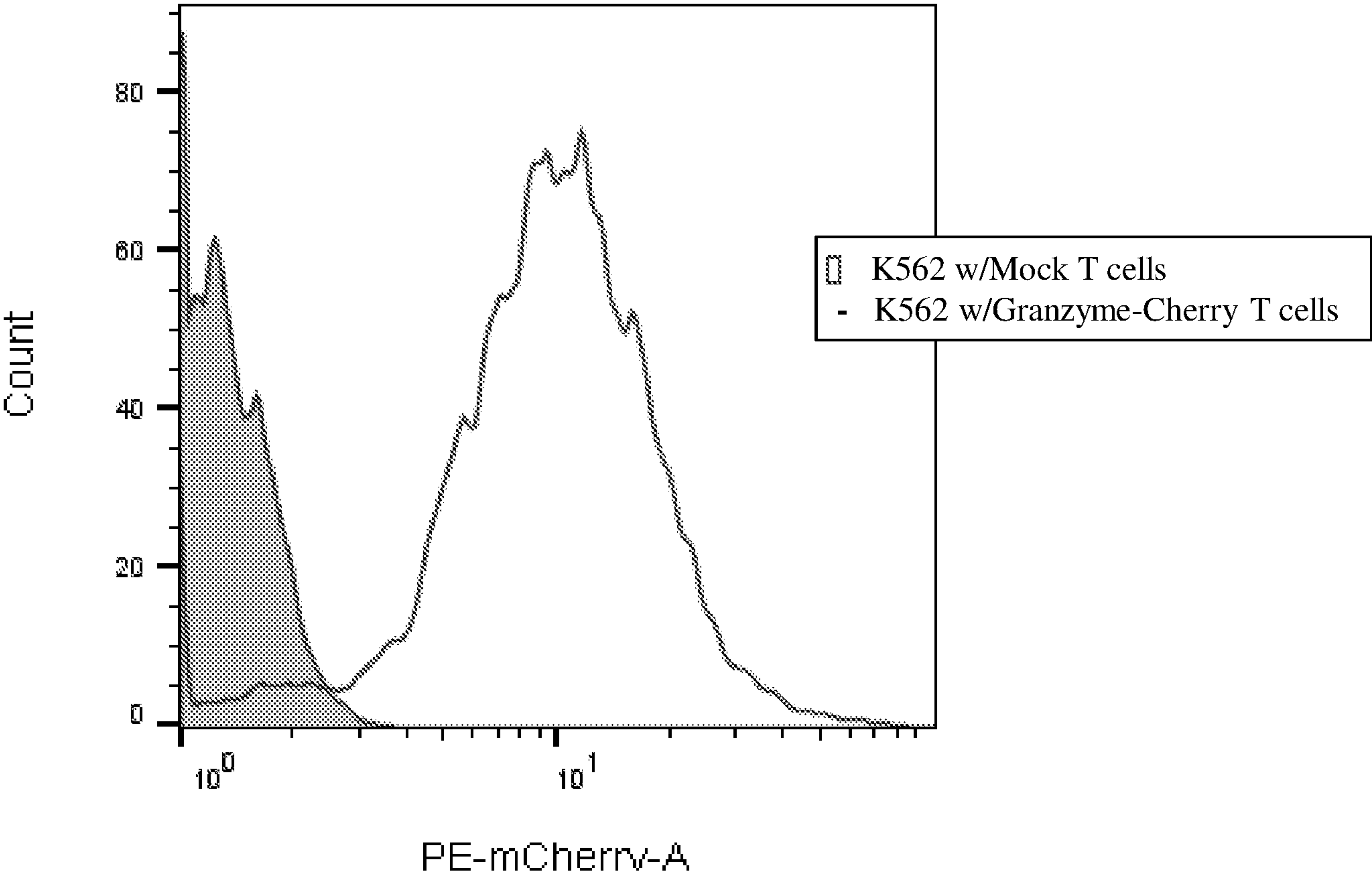


Figure 2D

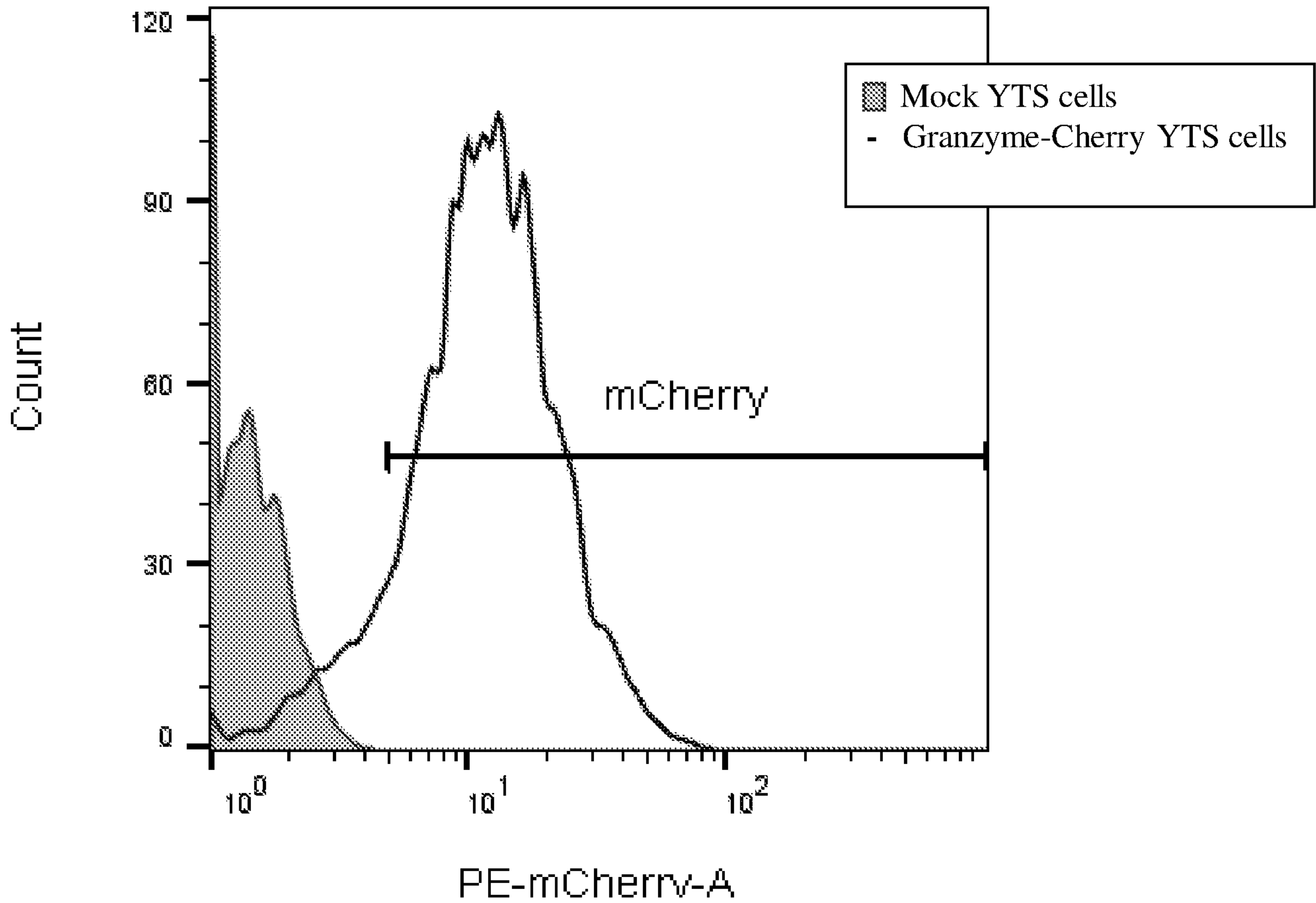


Figure 2E

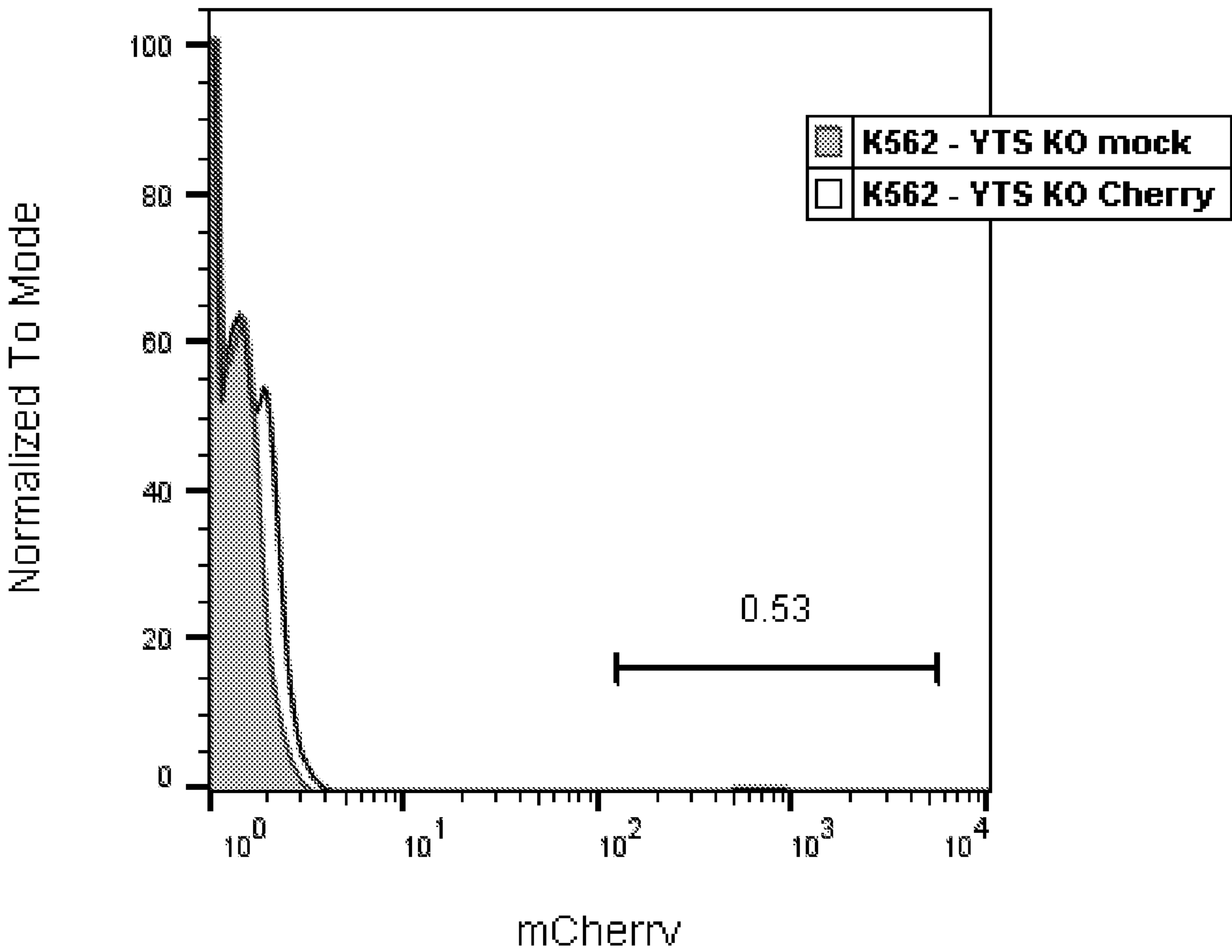


Figure 3A

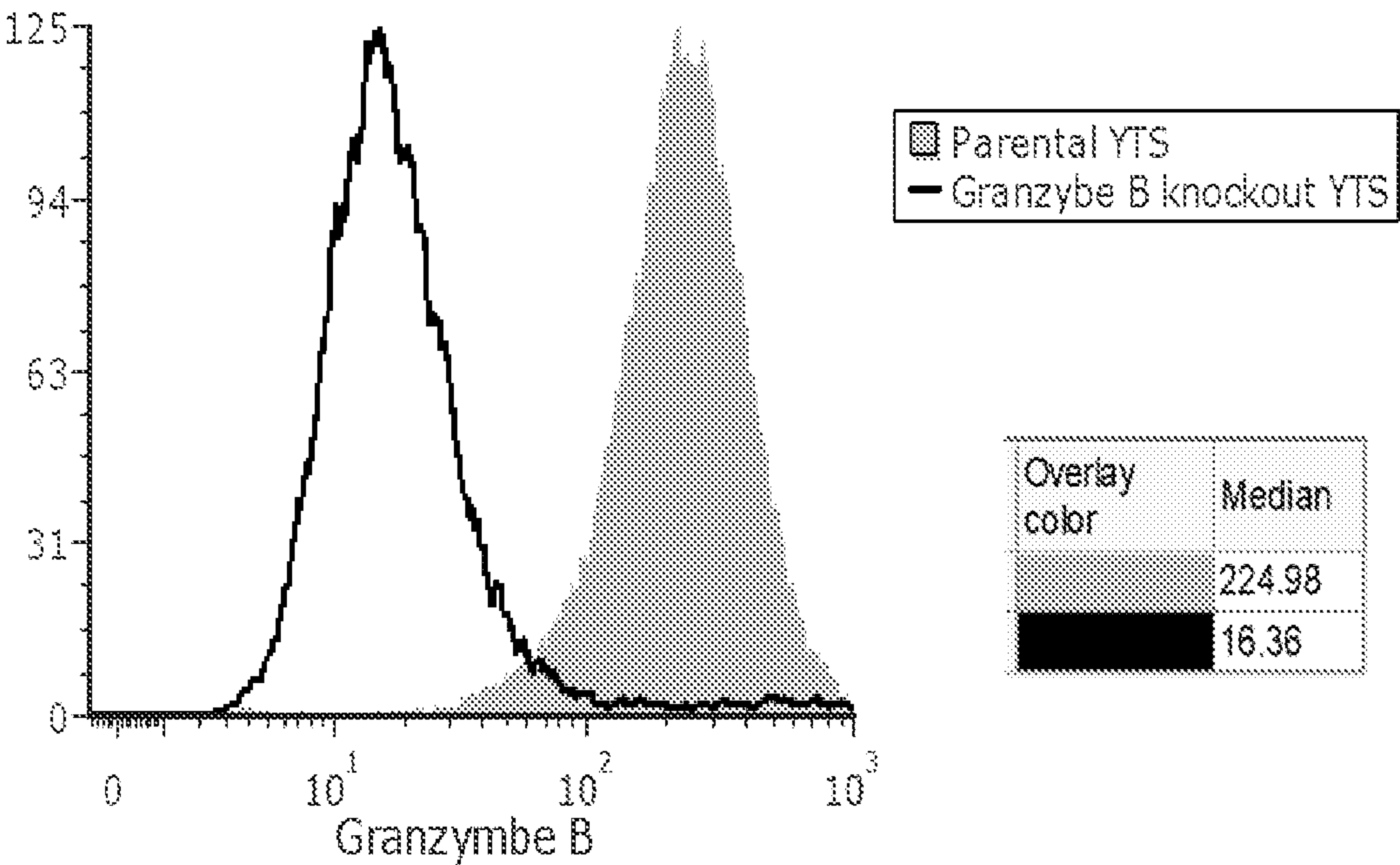


Figure 3B

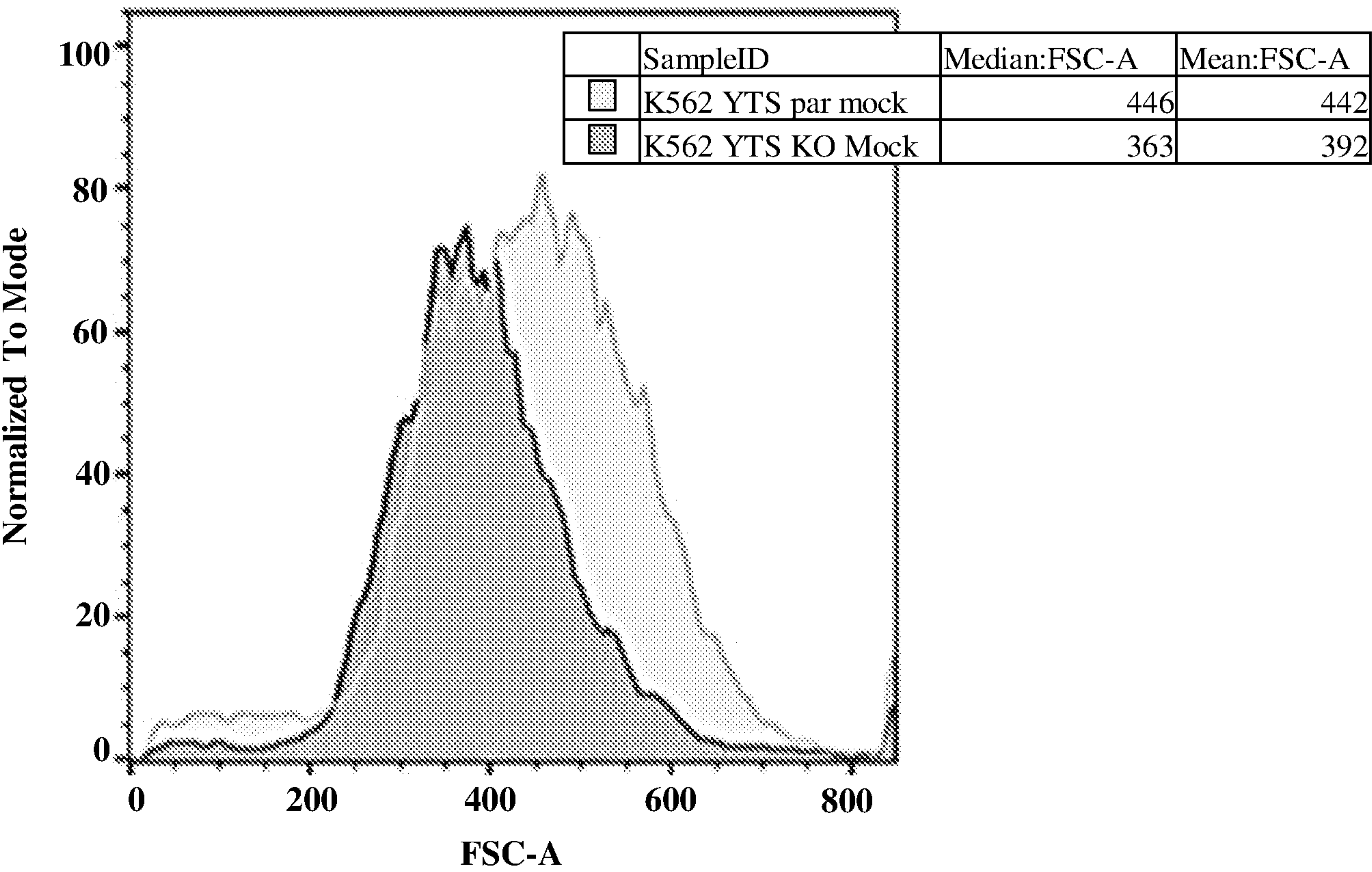


Figure 3C

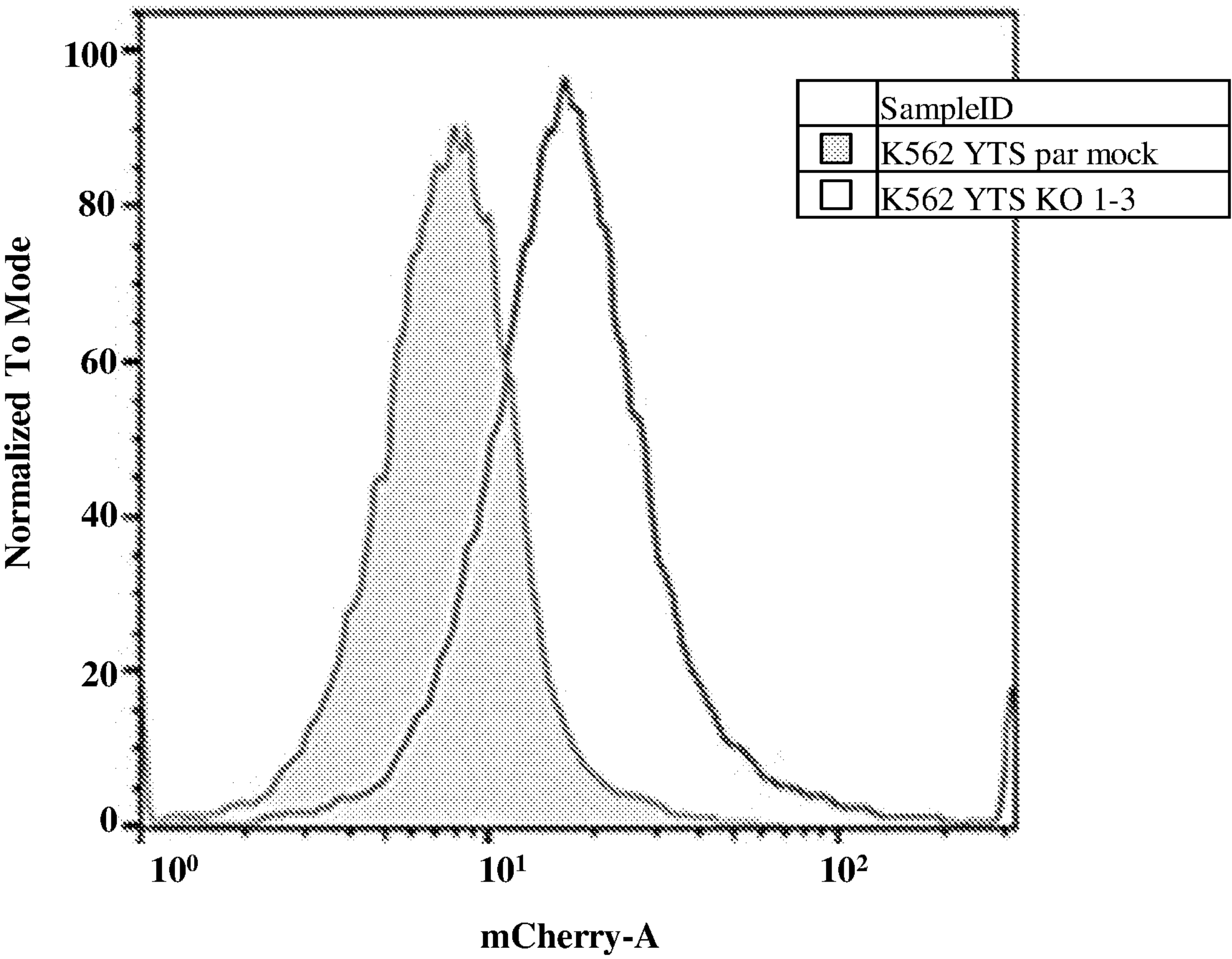


Figure 3C continued

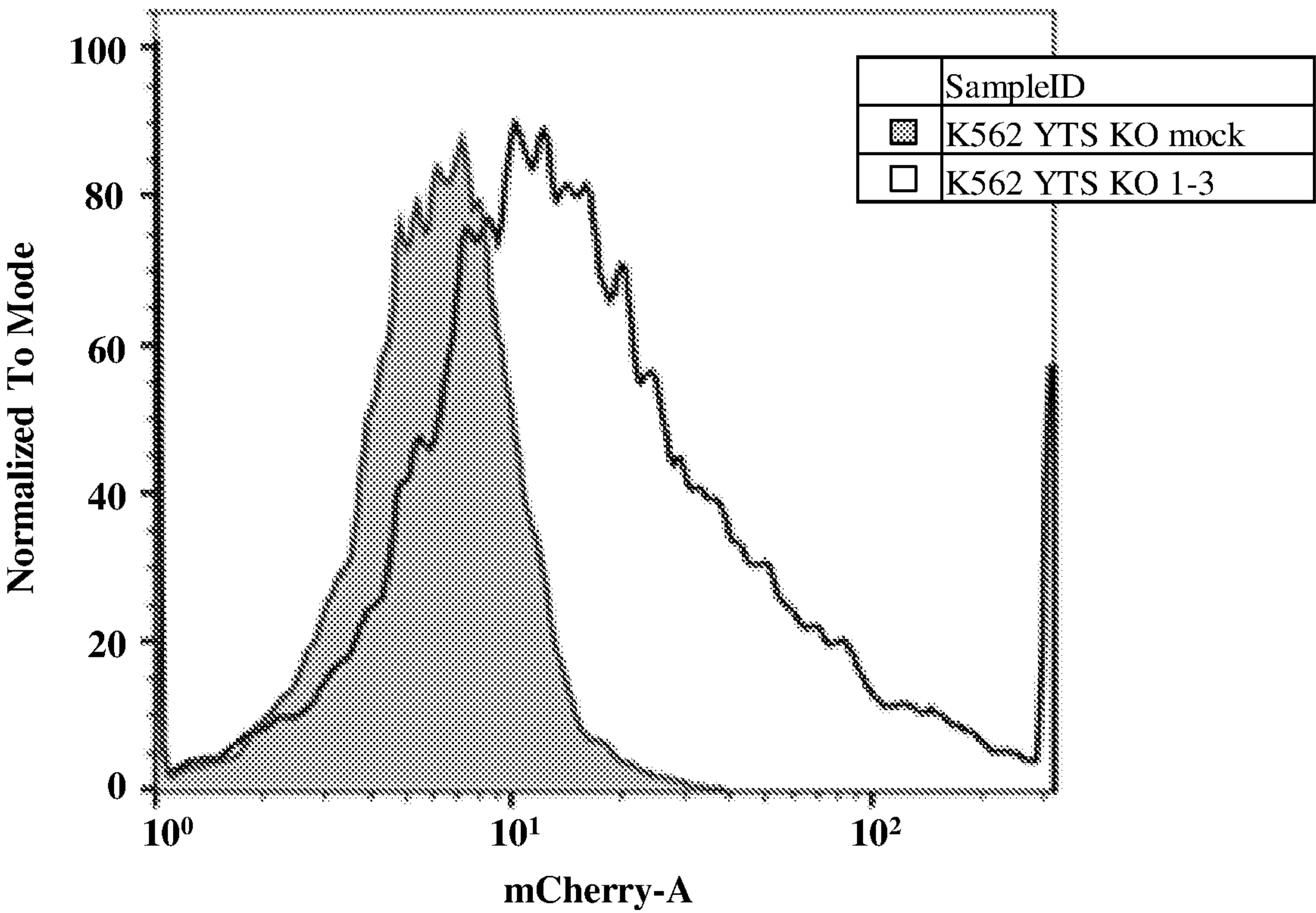
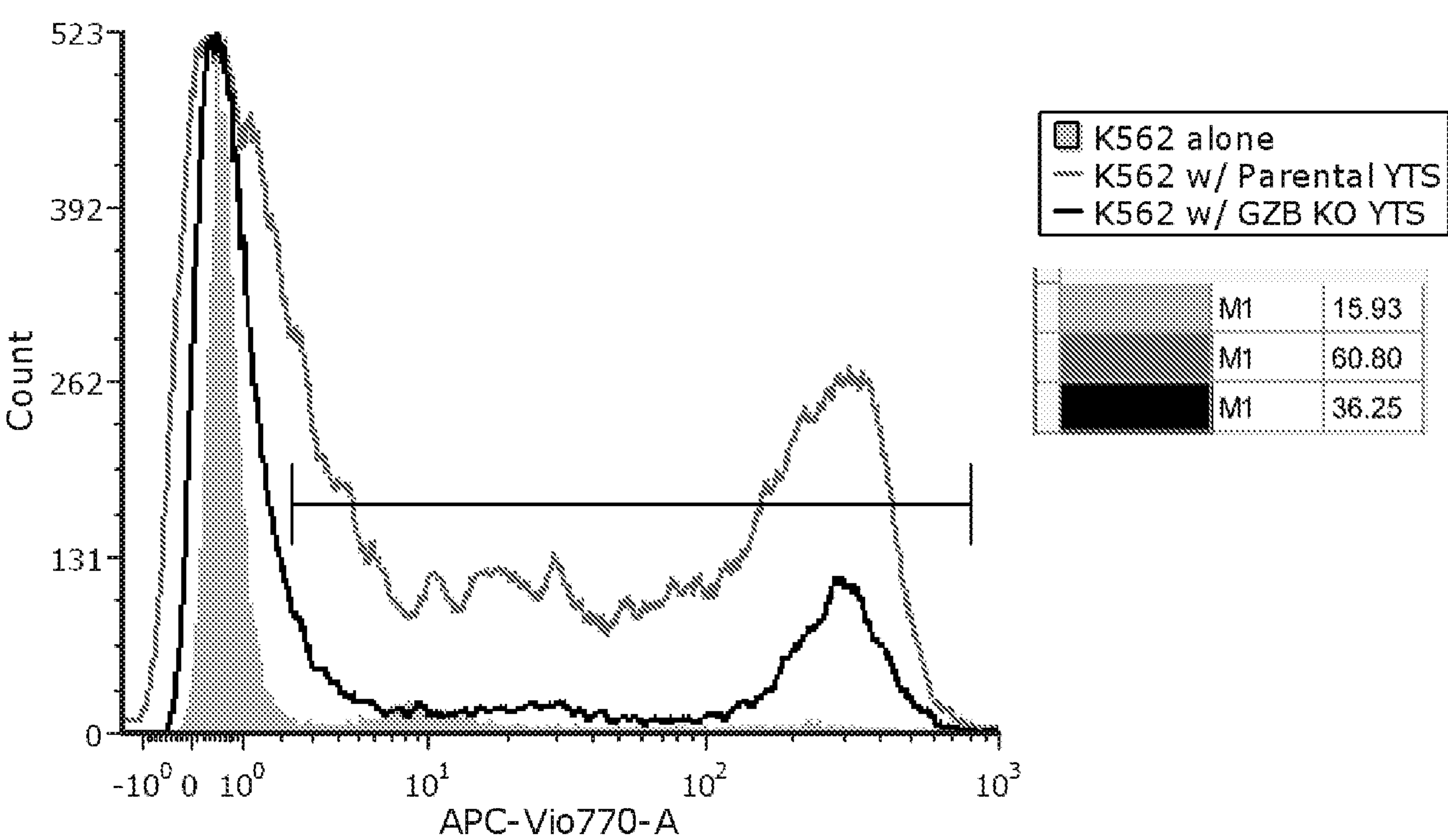


Figure 3D



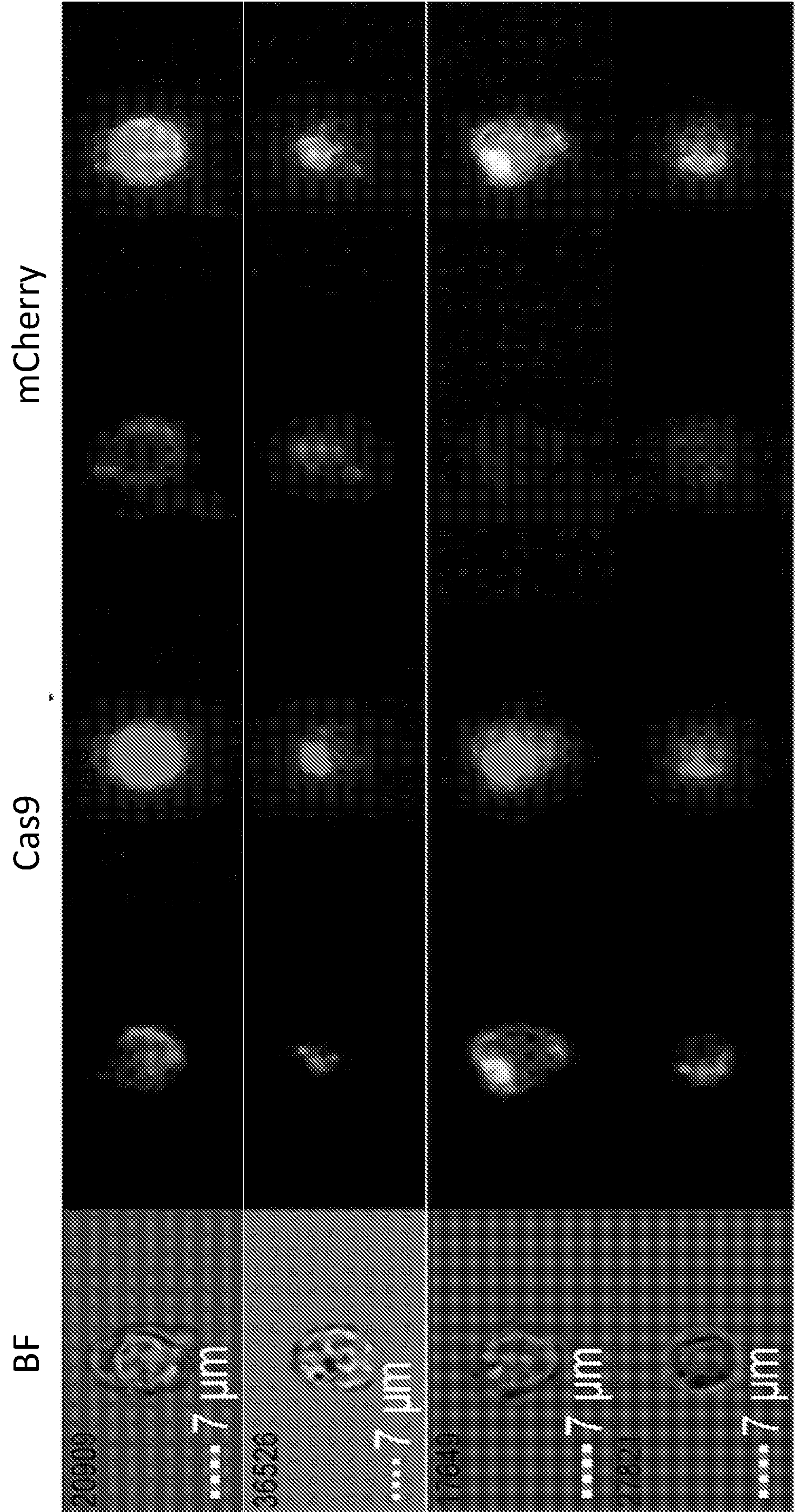


Figure 4A

Figure 4B

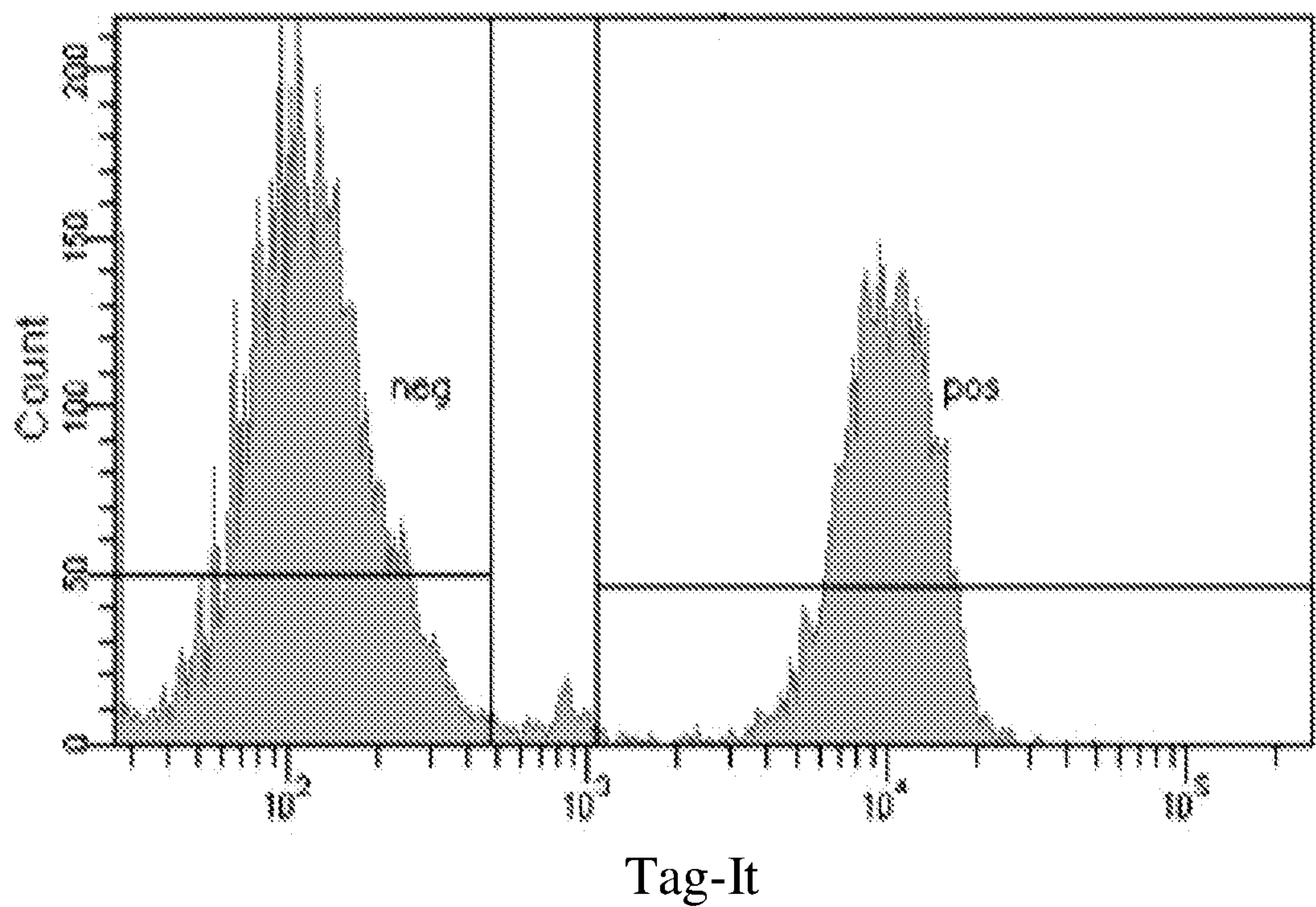


Figure 4C

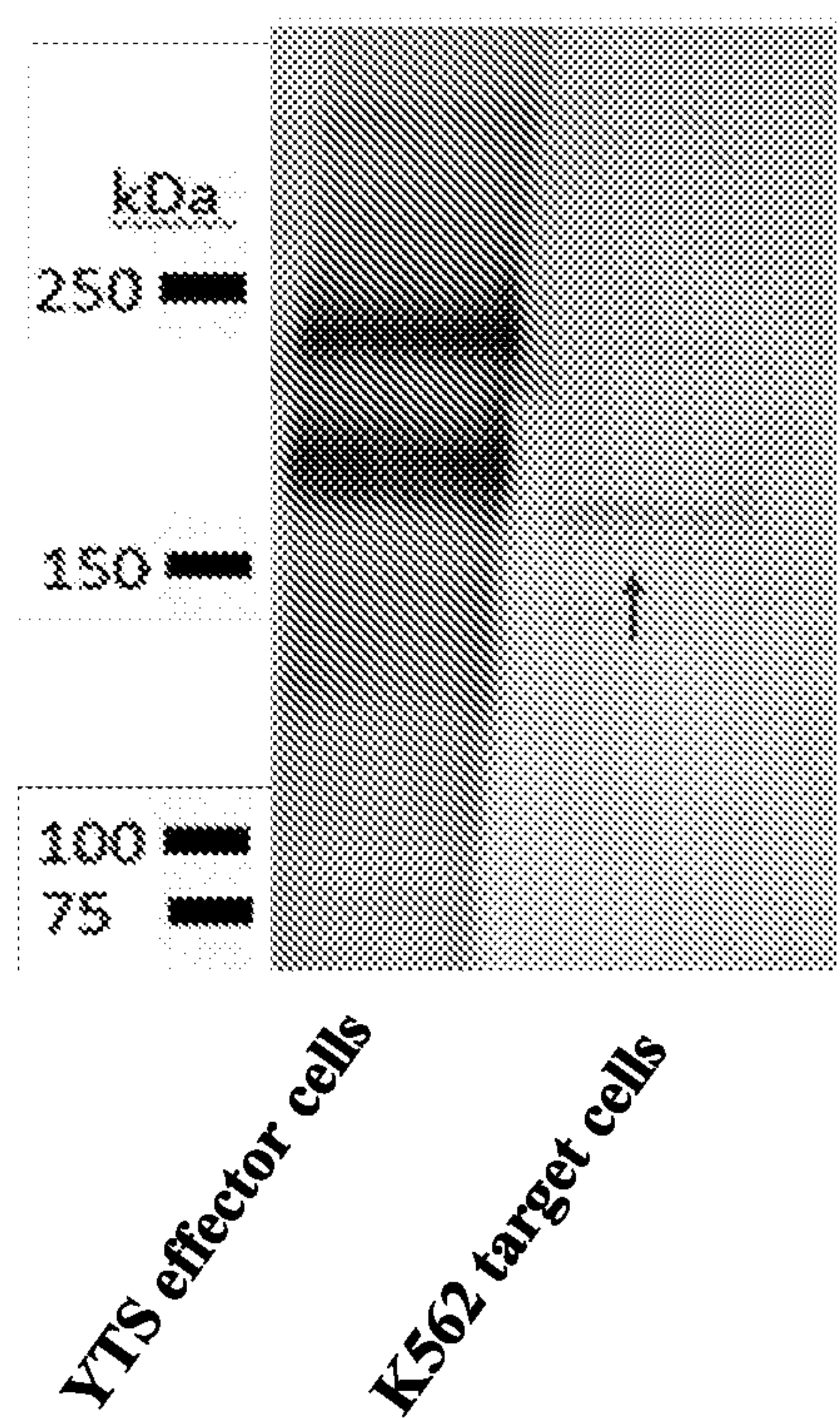
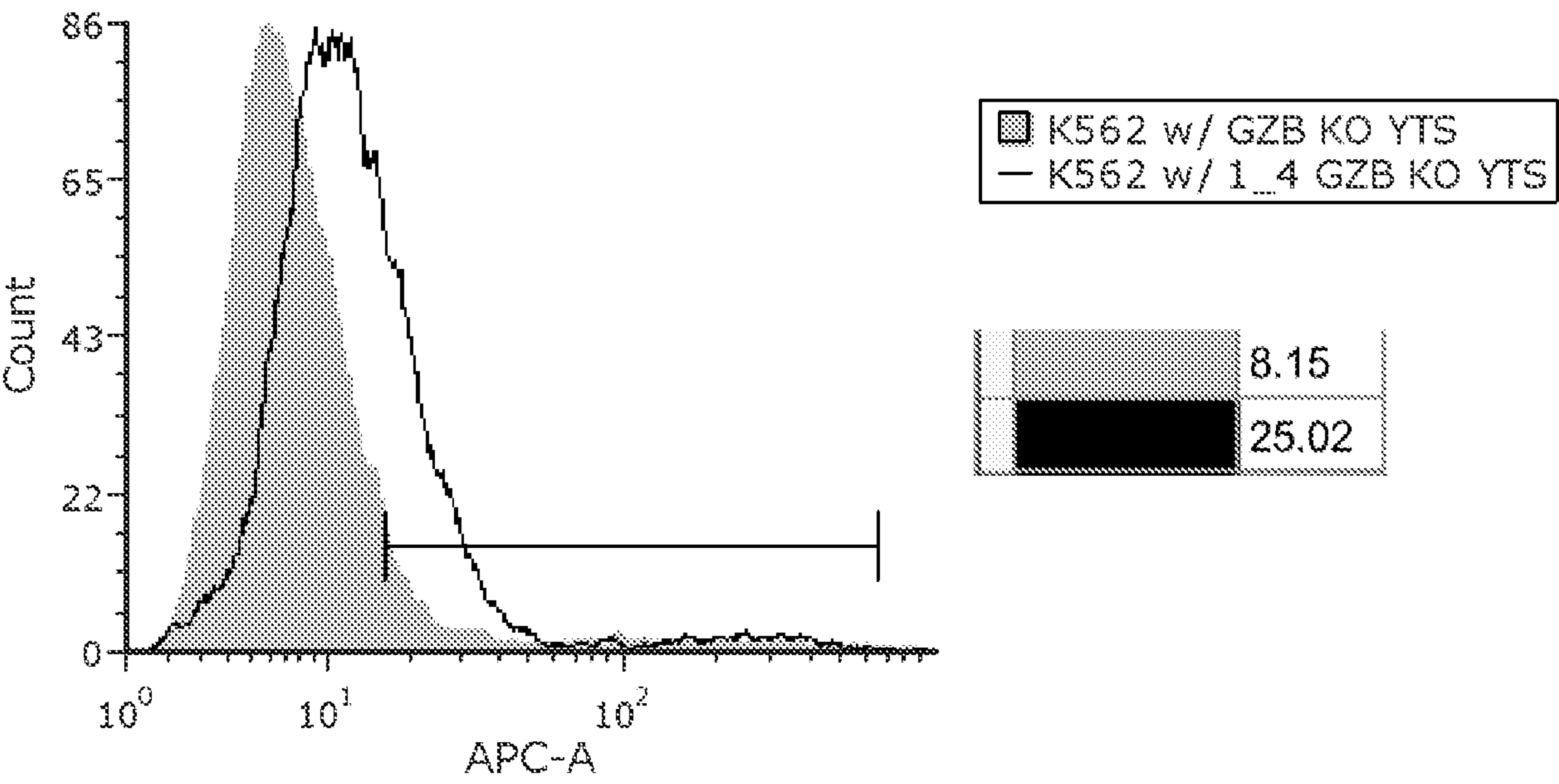


Figure 4D



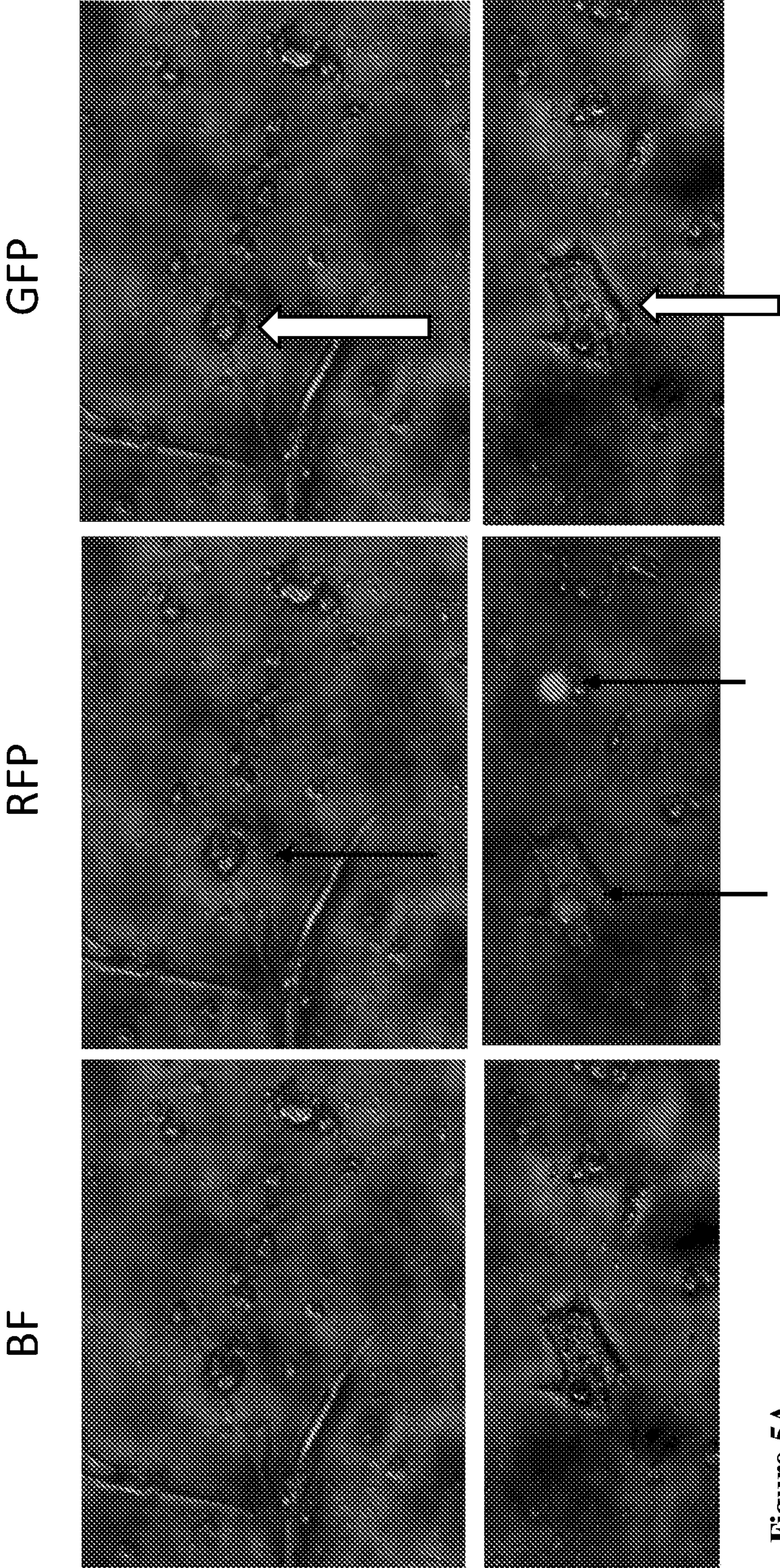


Figure 5A

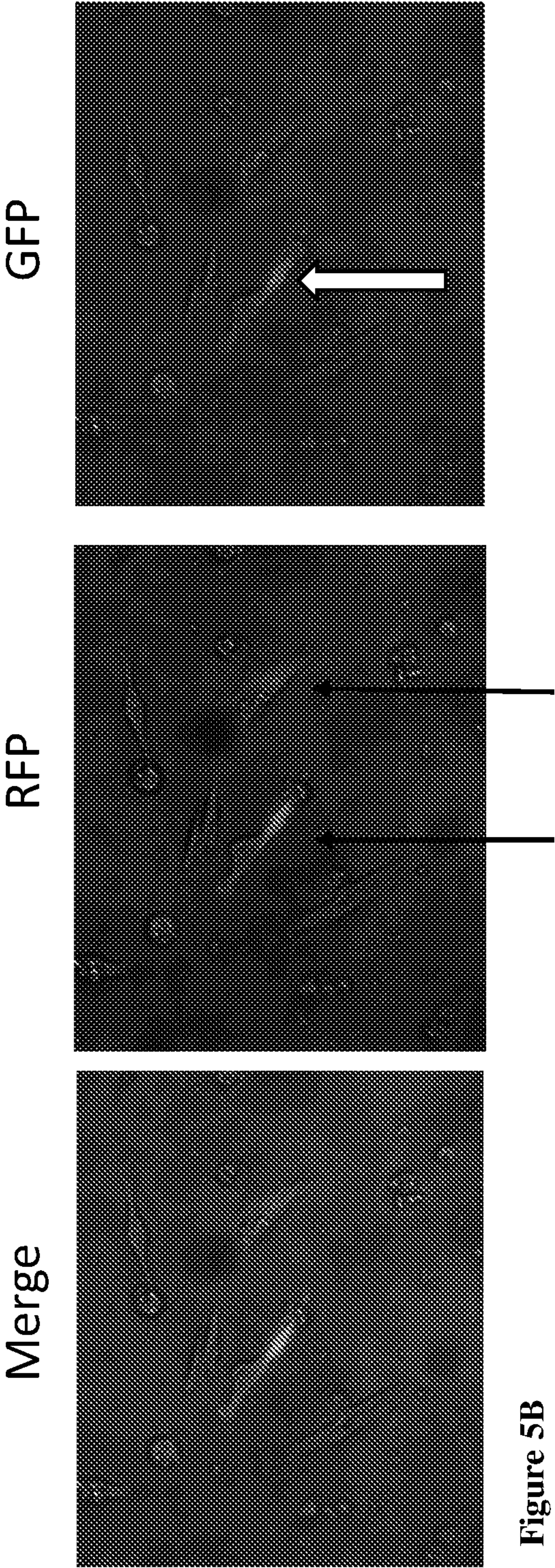


Figure 5B

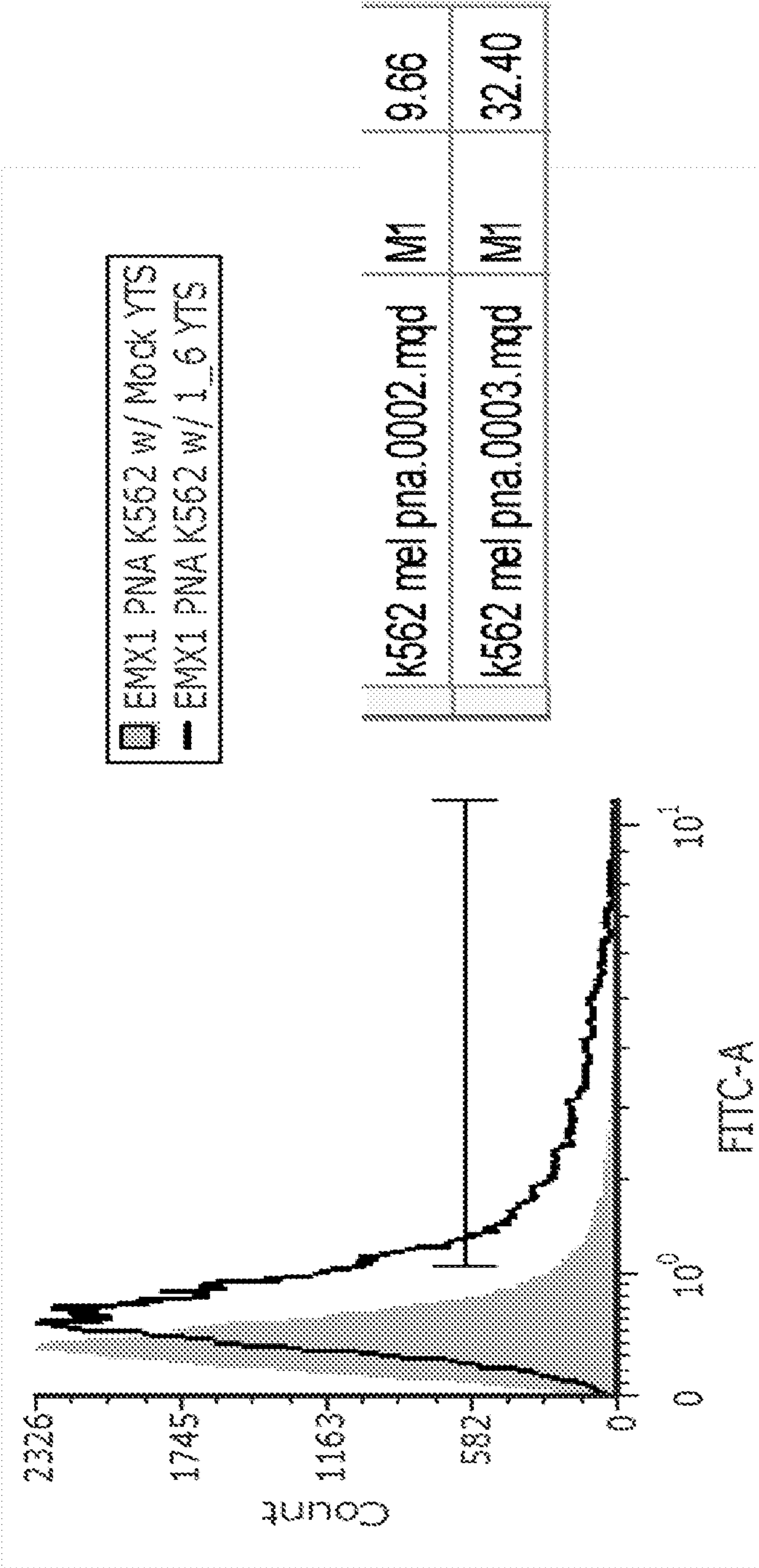


Figure 5C

Figure 5D

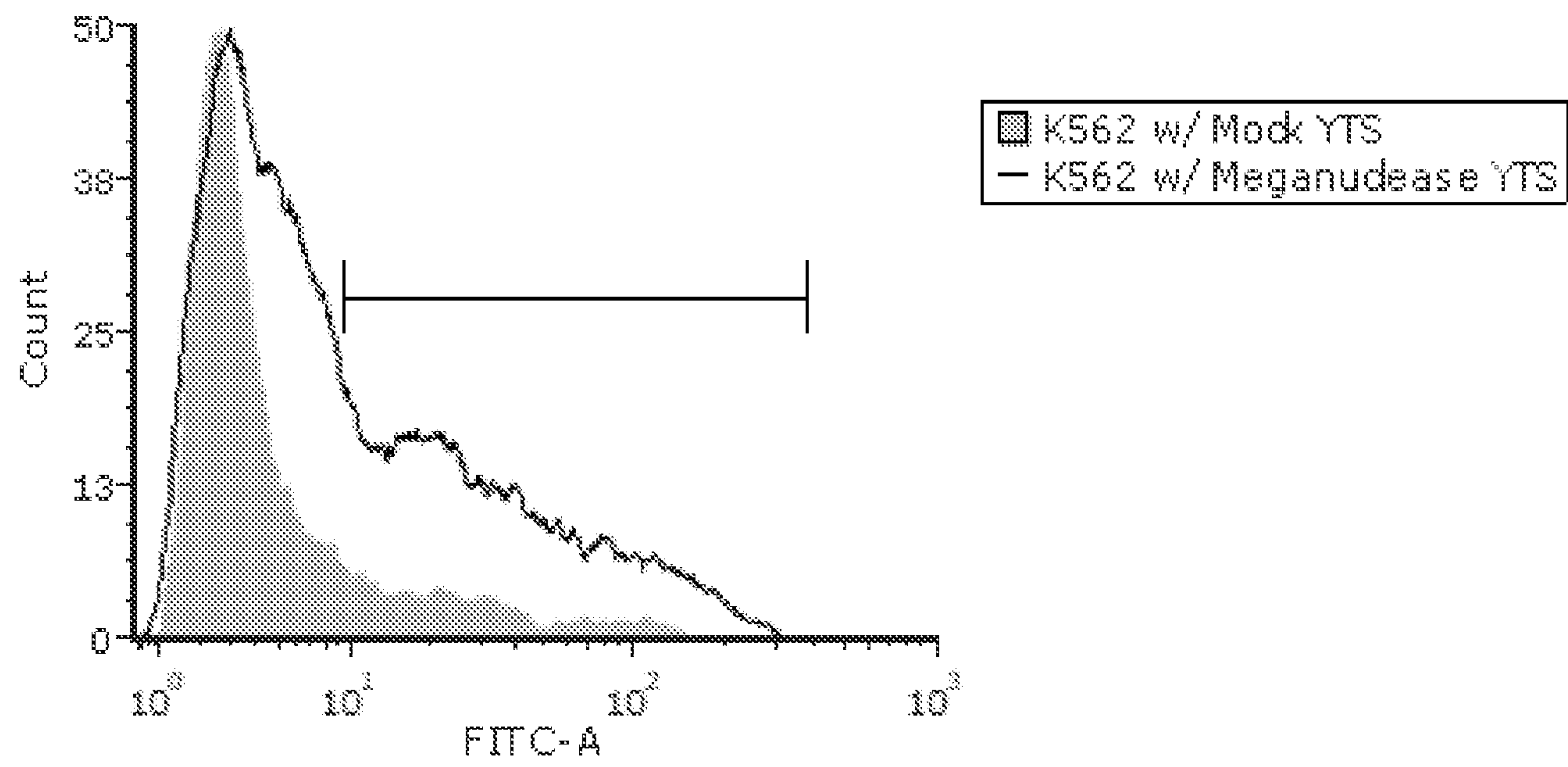


Figure 6A

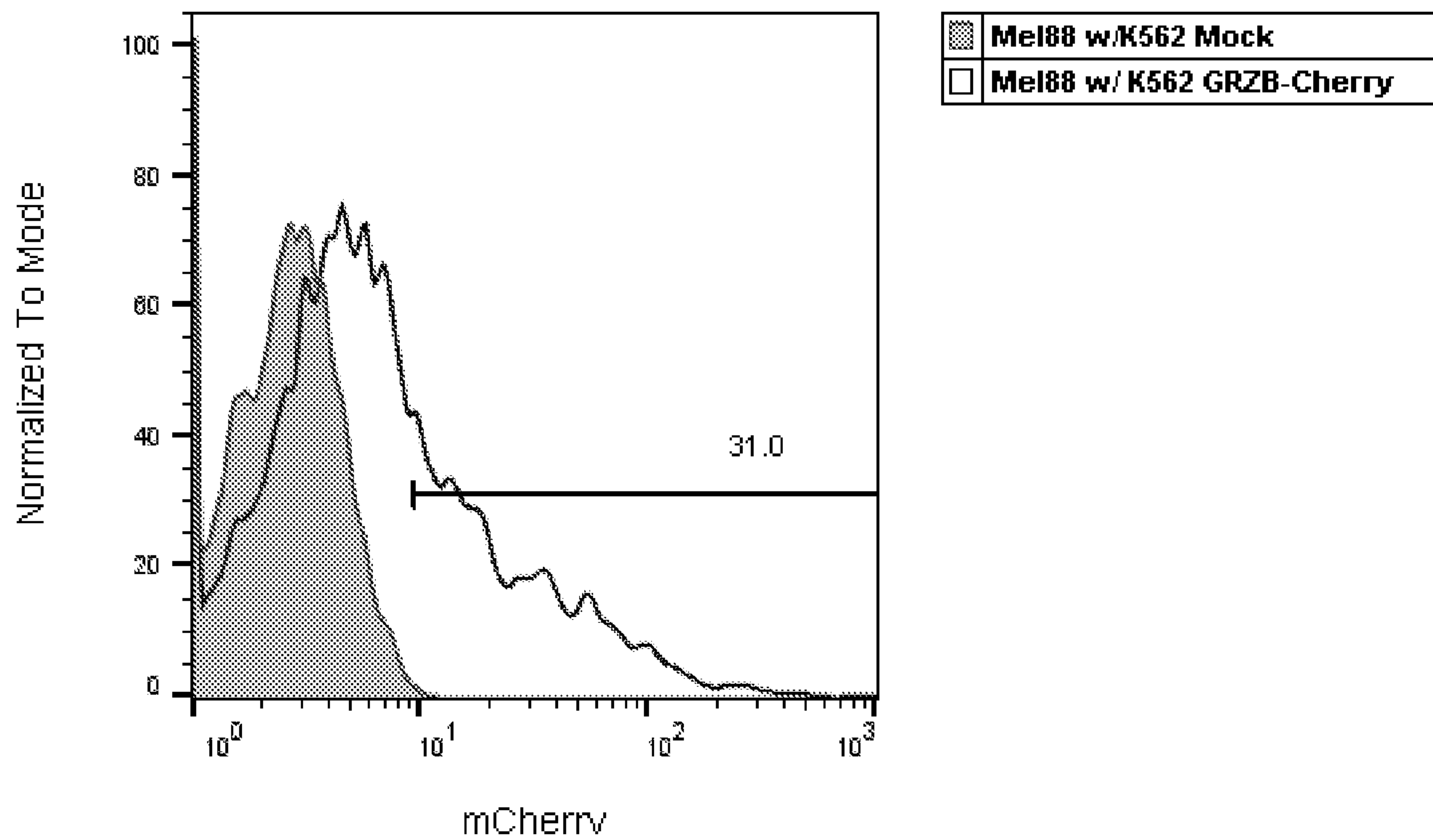


Figure 6B

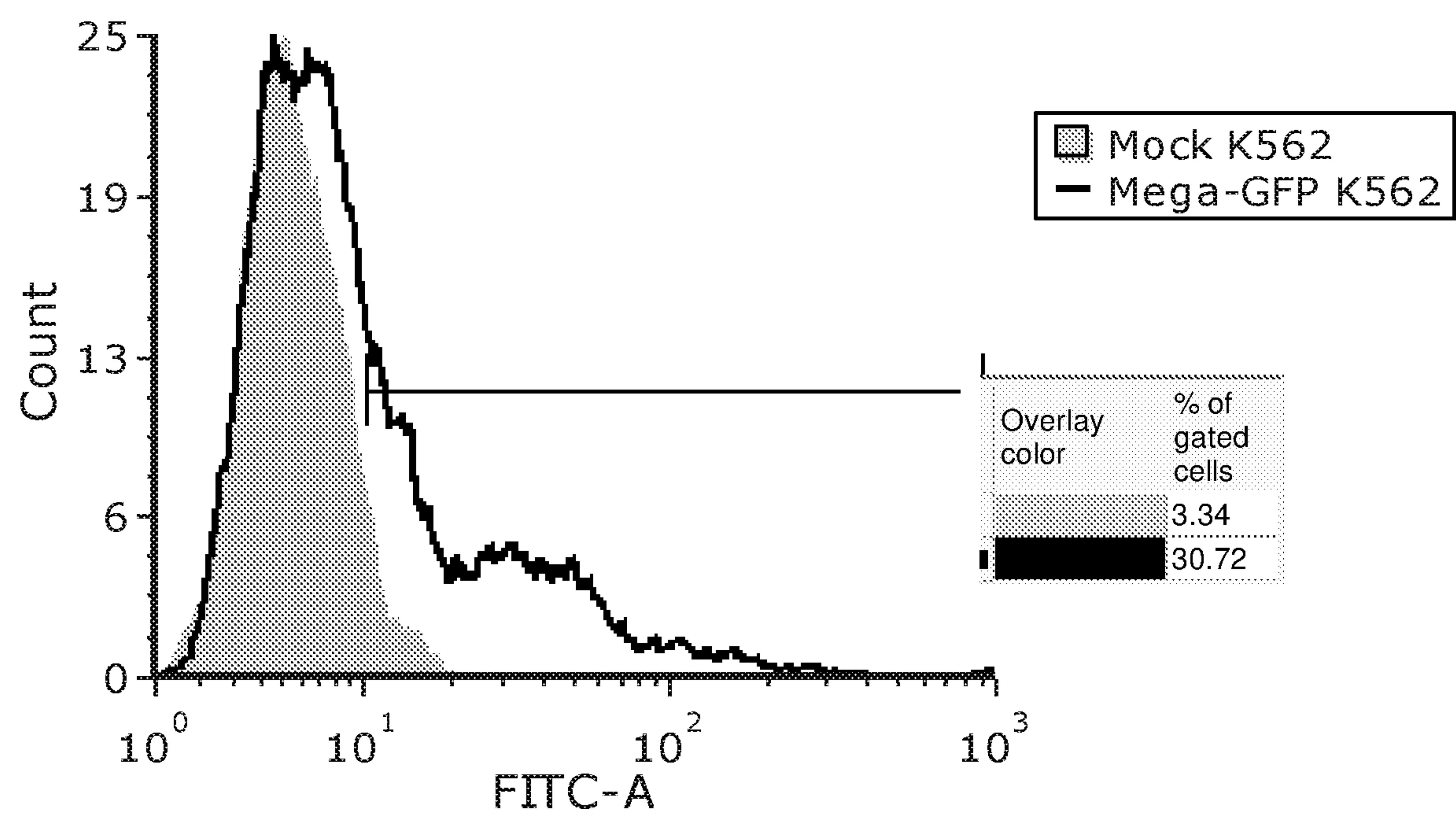


Figure 6C

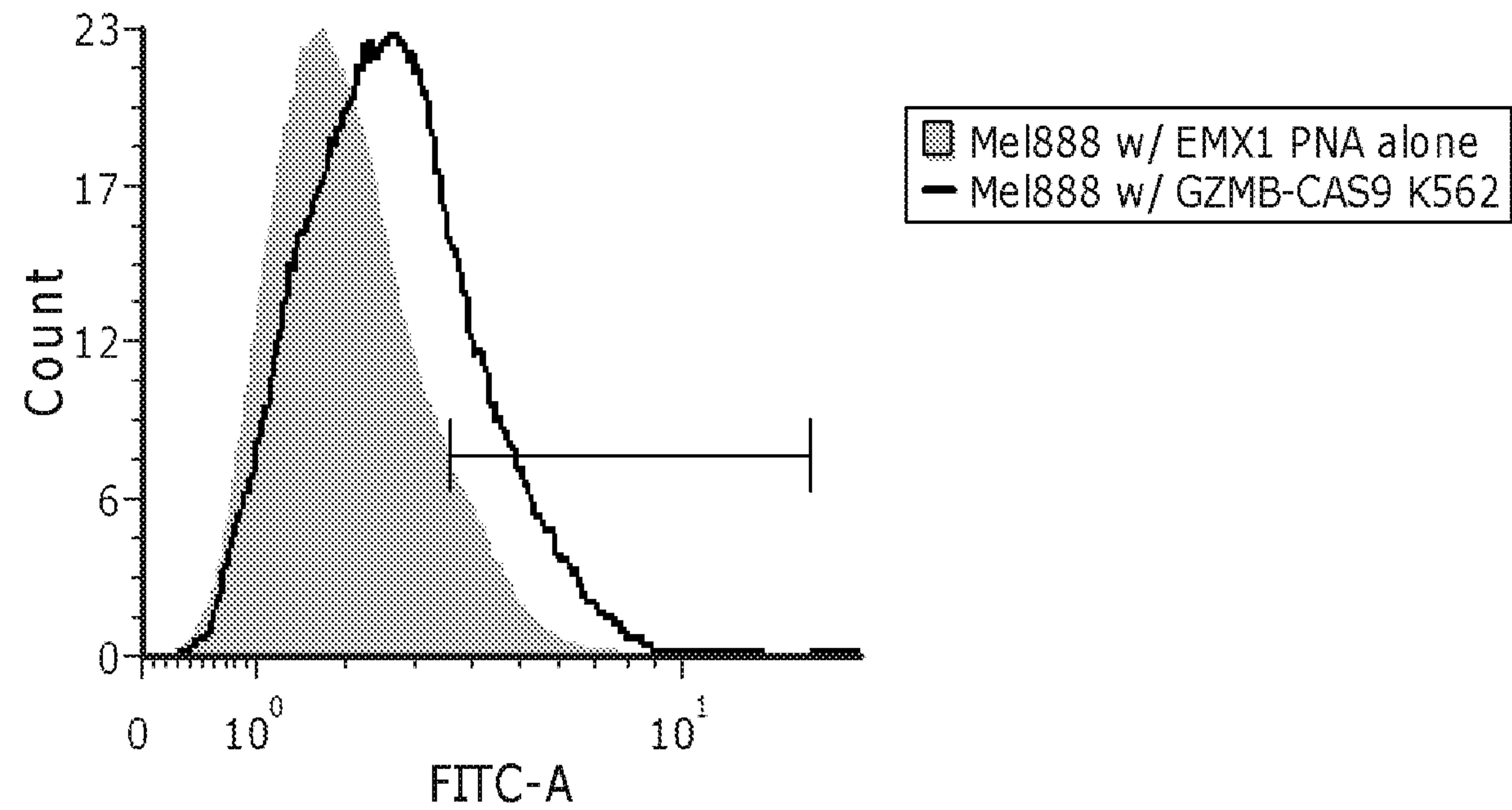


Figure 7A

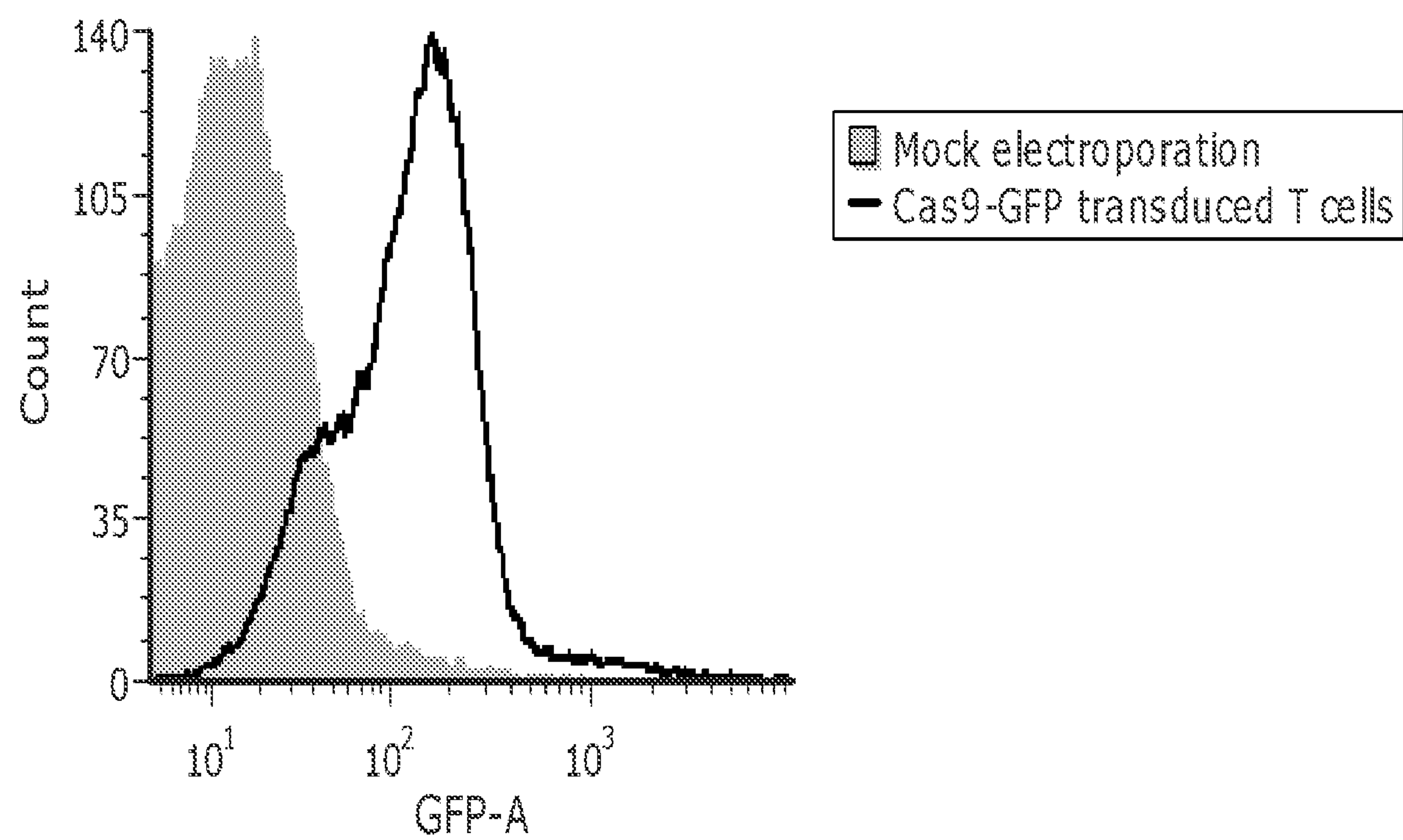


Figure 7B

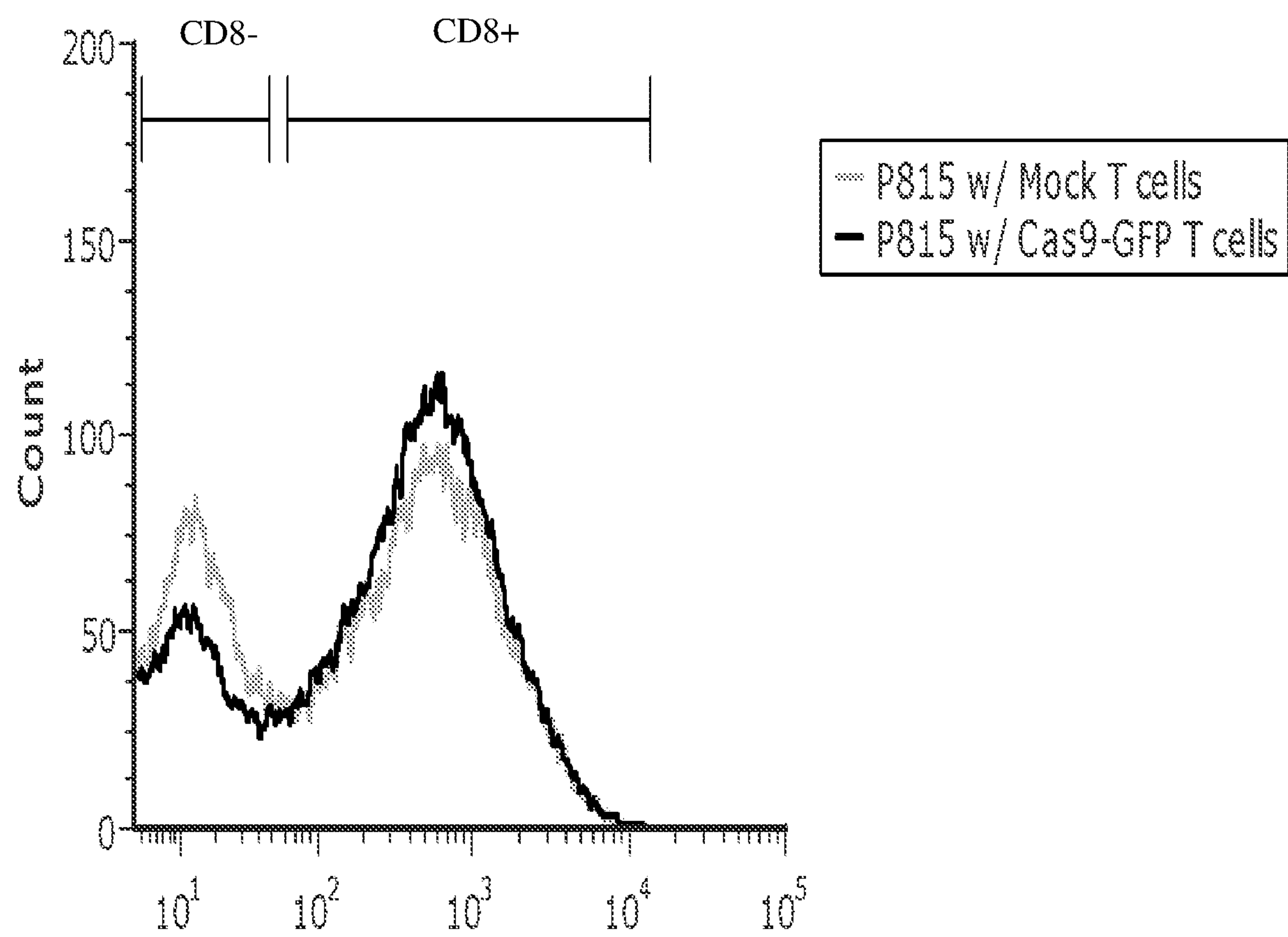


Figure 7C

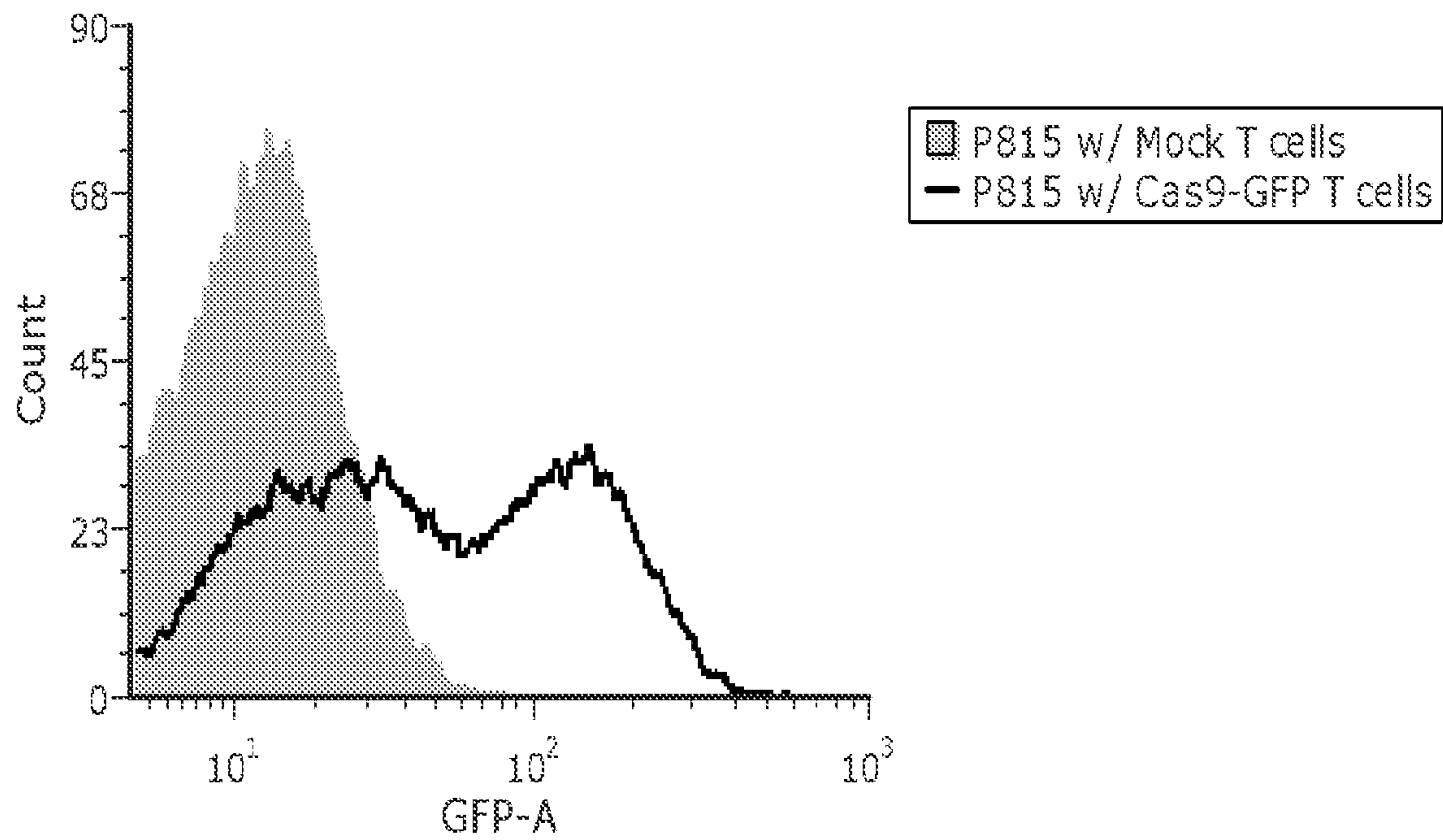


Figure 7D

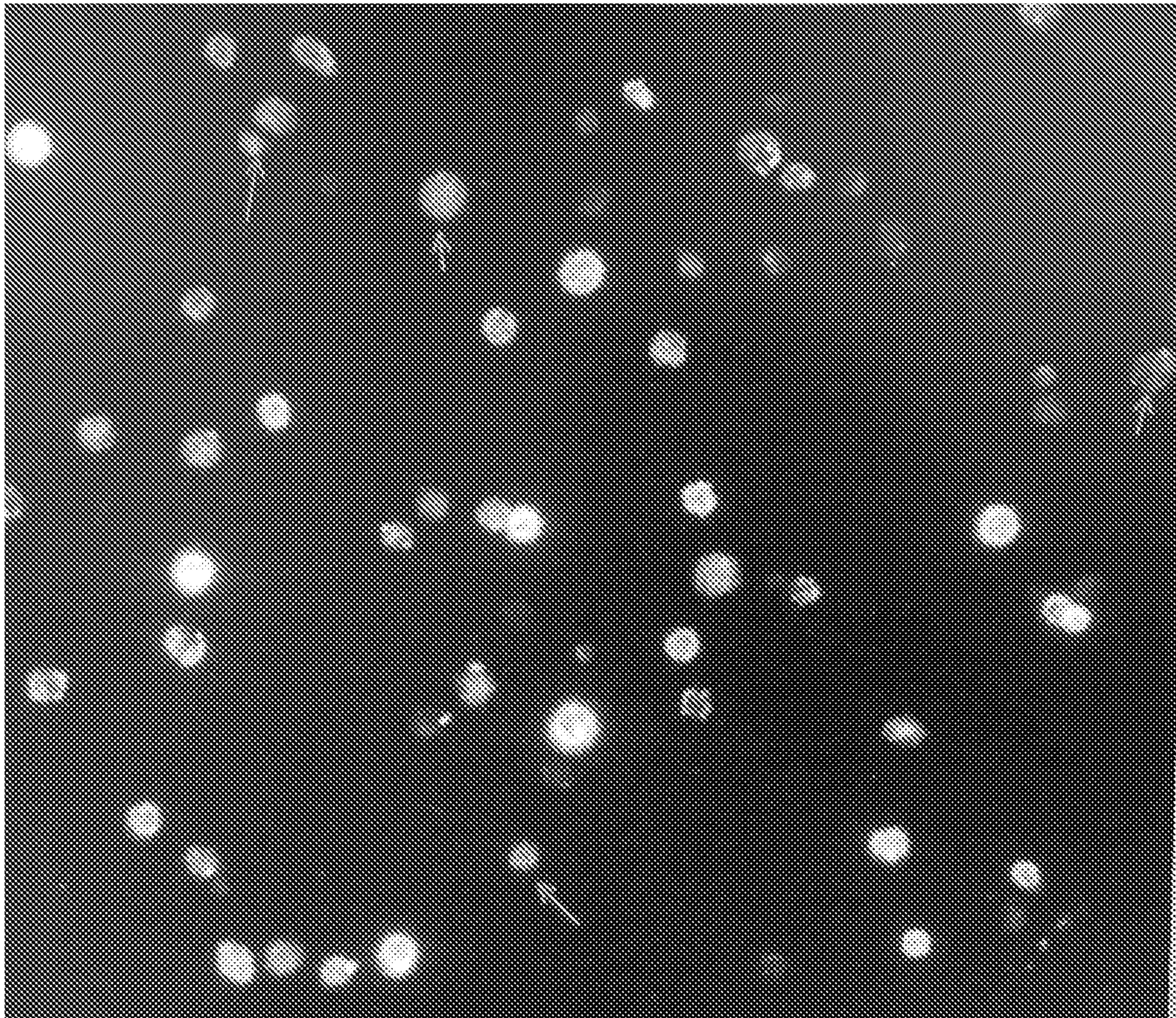


Figure 8A

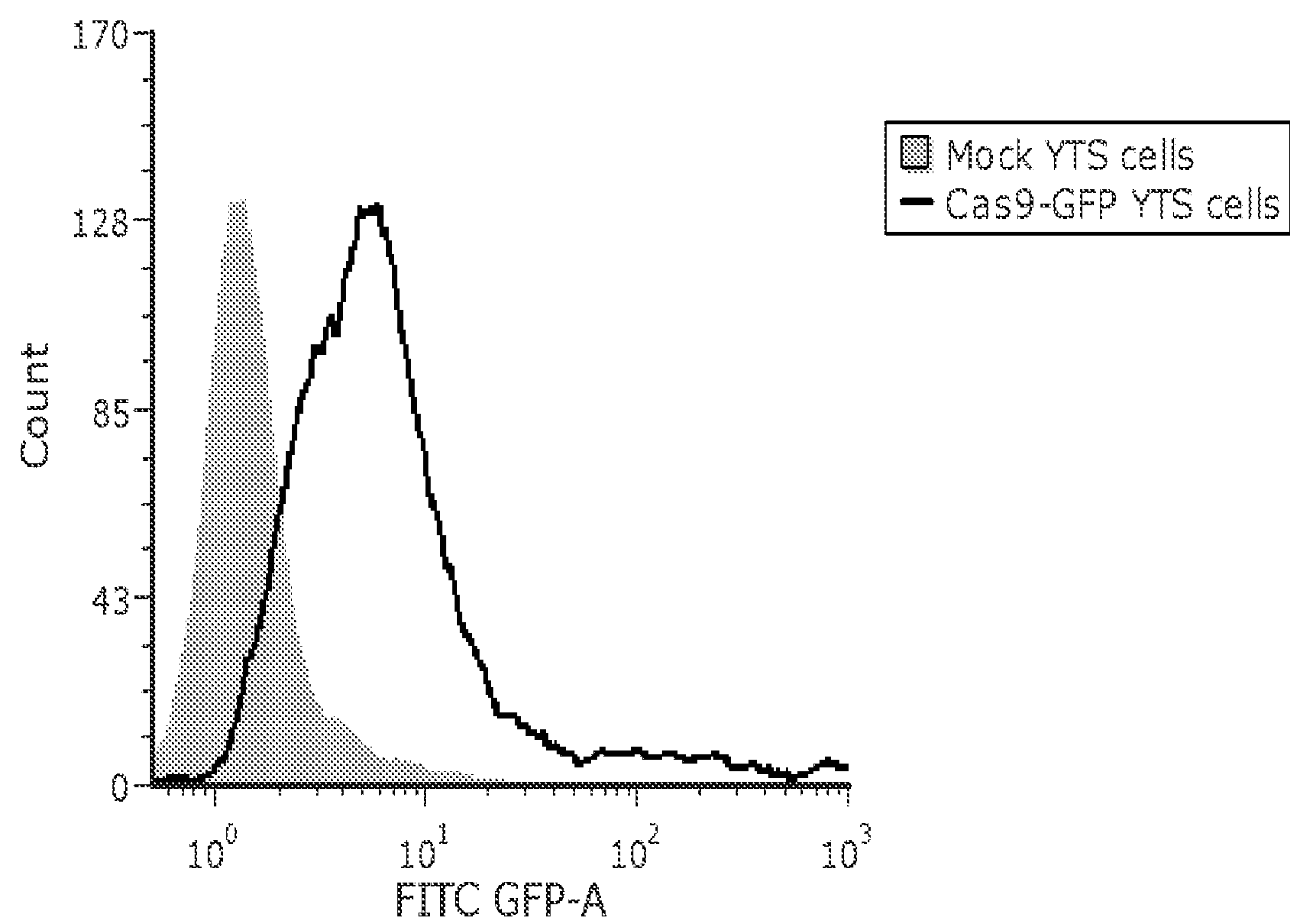


Figure 8B

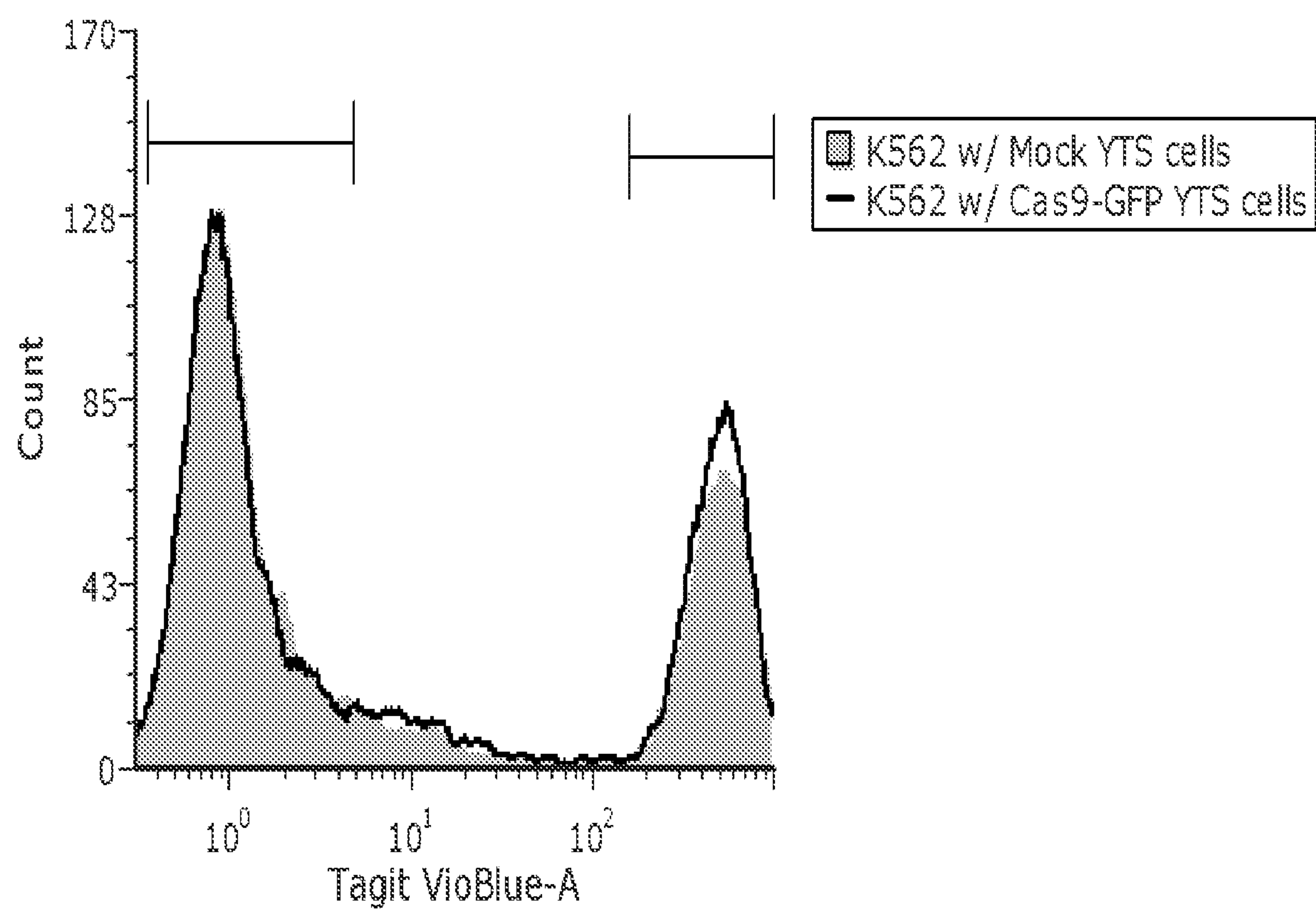


Figure 8C

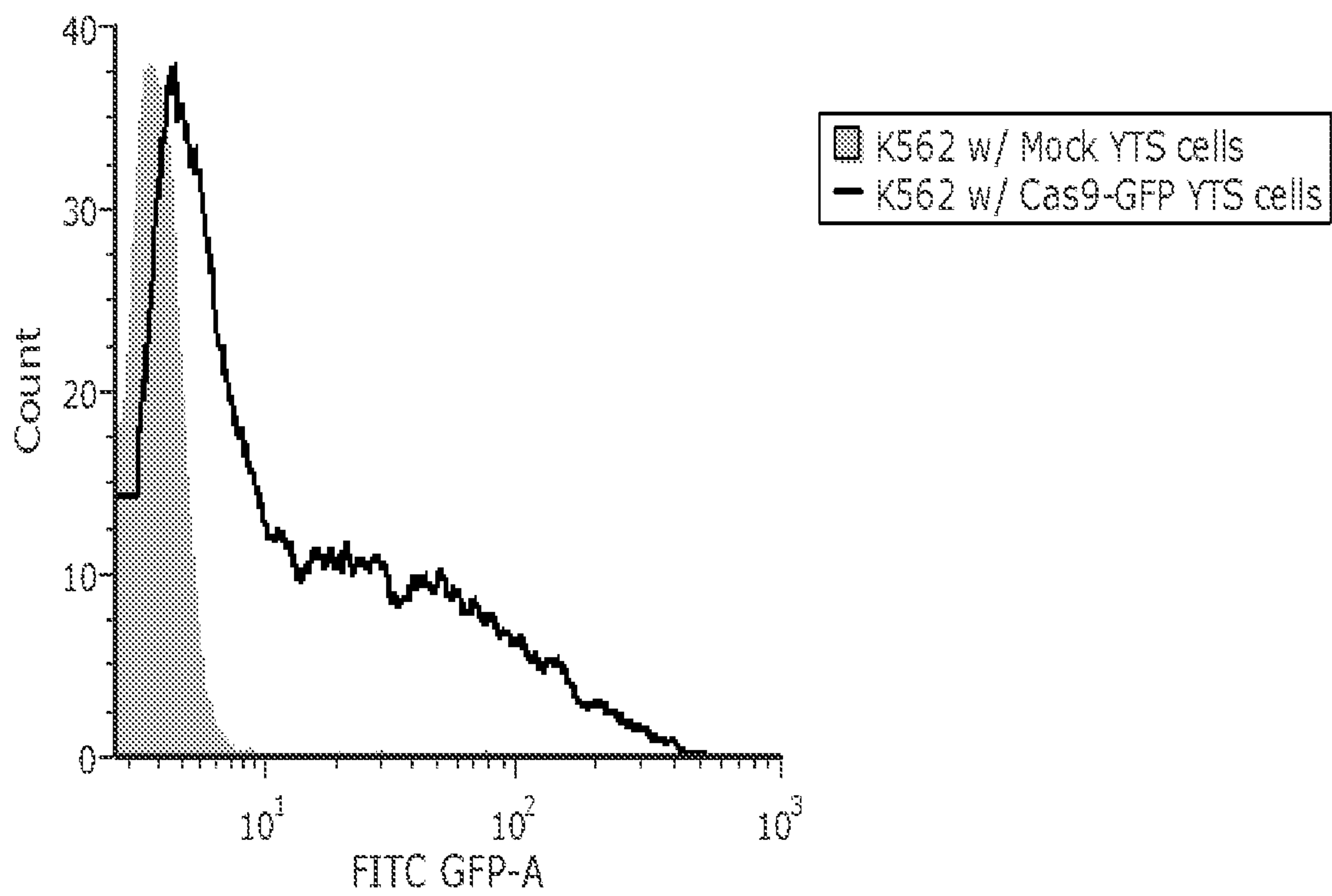


Figure 8D

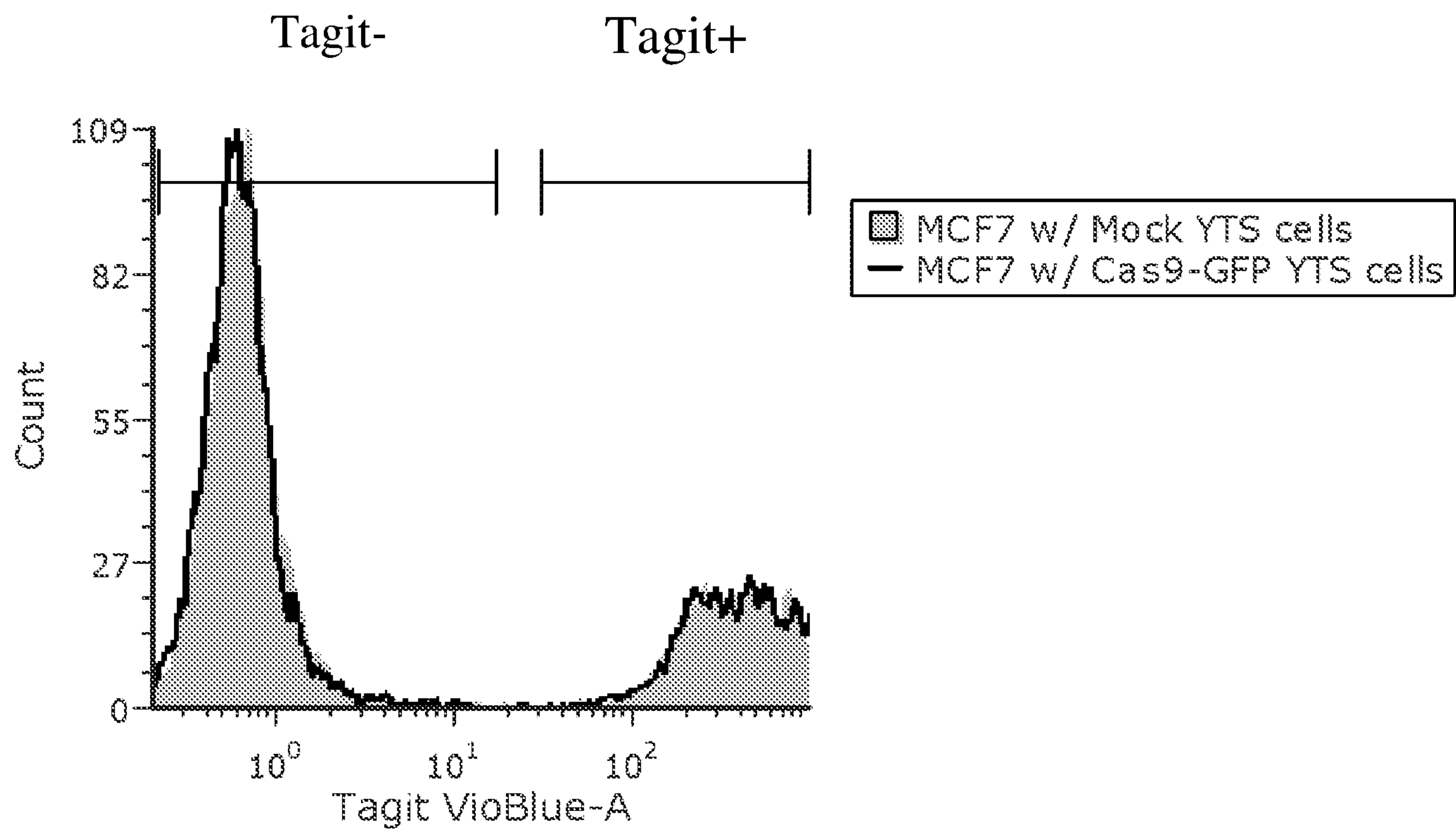


Figure 8E

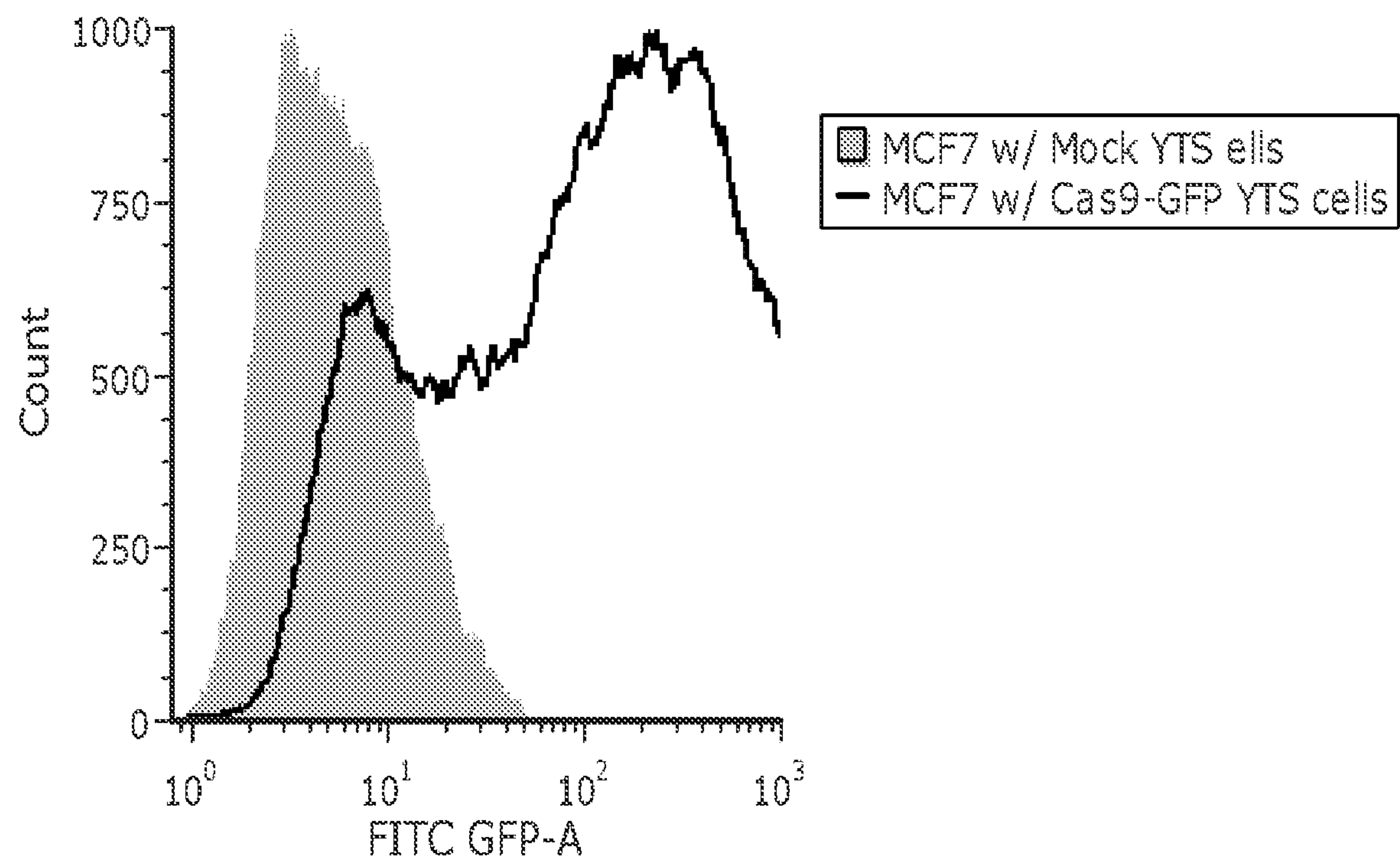


Figure 8F

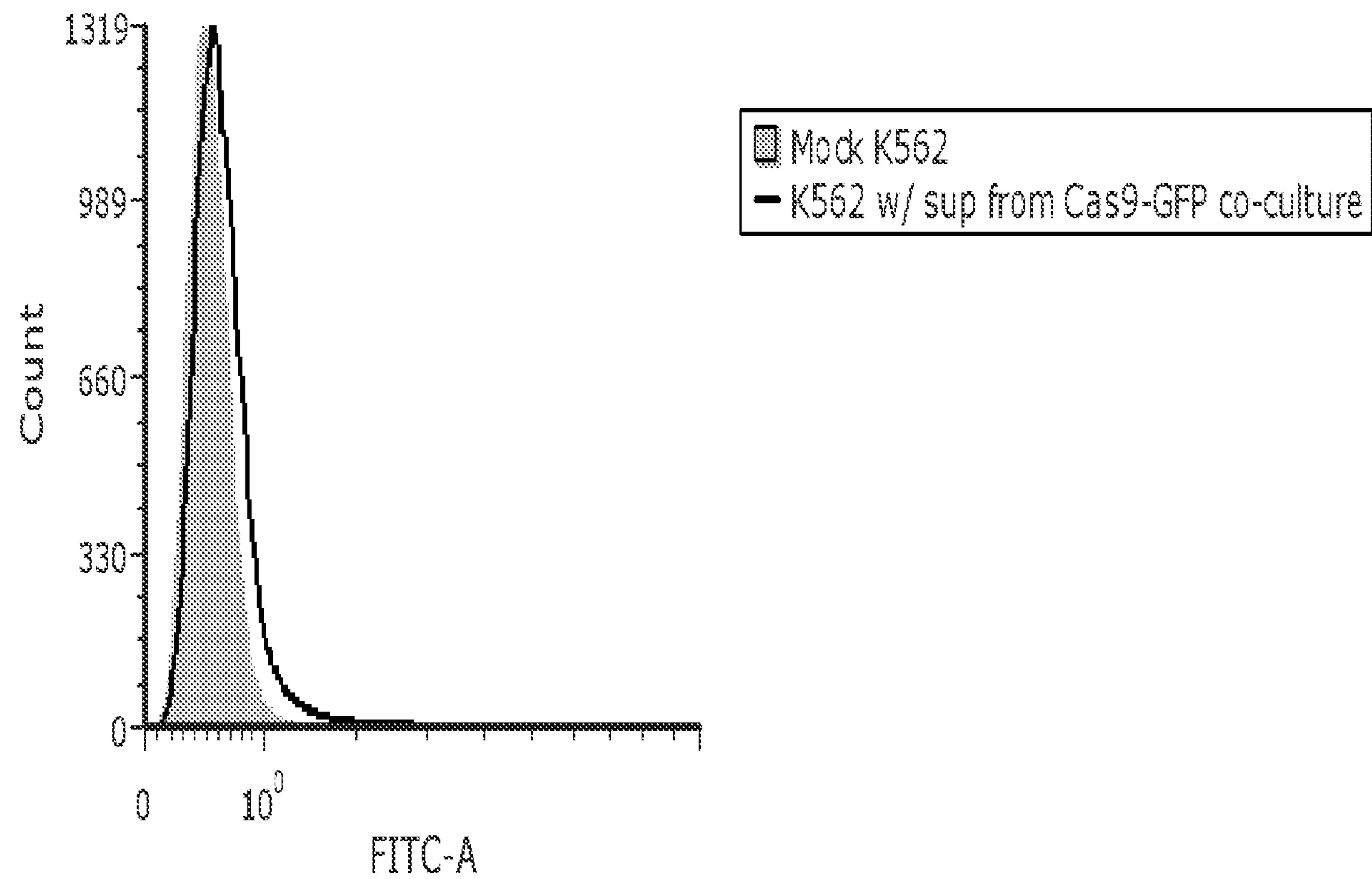


Figure 8G

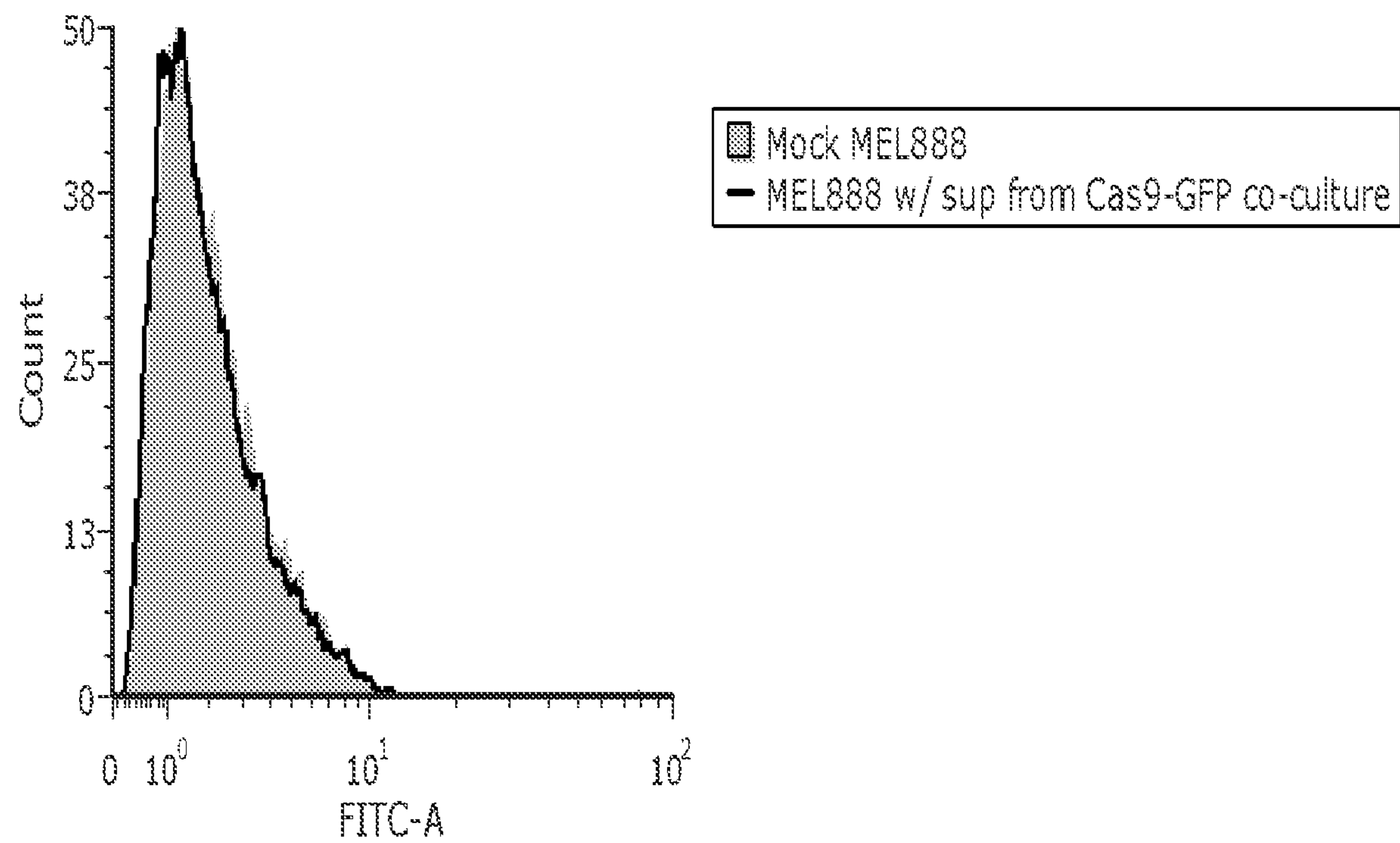
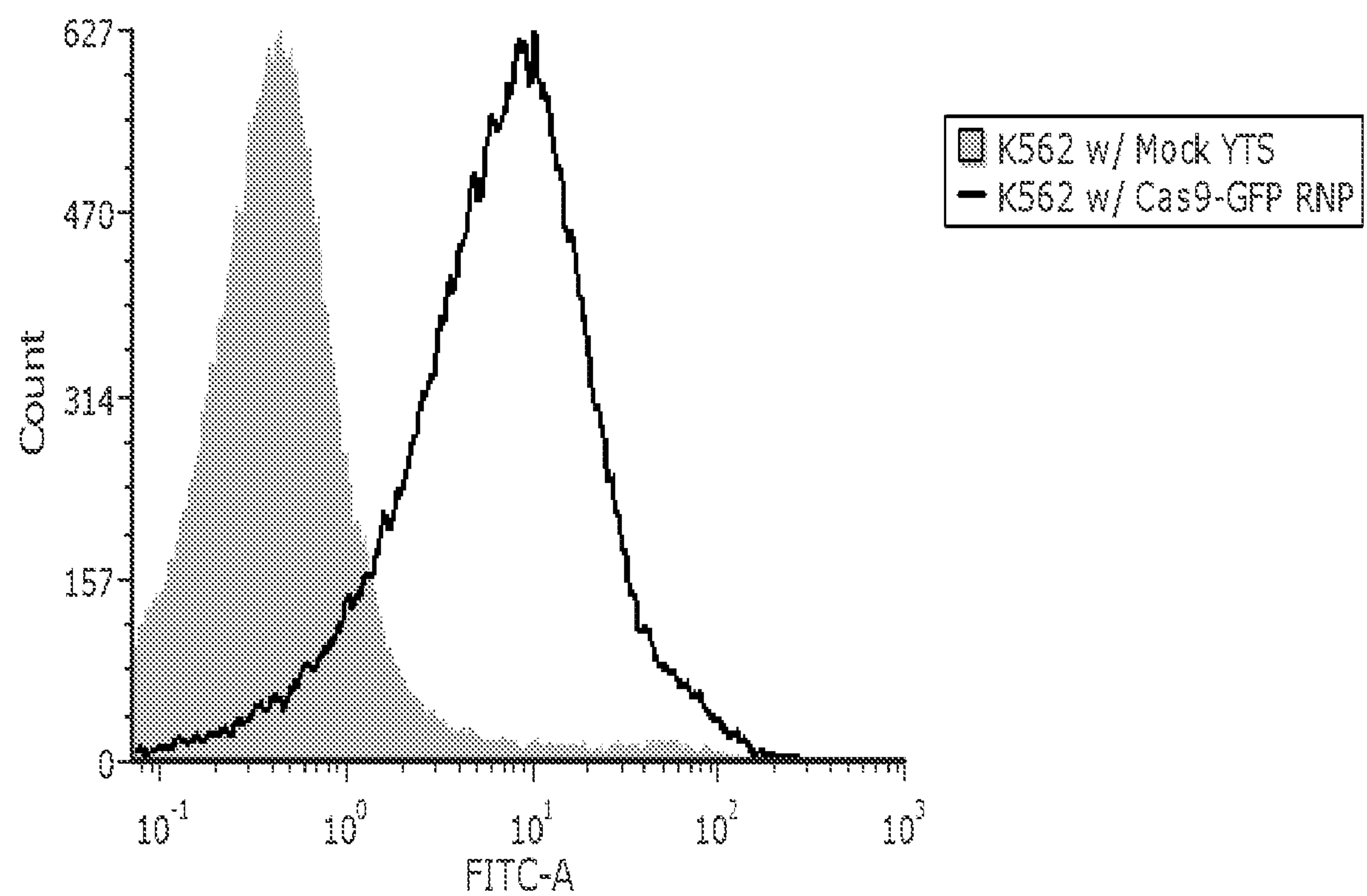


Figure 8H



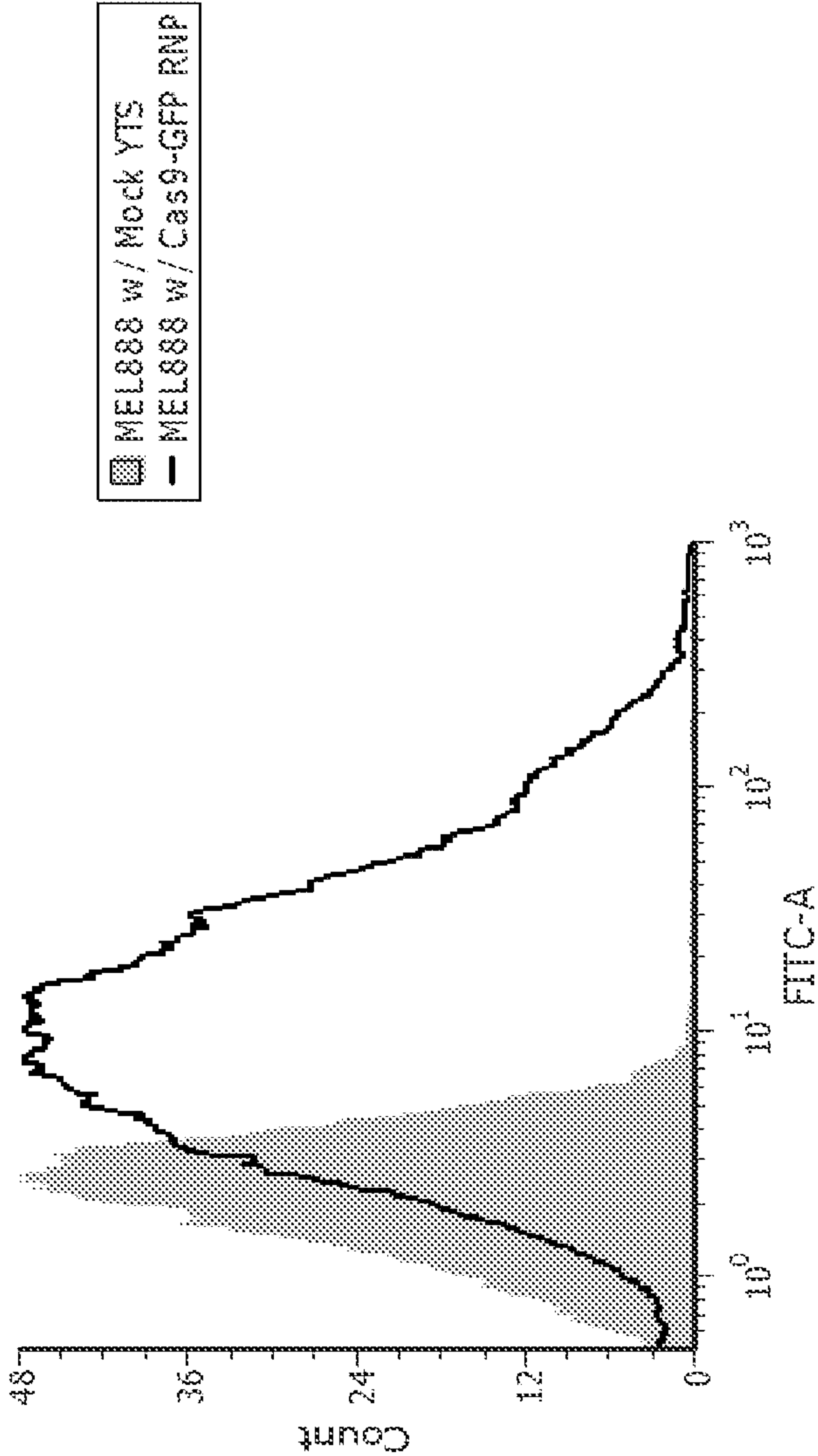


Figure 8I

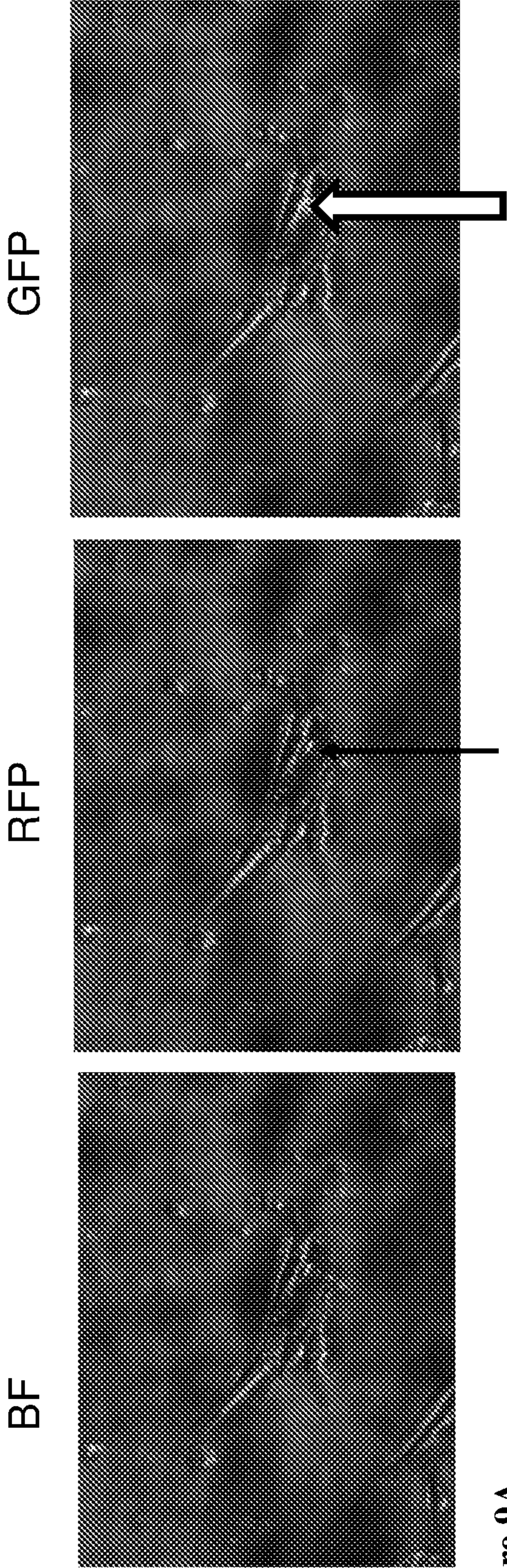


Figure 9A

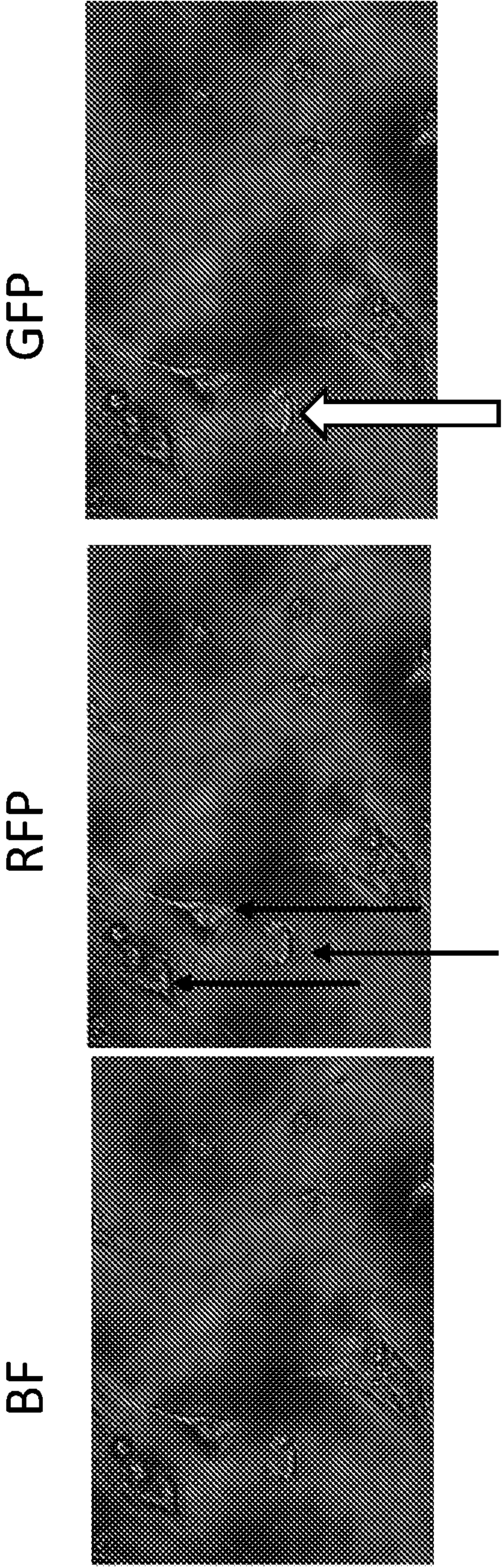


Figure 9B

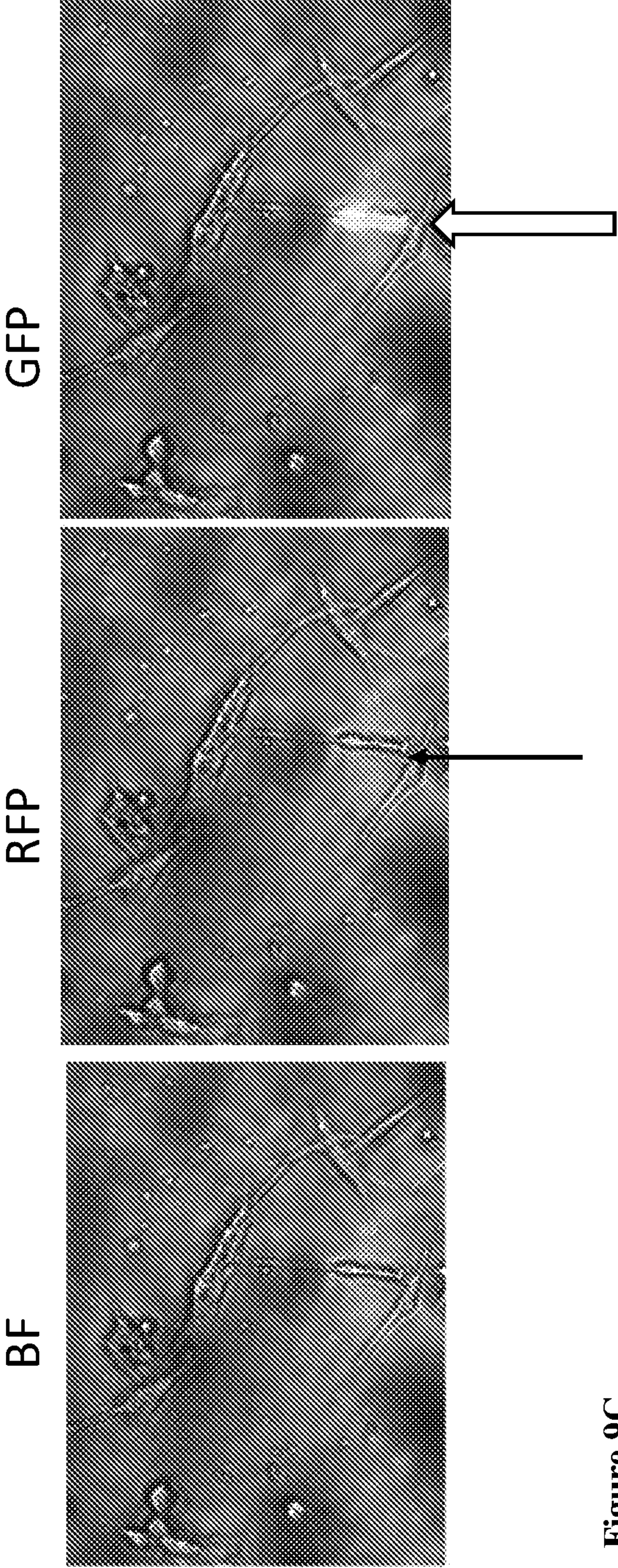


Figure 9C

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2021/050007

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/00 A61K35/17 A61P43/00 C12N9/22 C12N9/64
ADD.

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)

A61K C12N A61P

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)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/157864 A1 (BRITISH COLUMBIA CANCER AGENCY [CA]) 22 October 2015 (2015-10-22)	1-61
Y	abstract page 1, paragraph 0001 - page 26, paragraph 0074 examples claims	1-61

X	US 2019/169597 A1 (ASTRAKHAN SASHA [US] ET AL) 6 June 2019 (2019-06-06)	1-61
Y	abstract page 1, paragraph 0003 - page 24, paragraph 0371 examples claims	1-61

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Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"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

"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

"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

"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

"&" document member of the same patent family

Date of the actual completion of the international search

24 March 2021

Date of mailing of the international search report

01/04/2021

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax: (+31-70) 340-3016

Authorized officer

Camilleri, Alain

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	B DÄLKEN ET AL: "Targeted induction of apoptosis by chimeric granzyme B fusion proteins carrying antibody and growth factor domains for cell recognition", CELL DEATH & DIFFERENTIATION, vol. 13, no. 4, 1 January 2006 (2006-01-01), pages 576-585, XP055456242, GB ISSN: 1350-9047, DOI: 10.1038/sj.cdd.4401773 abstract page 576, left-hand column, paragraph 1 - page 584, left-hand column, paragraph 3 -----	1-61

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2021/050007

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IL2021/050007

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015157864	A1	22-10-2015	CA 2944663 A1 22-10-2015
			US 2017182096 A1 29-06-2017
			WO 2015157864 A1 22-10-2015

US 2019169597	A1	06-06-2019	AU 2017312132 A1 21-03-2019
			CA 3034101 A1 22-02-2018
			CN 109715171 A 03-05-2019
			EP 3500271 A1 26-06-2019
			JP 2019528691 A 17-10-2019
			US 2019169597 A1 06-06-2019
			WO 2018035423 A1 22-02-2018
