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(54) Title: PSMA-BASED MOLECULAR-GENETIC REPORTER SYSTEM

(57) Abstract: The presently disclosed subject matter provides methods, reporter gene constructs, and kits for using prostate-specific membrane antigen (PSMA) as an imaging reporter to image a variety of cells and tissues.

PSMA-BASED MOLECULAR-GENETIC REPORTER SYSTEM

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No.
5 61/954,947, filed March 18, 2014, and U.S. Provisional Application No. 61/972,833,
filed March 31, 2014, which are incorporated herein by reference in their entirety.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made in part with United States Government support under
10 EB005324 awarded by the National Institutes of Health (NIH). The U.S. Government
has certain rights in the invention.

BACKGROUND

Reporter gene imaging is a component of molecular imaging that can provide
15 noninvasive assessments of endogenous biologic processes in living subjects and that
can be performed using different imaging modalities (Brader et al, 2013). Reporter
genes were first developed using the bacterial β -galactosidase and chloramphenicol
acetyltransferase genes to study various cellular processes both *in vitro* and *in vivo*
(Forss et al, 1990; Overbeek et al. 1985). Visualization of these first reporter
20 enzymes required postmortem tissue sampling and processing (Forss et al, 1990;
Overbeek et al. 1985), but advances in cell biology led to the development of novel
noninvasive visualization systems that could provide accurate and sensitive
measurements in animal models of human disease (Brader et al, 2013). In particular,
gene reporter-probe systems currently offer a non-invasive means to monitor gene
25 therapy, to track the movement of cells or the activation of signal transduction
pathways, and to study protein-protein interactions and other aspects of signal
transduction (Brader et al, 2013).

A number of reporter genes, including the mutant herpes simplex virus
thymidine kinase (HSV-sr39tk) and the human sodium iodide symporter (hNIS) have
30 been clinically applied (Barton et al. 2008; Yaghoubi et al, 2001). However, in
general, current reporter-probe systems still face limitations for clinical translation.
Such limitations include, for example, the lack of biocompatibility due to
immunogenicity, low sensitivity due to insufficient reporter expression per cell or lack

of a signal amplification mechanism, and high background due to non-specific binding of probe.

SUMMARY

5 In one aspect, the presently disclosed subject matter provides a method for using prostate-specific membrane antigen (PSMA) as an imaging reporter, the method comprising: a) introducing a reporter gene construct comprising a PSMA gene operably linked to a transcriptional promoter to a cell; b) allowing the cell to express PSMA protein; c) adding an imaging probe that can detect the PSMA protein to the
10 cell; and d) imaging the imaging probe, thereby detecting the PSMA protein.

 In certain aspects, the presently disclosed subject matter provides an adenoviral reporter gene construct comprising a prostate-specific membrane antigen (PSMA) gene operably linked to a cytomegalovirus immediate-early gene (CMV) promoter.

15 In other aspects, the presently disclosed subject matter provides a non-viral, episomal reporter gene construct comprising a prostate-specific membrane antigen (PSMA) gene operably linked to a PEG-Promoter.

 In further aspects, the presently disclosed subject matter provides a kit comprising a presently disclosed construct.

20 Certain aspects of the presently disclosed subject matter having been stated hereinabove, which are addressed in whole or in part by the presently disclosed subject matter, other aspects will become evident as the description proceeds when taken in connection with the accompanying Examples and Figures as best described herein below.

25

BRIEF DESCRIPTION OF THE DRAWINGS

 Having thus described the presently disclosed subject matter in general terms, reference will now be made to the accompanying Figures, which are not necessarily drawn to scale, and wherein:

30 FIGS. 1A-1D show *in vitro* characterization of reporter adenovirus: (A) reporter expression was validated by western blot in HCT1 16 cells and by fluorescent cellular immunoassay in PC3-CAR cells 48 hours after infection of reporter adenovirus; and functionality of each reporter was verified in HCT1 16 and PC3-CAR

cells by (B) [³H]GCV uptake, (C) ¹²⁵I uptake, and (D) YC-I-27 binding. Data were normalized to cellular GFP expression and plotted as mean ± standard error of the mean of 3 replicates. *P < 0.01;

FIG. 2 shows *in vivo* GFP imaging of PC3-CAR cells infected *in vitro* with equal MOI of a PET imaging reporter or control adenovirus (AdTrack-Luciferase), separately mixed with matrigel, and implanted contralaterally into the upper flanks of a nude mouse (24 hours post-infection);

FIG. 3 shows the bioluminescent imaging of the matrigel suspension model from FIG. 2;

FIGS. 4A-4G show *in vivo* comparison of imaging reporters: time-activity curves for respective reporter-probe systems in matrigel suspension model for: (A) HSV-sr39TK:FHBG; (B) hNIS: ¹²⁵I; and (C) PSMA:DCFpyL. Reporter derived signal-to-control and reporter derived signal-to-muscle ratios for: (D) HSV-sr39TK; (E) hNIS; and (F) PSMA systems. (G) Peak signal-to-noise ratios of each reporter-probe pair where compared. Data are mean ± standard error of the mean of four animals. *P < 0.05;

FIGS. 5A-5B show PET/CT imaging of reporter-probe systems in which representative maximum intensity projections of reporter-probe systems of data were collected from 60-90 min after radiotracer injection and images were cropped above the liver and below the thyroid to display the ability of each reporter to sequester its respective probe (A); and uncropped images demonstrate background associated with each reporter-probe system (B). All images were decay corrected and scaled to the same maximum value;

FIGS. 6A-6D show (A) uptake of adenovirus expressing PSMA or a luciferase gene as compared to muscle (control); (B) target to background ratio of PSMA relative to luciferase and muscle; (C) PSMA imaging using ¹⁸F-DCFBC as a probe at 60 min post injection; and (D) PSMA imaging using ¹⁸F-DCFBC as a probe at 4 hr post injection. Mice were implanted with matrigel suspensions of cells expressing either PSMA (left) or a negative control gene (luciferase, right) in the upper flanks;

FIGS. 7A-7B show monitoring of hepatic infection and transgene expression by imaging PSMA in a murine model: athymic nude mice were administered adenovirus expressing PSMA or a luciferase (negative control) gene intravenously

and specific uptake was observed in the liver of animals by (A) optical and (B) SPECT imaging (obtained at 15 min post-injection of radiotracer);

FIGS. 8A-8E show an embodiment of a non-viral expression system for PSMA: (A) basic expression vector using the promoter for rat progression elevated gene-3 (PEG-Prom); (B) clinically compatible version of vector (A) equipped with
5 CpG-free vector backbone and CpG-free PSMA cDNA; (C) episomal amplisome vector: GAL4, yeast Gal4 DNA binding protein; VP16; transcriptional enhancer from CMV; 5x UAS (5 repetitions of the upstream activation sequence); (D) schematic diagram of PSMA: CD, cytoplasmic domain; TM, transmembrane domain; ED,
10 extracellular domain; and (E) various truncations of PSMA: FL, full length; W2G, point mutation at the second amino acid from tryptophan to glycine; C-625, truncations that lacks C-terminal part of the gene from amino acid 625; N-11, lacks N-terminal 11 amino acids;

FIGS. 9A-9B show that PSMA was expressed on the cell surface of the
15 transfected cells: (A) FACS analyses of transfected cells using fluorescent labeled PSMA-targeted compound (YC-VIII-46); and (B) immunofluorescent analyses of transfected cells using anti-PSMA antibody;

FIG. 10 shows an *in vitro* radio uptake assay using MDA-MB-231 cell lines transiently transfected with the basic non-viral vector with full-length and truncations
20 of PSMA;

FIG. 11 shows a map of a representative CpG-free-PEG-PSMA vector. This clinically compatible vector contains CpG-free PSMA cDNA, a CpG-free neo^r selection marker, and a CpG-free S/MAR sequence; and

FIG. 12 shows a representative cDNA sequence of a CpG-free PSMA gene
25

DETAILED DESCRIPTION

The presently disclosed subject matter now will be described more fully hereinafter with reference to the accompanying Figures, in which some, but not all embodiments of the inventions are shown. Like numbers refer to like elements
30 throughout. The presently disclosed subject matter may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Indeed, many modifications and other embodiments of

the presently disclosed subject matter set forth herein will come to mind to one skilled in the art to which the presently disclosed subject matter pertains having the benefit of the teachings presented in the foregoing descriptions and the associated Figures.

Therefore, it is to be understood that the presently disclosed subject matter is not to be
5 limited to the specific embodiments disclosed and that modifications and other
embodiments are intended to be included within the scope of the appended claims.

Molecular-genetic imaging is the visualization, characterization, and measurement of biological processes at the molecular and cellular levels by using reporter gene/reporter probe systems. The prostate-specific membrane antigen
10 (PSMA) is a human transmembrane protein that possesses many properties that are desirable as a reporter for an imaging system. PSMA is internalized upon the binding of certain ligands (Liu et al, 1998), providing a mechanism to accumulate PSMA-targeting agents within cells. The expression of PSMA is limited to the prostate, the proximal tubules of the kidney, and the brain (Rajasekaran et al, 2005). Alternative
15 processing results in both cell surface and cytoplasmic isoforms, the latter of which is primarily expressed in the healthy prostate (Mlcochova et al, 2009). PSMA expression is low and isolated in healthy tissues, allowing imaging at high sensitivity and specificity for metastatic prostate tumors that overexpress the cell surface isoform (Afshar-Oromieh et al, 2013; Barrett et al, 2013; Cho et al, 2012). There are several
20 PSMA-binding ligands that have been developed for the delivery of imaging and therapeutic agents for prostate cancer, including low-molecular-weight nuclear, fluorescent, and multi-modality imaging probes (Chen et al, 2009; Banerjee et al, 2011). This combination of tissue-restricted expression, human biocompatibility, available probe diversity, and proven clinical utility are some of the advantages of
25 using PSMA as an imaging reporter. The presently disclosed subject matter provides a PSMA-based gene reporter/probe system that can be generalized for a variety of cell and tissue types.

I. PSMA REPORTER GENE CONSTRUCTS AND KITS

30 In some embodiments, the presently disclosed subject matter provides a reporter gene construct comprising a PSMA gene. The PSMA gene may be from a variety of organisms, although in preferred embodiments, the gene will be a human gene (e.g., GenBank Accession No. DD461260 or NG_029170) and the PSMA

protein expressed from the gene will be a human protein (e.g., GenBank Accession No. NP_004467). In some embodiments, the PSMA gene will have substantial percent identity to the sequence identified as GenBank Accession No. DD461260 NG_029170 and the PSMA protein will have substantial percent identity to the
5 sequence identified as GenBank Accession No. NP_004467.

The term "percent identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case
10 may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: Computational Molecular Biology (Lesk, A. M., ed.) Oxford University Press, New York (1988); Biocomputing: Informatics and Genome Projects (Smith, D. W., ed.) Academic Press, New York (1993); Computer Analysis
15 of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., eds.) Humana Press, New Jersey (1994); Sequence Analysis in Molecular Biology (von Heinje, G., ed.) Academic Press (1987); and Sequence Analysis Primer (Gribskov, M. and Devereux, J., eds.) Stockton Press, New York (1991). Preferred methods to determine identity are designed to give the best match between the sequences tested. Methods to
20 determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, Wis.). Multiple alignments of the sequences may be performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS*.
25 5:151-153) with the default parameters, including default parameters for pairwise alignments.

The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" may be commercially available or independently
30 developed. Typical sequence analysis software will include but is not limited to the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wis.), BLASTP, BLASTN, BLASTX (Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410, and DNASTAR (DNASTAR, Inc., Madison, Wis.). Within

the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters which originally
5 load with the software when first initialized.

It is well understood by one skilled in the art that many levels of sequence identity are useful in identifying genes and proteins for use in the presently disclosed subject matter, for example wherein genes and proteins from a different species but have the same or similar function or activity. The term "substantial identity" or
10 "substantial percent identity" in its various grammatical forms comprises a sequence that has a desired percent identity, for example, at least 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% compared to a reference sequence using one
15 of the alignment programs described using standard parameters.

The term "imaging reporter", as used herein, refers to a protein that, in combination with an imaging probe, can be used to mark cells, intracellular organelles, and the like. Examples of reporter systems include reporters that encode β -galactosidase, chloramphenicol acetyltransferase, luciferase, and green fluorescent
20 protein (GFP). In some embodiments, the imaging reporter may act as both an imaging probe to visualize the target molecule, as well as a therapeutic agent, such as to inhibit a disease process, for example.

The term "reporter gene construct", as used herein, refers to a vector comprising the gene encoding for the imaging reporter. The reporter gene construct
25 can contain a number of components, including, but not limited to one or more of the following: an origin of replication; a selectable marker gene; one or more expression control elements, such as a transcriptional control element (e.g., a promoter, an enhancer, a terminator), and/or one or more translation signals; and a signal sequence or leader sequence for targeting to the secretory pathway in a selected host cell. In
30 some embodiments, the presently disclosed construct comprises all or part of a PSMA gene.

The term "promoter" or "transcriptional promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In

general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may
5 direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters that cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters." Promoters that cause a gene to be expressed in a specific cell type are commonly referred to as "cell-specific promoters"
10 or "tissue-specific promoters." Promoters that cause a gene to be expressed at a specific stage of development or cell differentiation are commonly referred to as "developmentally-specific promoters" or "cell differentiation-specific promoters." Promoters that are induced and cause a gene to be expressed following exposure or treatment of the cell with an agent, biological molecule, chemical, ligand, light, or the
15 like that induces the promoter are commonly referred to as "inducible promoters" or "regulatable promoters." In some embodiments, the promoter is tumor-specific, such as the promoter from a minimal promoter region of progression elevated gene-3 (*Peg-3*), which allows cancer-selective expression. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely
20 defined, DNA fragments of different lengths may have identical promoter activity.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then spliced (if the coding sequence contains introns) and translated into the protein encoded by the coding sequence.

25 "Transcriptional and translational control sequences" are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

The term "operably linked" refers to the association of nucleic acid sequences
30 on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is

under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

In some embodiments, the reporter gene construct is based on a viral vector, such as a retroviral vector including a lentiviral vector, an adenoviral vector, an adeno-associated viral vector, and the like. In some embodiments, the reporter gene construct is based on an adenoviral vector. Advantages of adenoviral vectors include, but are not limited to, that they are a means to transfer equal amounts of transgene reporters, the adenovirus genome is maintained episomally and is not influenced by integration site or copy number, and adenoviruses have been applied successfully in imaging reporter gene comparisons (Gil et al, 2012; Min et al, 2003; Miyagawa et al, 2005).

In some embodiments, the presently disclosed subject matter provides an adenoviral reporter gene construct comprising a prostate-specific membrane antigen (PSMA) gene operably linked to a cytomegalovirus immediate-early gene (CMV) promoter. The CMV promoter induces high-level constitutive expression in a variety of mammalian cell lines.

In some embodiments, the presently disclosed subject matter provides a non-viral, episomal reporter gene construct. In general, in eukaryotes, episomes are closed circular DNA molecules that are replicated in the nucleus and remain physically separate from host cell chromosomes. In other embodiments, the presently disclosed subject matter provides a non-viral, episomal reporter gene construct comprising a prostate-specific membrane antigen (PSMA) gene operably linked to a PEG-Promoter, a tumor-specific promoter from a minimal promoter region of progression elevated gene-3 (*Peg-3*), which allows cancer-selective expression of PSMA.

In some embodiments, the non-viral, episomal reporter gene construct further comprises a Gal4-VP16 and Upstream Activation Sequence (UAS) transcriptional amplification system. In this system, the Gal4-VP16 component, a fusion protein of yeast transcriptional activator protein Gal4 and herpes simplex virus VP16, acts as an activator and the UAS component acts as an enhancer to which Gal4 specifically binds to activate gene transcription. In other embodiments, each UAS is 17 base pairs long, roughly palindromic, and in the form of CGG-N 11-CCG. In other embodiments, the UAS is 5' -(T/C)GGAGTACTGTCCTCCG-3' (SEQ ID NO: 1). Multiple copies of the UAS can be inserted onto one construct, such as 2, 3, 4, 5, 6, 7,

8, 9, 10, or more. These copies may be separated by other nucleotides. In further embodiments, the transcriptional amplification system comprises five copies of the UAS. In still further embodiments, the five copies of the UAS has the sequence:

CGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTC
 5 CTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAG (SEQ
 ID NO: 2).

In some embodiments, the non-viral, episomal reporter gene construct further comprises the *EBNA-1* (Epstein-Barr nuclear antigen 1) gene, which encodes a viral protein associated with the Epstein-Barr virus, for prolonged action in transfected
 10 cells. In other embodiments, the non-viral, episomal gene construct further comprises OriP, a viral origin of plasmid replication.

In some embodiments, the PSMA gene comprises the coding sequence of the full-length PSMA protein. In other embodiments, the PSMA gene is truncated at the 5' end of the coding sequence of the PSMA protein. It has been found that N-
 15 terminal truncations of the PSMA protein, such as truncation of the first 11 amino acids, still allows the N-terminally modified PSMA to successfully localize to the cell membrane and centrosome. In still other embodiments, the truncation results in a mutated PSMA protein that is lacking the first 11 amino acids of the wild-type PSMA protein. In addition, substitution of the second amino acid in the PSMA protein from
 20 glycine to tryptophan also allows the modified PSMA protein to localize correctly. In further embodiments, the PSMA protein is mutated such that its second amino acid is glycine instead of tryptophan. In still further embodiments, the PSMA gene is mutated such that the construct expresses a mutated PSMA protein that has glycine as
 a second amino acid instead of tryptophan.

25 As used herein, the term "mutation" or "mutated" is used herein to refer to an alteration in a polynucleotide sequence, which results in an altered gene product or protein. A mutation according to the presently disclosed subject matter can involve substitution, insertion or deletion. A polynucleotide or protein in which a mutation has occurred is called a "mutant". Mutation may be introduced to one or both strands
 30 of a double-stranded polynucleotide. The strand of a double-stranded polynucleotide in which a mutation has occurred is referred to as a "mutant strand"; the strand with no mutation introduced is called a "non-mutant strand". The term "mutagenesis" according to the invention refers to the introduction of mutations into a polynucleotide

sequence. The term "wild-type" as used herein refers to the normal, non-mutated version of a gene or protein.

Previous efforts have shown that PSMA expression has not significantly altered cellular phenotype *in vitro* in multiple mammalian cell types. In still further
5 embodiments, functional modifications that alter PSMA enzymatic activity, ligand binding ability, cellular internalization, and the like are employed, for example in cases of negative effects of transgene expression (Anilkumar et al, 2003; Rajasekaran et al, 2003).

In some embodiments, a CpG-free version of PSMA is used that has a
10 modified cDNA sequence that contains no CpG sequences. This modification at the DNA level does not change the protein sequence. Therefore, the CpG-free PSMA also has substantial percent identity to the protein sequence identified as GenBank Accession No. NP_004467. Accordingly, in some embodiments, the PSMA gene is CpG-free.

15 In other embodiments, the reporter gene construct comprising a PSMA gene is CpG-free. In still other embodiments, a presently disclosed clinically compatible vector is equipped with CpG-free sequences. In further embodiments, a CpG-free-PEG-PSMA vector comprises a CpG-free R6K gamma origin of replication, a CpG-free polyadenylation signal, a CpG-free Neomycin/Kanamycin resistant gene, a CpG-
20 free S/MAR sequence and a CpG-free PSMA. Using the CpG-free DNA prevents harmful immune reaction in human via Toll-like receptor 9 (Bauer et al, 2001) and using a Neomycin/Kanamycin resistant gene allows the presently disclosed vectors to be employed for clinical use (FDA, 1996).

As used herein, a "CpG site" is a region of DNA where a cytosine nucleotide
25 occurs next to a guanine nucleotide. Cytosines in CpG sites can be methylated to form 5-methylcytosine. As used herein, the term "CpG-free" means that the gene and/or construct does not have any CpG sites.

In some embodiments, the presently disclosed subject matter provides a kit comprising a presently disclosed construct. In general, a presently disclosed kit
30 contains some or all of the components, reagents, supplies, and the like to practice a method according to the presently disclosed subject matter. In other embodiments, the term "kit" refers to any intended article of manufacture (e.g., a package or a container) comprising a presently disclosed reporter gene construct and a set of

particular instructions for practicing the methods of the presently disclosed subject matter.

II. METHODS FOR USING PSMA AS AN IMAGING REPORTER

5 In some embodiments, the presently disclosed subject matter provides a method for using prostate-specific membrane antigen (PSMA) as an imaging reporter, the method comprising: a) introducing a reporter gene construct comprising a PSMA gene operably linked to a transcriptional promoter to a cell; b) allowing the cell to express PSMA protein; c) adding an imaging probe that can detect the PSMA protein
10 to the cell; and d) imaging the imaging probe, thereby detecting the PSMA protein.

The presently disclosed constructs can be used in the presently disclosed methods and are described in detail hereinabove.

In some embodiments, the reporter gene construct is a viral reporter gene construct. In other embodiments, the viral reporter gene construct is an adenoviral
15 reporter gene construct. In still other embodiments, the reporter gene construct is a non-viral, episomal reporter gene construct.

In some embodiments, the transcriptional promoter is a constitutive promoter. In other embodiments, the constitutive promoter is the cytomegalovirus immediate-early gene (CMV) promoter. In still other embodiments, the transcriptional promoter
20 is the PEG-Promoter.

In some embodiments, the non-viral, episomal reporter gene construct further comprises a Gal4-VP16 and Upstream Activation Sequence (UAS) transcriptional amplification system. In other embodiments, the transcriptional amplification system
comprises five copies of the UAS.

25 In some embodiments, the non-viral, episomal reporter gene construct further comprises the *EBNA-1* gene. In other embodiments, the non-viral, episomal reporter gene construct further comprises OriP.

In some embodiments, the PSMA gene comprises the coding sequence of the full-length PSMA protein. In other embodiments, the PSMA gene is truncated at the
30 5' end of the coding sequence of the PSMA protein. In still other embodiments, the truncation results in a mutated PSMA protein that is lacking the first 11 amino acids of the wild-type PSMA protein. In further embodiments, the PSMA protein is mutated such that its second amino acid is glycine instead of tryptophan.

In some embodiments, the PSMA gene is CpG-free. In other embodiments, the reporter gene construct comprising a PSMA gene is CpG-free. In still other embodiments, the reporter gene construct comprising a PSMA gene further comprises a neomycin or kanamycin resistant gene.

5 In some embodiments, the presently disclosed method comprises introducing a reporter gene construct comprising a PSMA gene operably linked to a transcriptional promoter to a cell. Introducing the reporter gene construct to a cell may be performed using electroporation, DEAE Dextran transfection, calcium phosphate transfection, cationic liposome fusion, protoplast fusion, creation of an *in vivo* electric field, DNA-
10 coated microprojectile bombardment, injection with recombinant replication-defective viruses, homologous recombination, *in vivo* gene therapy, *ex vivo* gene therapy, viral vectors, naked DNA transfer, and any other method that will allow the reporter gene construct to enter the cell.

In some embodiments, the cell is found in a subject, in which case introducing
15 the reporter gene construct to a cell may also occur by any suitable route of administration, including orally, nasally, transmucosally, ocularly, rectally, intravaginally, parenterally, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intra-articular, intra-sternal, intra-synovial, intra-hepatic, intralesional, intracranial, intraperitoneal,
20 intranasal, or intraocular injections, intracisternally, topically, as by powders, ointments or drops (including eyedrops), including buccally and sublingually, transdermally, through an inhalation spray, or other modes of delivery known in the art.

In those embodiments where the cell is found in a subject, the cell is desirably
25 a human cell, although it is to be understood that the methods described herein are effective with respect to the cells of all vertebrate species, which are intended to be included in the term "subject." Suitable animal cells include mammals including, but not limited to, primates, e.g., humans, monkeys, apes, gibbons, chimpanzees, orangutans, macaques and the like; bovines, e.g., cattle, oxen, and the like; ovines, e.g., sheep and the like; caprines, e.g., goats and the like; porcines, e.g., pigs, hogs,
30 and the like; equines, e.g., horses, donkeys, zebras, and the like; felines, including wild and domestic cats; canines, including dogs; lagomorphs, including rabbits, hares, and the like; and rodents, including mice, rats, guinea pigs, and the like. An animal

cell may be a transgenic animal cell. In some embodiments, the cell is a human cell including, but not limited to, fetal, neonatal, infant, juvenile, and adult human cells. Further, a "cell" can include a diseased cell, such as a cancer cell. In other embodiments, the subject is human.

5 In some embodiments, after introducing a reporter gene construct comprising a PSMA gene operably linked to a transcriptional promoter of a cell, the methods comprise allowing the cell to express the PSMA protein. By "express" or "expressing", it is meant the process by which information from a gene is used in the synthesis of a functional gene product, such as a protein.

10 In some embodiments, an imaging probe is then added to detect the PSMA protein. By "detect" or "detecting", it is meant that the imaging probe binds, is cleaved by, or in some way interacts with the expressed PSMA protein. A variety of imaging probes can be used, although in a preferred embodiment, the imaging probe allows for sensitive, non-invasive imaging. In another preferred embodiment, the
15 imaging probe is compatible with human use (non-immunogenic).

 In some embodiments, PSMA-binding ligands can be used as imaging probes, such as low-molecular-weight nuclear, fluorescent, and multi-modality imaging probes. In other embodiments, the imaging probe is chosen depending on the pharmacokinetics and sensitivity required for a particular indication. In still other
20 embodiments, a high-affinity imaging probe is used that does not serve as a substrate. In still further embodiments, the imaging probe may bind the active site of PSMA electrostatically, treating it essentially as a receptor ("receptor" probe). In further embodiments, the imaging probe may be a PSMA inhibitor.

 Accordingly, in some embodiments, the imaging probe detects the PSMA
25 protein by binding to the active site of the PSMA protein. In other embodiments, (aminostyryl)pyridinium (ASP) dyes are used as imaging probes. In still other embodiments, the imaging probe is 2-(3-{1-carboxy-5-[(6-[¹⁸F]fluoro-pyridine-3-carbonyl)-amino]-pentyl}-ureido)-pentanedioic acid (DCFpyL) or N-[N-[(S)-1,3-dicarboxypropyl]carbamoyl]-4-[¹⁸F]fluorobenzyl-L-cysteine (DCFBC).

30 An advantage of using PSMA as an imaging reporter is that it possesses enzymatic activity. Therefore, in some embodiments, the imaging probe may be an "enzymatic" probe, the cleavage products of which can be fluorescent and/or radioactive. In other embodiments, the cleavage products of the probe intercalate

within the target cell membrane. In still other embodiments, an enzymatic imaging probe is used for the detection of cellular events. Accordingly, in some embodiments, the presently disclosed methods further comprise the cleavage of the imaging probe by the PSMA protein. In other embodiments, cleavage of the imaging probe results in
5 at least one cleavage product that is fluorescent and/or radioactive.

In some embodiments, after adding the imaging probe, the presently disclosed methods comprise imaging the probe, thereby detecting the PSMA protein. Molecular imaging can be performed with a range of instruments, most of which utilize a specific region of the electromagnetic spectrum. These imaging modalities
10 include, but are not limited to, magnetic resonance imaging (MRI), x-ray computed tomography (CT), positron emission tomography (PET), single photon emission computed tomography (SPECT), and optical (bioluminescence and fluorescence imaging). In other embodiments, imaging the PSMA protein occurs by at least one method selected from the group consisting of near-infrared (NIR) optical tomography,
15 positron emission tomography (PET), and single photon emission computed tomography (SPECT).

In some embodiments, the PSMA protein is detected on the surface of the cell. In other embodiments, the PSMA protein is detected inside the cell. In still other
20 embodiments, the imaging probe is imaged on the cell surface. In further embodiments, the imaging probe is imaged inside the cell. The term "surface of the cell" or "cell surface" as used herein means the outer surface of the cell, including the cell or plasma membrane. In further embodiments, the PSMA protein or imaging probe may intercalate within the cell membrane, which means herein that the PSMA
protein or imaging probe is still on the cell surface.

25 In some embodiments, the presently disclosed subject matter enables non-invasive imaging of *in vivo* gene expression, such as for the study of signal transduction pathways and protein interaction networks, the study of the underlying physiology or pathophysiology of disease, such as cancer and neurodegenerative diseases, help in developing new, selective drugs for various diseases, and the like.

30 Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly

understood by one of ordinary skill in the art to which this presently described subject matter belongs.

Following long-standing patent law convention, the terms "a," "an," and "the" refer to "one or more" when used in this application, including the claims. Thus, for
5 example, reference to "a subject" includes a plurality of subjects, unless the context clearly is to the contrary (e.g., a plurality of subjects), and so forth.

Throughout this specification and the claims, the terms "comprise,"
"comprises," and "comprising" are used in a non-exclusive sense, except where the
context requires otherwise. Likewise, the term "include" and its grammatical variants
10 are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that can be substituted or added to the listed items.

For the purposes of this specification and appended claims, unless otherwise indicated, all numbers expressing amounts, sizes, dimensions, proportions, shapes, formulations, parameters, percentages, parameters, quantities, characteristics, and
15 other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term "about" even though the term "about" may not expressly appear with the value, amount or range. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are not and need not be exact, but may be approximate and/or larger
20 or smaller as desired, reflecting tolerances, conversion factors, rounding off, measurement error and the like, and other factors known to those of skill in the art depending on the desired properties sought to be obtained by the presently disclosed subject matter. For example, the term "about," when referring to a value can be meant to encompass variations of, in some embodiments, $\pm 100\%$ in some
25 embodiments $\pm 50\%$, in some embodiments $\pm 20\%$, in some embodiments $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$, and in some embodiments $\pm 0.1\%$ from the specified amount, as such variations are appropriate to perform the disclosed methods or employ the disclosed compositions.

Further, the term "about" when used in connection with one or more numbers
30 or numerical ranges, should be understood to refer to all such numbers, including all numbers in a range and modifies that range by extending the boundaries above and below the numerical values set forth. The recitation of numerical ranges by endpoints includes all numbers, e.g., whole integers, including fractions thereof, subsumed

within that range (for example, the recitation of 1 to 5 includes 1, 2, 3, 4, and 5, as well as fractions thereof, e.g., 1.5, 2.25, 3.75, 4.1, and the like) and any range within that range.

5

EXAMPLES

The following Examples have been included to provide guidance to one of ordinary skill in the art for practicing representative embodiments of the presently disclosed subject matter. In light of the present disclosure and the general level of skill in the art, those of skill can appreciate that the following Examples are intended to be exemplary only and that numerous changes, modifications, and alterations can be employed without departing from the scope of the presently disclosed subject matter. The following Examples are offered by way of illustration and not by way of limitation.

15

EXAMPLE 1

Genetic reporters provide a non-invasive method to monitor and evaluate a population of cells. The ideal properties of a gene reporter-probe system include biocompatibility, lack of immunogenicity, low background expression or signal, and high sensitivity of detection. The prostate-specific membrane antigen (PSMA) is an attractive candidate for a genetic reporter as it is a human transmembrane protein with a very selective expression pattern and there are several PSMA imaging agents available for clinical and pre-clinical applications. The present studies evaluated the use of PSMA as a genetic imaging reporter by comparison to two clinically established reporters, the mutant herpes simplex virus type I thymidine kinase and the human sodium-iodide symporter. Adenoviruses expressing each reporter were constructed and validated *in vitro* for expression and function. To compare PSMA to existing imaging reporters, a bilateral matrigel suspension model was established with nude mice bearing cells equally infected with each reporter or control adenovirus. Dynamic PET scans were performed and time-activity curves were generated for each reporter-probe pair. A comparison of peak target-to-background ratios revealed that PSMA offered the highest ratio relative to the control matrigel suspension as well as muscle. Further, PSMA was applied as an imaging reporter to monitor adenoviral liver transduction with both nuclear and optical imaging probes.

Materials and Methods

Chemicals and primers were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture reagents were purchased from Mediatech, Inc. (Manassas, VA), Gemini
5 Bio-Products (Woodland, CA), and Invitrogen (Carlsbad, CA). Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs (Ipswich, MA). Plasmid and gel purification components were purchased from Qiagen, Inc. (Valencia, CA) or Sigma-Aldrich. Transfection reagent was purchased from Invitrogen. Adenovirus purification kits were purchased from Puresyn, Inc. (Malvern,
10 Pennsylvania). [³H]Gancyclovir ([³H]GCV) was purchased from Moravек Biochemicals (Brea, CA). [¹²⁵I]NaI was purchased from MP Biomedicals (Solon, OH). The J591 antibody was kindly provided by Dr. Neil Bander (Weill Cornell Medical College, NY).

Cell Culture. Cell culture media contained 50 µg/mL gentamycin and 5
15 µg/mL ciprofloxacin to prevent bacterial contamination. HCT116 cells were obtained from Dr. Bert Vogelstein (Johns Hopkins University, MD) and grown in McCoy's 5A supplemented with 10% FBS. DPL cells were generated in the lab of Dr. Ronald Rodriguez and grown in DMEM supplemented with 10% FBS (Hoti et al, 2007). PC3-CAR cells were obtained from Dr. J.T. Hsieh (University of Texas Southwestern
20 Medical School, TX) and grown in RPMI 1640 medium supplemented with 10% FBS and 300 µg/mL G418.

Cloning of Adenoviral Vectors. Viruses were generated using the AdEasy vector system (Luo et al, 2007). PSMA was sub-cloned from pDonor-CMV-PSMA into pAd-Track-CMV using BglII and NotI. hNIS was sub-cloned from pDonor-
25 CMV-hNIS into pAd-Track-CMV using BglII and NotI. The CMV promoter was sub-cloned by PCR using primers 5'-CMV (ACGAAGATCTTAATAGTAATCAATTACGGGG) (SEQ ID NO: 3) and 3'-CMV (TCCGGAATTCCTAGCGGATCTGACGGTTCAC) (SEQ ID NO: 4) upstream of sr39tk-GFP in the shuttle plasmid pGal-mNLS-sr39tk-EGFP using BglII and EcoRI
30 restriction sites. The expression cassette from pCMV-mNLS-sr39tk-EGFP was then cloned into RpSWCl using NotI and Sail restriction sites. The resulting plasmids, pAd-Track-CMV- PSMA, pAd-Track-CMV-NIS, or RpS-CMV-mNLS-sr39tk-EGFP were recombined with pAdEasy-1 vector in BJ5183 *Escherichia coli* and selected for

kanamycin resistance. Each resulting viral genome was linearized with Pac I and transfected into DPL cells for virus production. The resulting adenoviruses were column purified using Adenopure adenovirus purification kit and titered in HEK293 cells by GFP and Hexon staining with Adeno-X rapid titer kit (Clontech, Mountain View, CA) 36 hours post-infection.

Preparation of J591 Antibody Conjugated to IRDye 800CW. The J591 antibody was labeled by using IRDye® 800CW NHS Ester (LI-COR, Lincoln, NE). Briefly, 20 µL of 3 mM IRDye® 800CW NHS Ester (-10 molar fold excess in DMSO) were added to 200 µL of J591 antibody (5 mg/mL in PBS) and kept 1 h at ambient temperature. The unreacted dye was removed by passing through Zeba desalting column (Pierce, Thermo Fisher Scientific, Rockford, IL). The conjugation of dye was confirmed by measuring Absorbance at 280 and 750 nm and by SDS-PAGE.

Western Blot. Cells were washed with 1x PBS and re-suspended in RIPA lysis buffer supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN) and incubated on ice for 30 minutes and then centrifuged for 10 minutes at 4°C. Total protein concentration was measured using the Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. Equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF membrane, blocked with 5% nonfat dry milk in PBS for 1 hour at room temperature, and then incubated with primary antibody (mouse monoclonal anti-hNIS from ABCAM [SPM186], mouse monoclonal anti-PSMA J591, rabbit polyclonal anti-HSV TK obtained from Dr. William Summers (Yale University, New Haven, CT) overnight at 4°C. The membrane was then probed with anti-mouse IR800 conjugated secondary antibody or anti-rabbit IR680 conjugated secondary antibody for 1 hour and scanned on the Odyssey Infrared Imager (LI-COR, Lincoln, NE) using the manufacturer's protocol.

Fluorescent Cellular Immunoassay. Control cells or cells infected with reporter virus were plated on 96-well plates. When 90% confluent, cells were washed with 1x PBS and fixed with 4% paraformaldehyde for 30 minutes at room temperature. After fixation, cells were washed with 1x PBS, blocked with 1% BSA in PBS for 1 hour at room temperature, and incubated with primary antibodies described above at 37°C for 1 hour. Cells were then probed with secondary antibody conjugated with IR800 for 1 hour and scanned on the Odyssey Infrared Imager using

the manufacturer's protocol.

Cell Uptake Assays. Cellular uptake of ^{125}I was measured in a fashion similar to that of De la Vieja (De la Vieja et al, 2005), while HSV-SR39TK expression was measured as described by Yaghoubi et al. (2006). PSMA expression was assessed by
5 measuring cellular uptake of the known, high-affinity PSMA targeting ligand, 2-[3-[1-carboxy-5-(4-[^{125}I]iodo-benzoylamino)-pentyl]-ureido]-pentanedioic acid (YC-I-27) (Chen et al, 2008). For uptake of ^{125}I , cells were harvested in PBS using a cell scraper and placed in a FACS tube for quantification in an automated γ -counter (1282 Compugamma CS, Pharmacia/LKB Nuclear, Inc., Gaithersburg, MD). For [^3H]GCV,
10 the amount of radiolabeled substrate retained was quantified by scintillation counting on a Microbeta Wallac (Perkin-Elmer, Waltham, MA). YC-I-27 uptake was also measured using the automated γ -counter. In each case, uptake was calculated as amount of radioactivity in the cells divided by radioactivity in the media and normalized by the degree of expression of green fluorescent protein (GFP).

In Vivo Imaging. Studies were performed according to protocols approved by
15 the Animal Care and Use Committee at Johns Hopkins University. A matrigel suspension model was developed to enable imaging of similar numbers of cells *in vivo*. Four- to six-week-old athymic male nude mice (Harlan Laboratories Inc., Frederick, MD) were used for all experiments. 4×10^6 infected cells were injected
20 subcutaneously in the presence of Matrigel (BD Biosciences, Bedford, MA) at a 2:1 (celkmatrigel) ratio.

PET was performed for *in vivo* reporter comparison. 9-(4-[^{18}F]Fluoro-3-[hydroxymethyl]butyl)guanine (FHBG) and 2-(3-{1-carboxy-5-[(6-[^{18}F]fluoro-pyridine-3-carbonyl)-amino]-pentyl}-ureido)-pentanedioic acid (DCFPyL) were
25 synthesized as previously described (Ponde et al, 2004; Chen et al, 2011). [^{124}I]NaI was kindly provided by Dr. Jason Lewis (Memorial Sloan-Kettering Cancer Center, NY). Athymic nude mice contralaterally implanted with reporter or control expressing matrigel suspensions in the upper flanks were used for imaging. Mice were anesthetized with 3% isoflurane in oxygen for induction and were maintained
30 under 1.5% isoflurane in oxygen at a flow rate of 0.8 L/min. Mice were then placed in the prone position on the gantry of a GE eXplore VISTA small animal PET scanner (GE Healthcare Life Sciences, Pittsburgh, PA) and injected intravenously with ~ 7.4 MBq (200 μCi) in 200 μL of PBS of each respective radiotracer. Images were

acquired as a dynamic scan of 18 five-minute frames at 250 to 700 keV for ^{18}F and 400-700keV for ^{124}I . Images were reconstructed using the FORE/2D-OSEM method (1 iteration, 16 subsets) and included correction for radioactive decay, scanner dead time, and scattered radiation. After PET imaging, the mobile mouse holder was placed on the gantry of an X-SPECT (Gamma Medica-Ideas, Salem, NH) small animal imaging device to acquire the corresponding CT. PET and CT data were co-registered using AMIDE software (sourceforge.net).

To apply PSMA as a reporter by imaging transduced hepatocytes, near-infrared (MR) optical and single photon emission computed tomographic (SPECT) imaging were performed. Nude mice were injected intravenously with 1×10^7 IU of Ad-Track-PSMA or Ad-Track-Luciferase in a 200 μL volume. Luciferase was not used for imaging in this instance but as a control gene. For NIR imaging, 24 hours after Ad-Track-PSMA or Ad-Track-Luciferase animals were administered 15 μg or 30 μg of Anti-PSMA Antibody, J591 conjugated with IR800 in 200 μL of PBS via the lateral tail vein. For SPECT animals received $\sim 7.4\text{MBq}$ (200 μCi) of the known, high-affinity PSMA targeting ligand, 2-(3-[1-carboxy-5-[(5-[^{125}I]iodo-pyridine-3-carbonyl)-amino]-pentyl]-ureido)-pentanedioic acid (YC-VI-11) (Chen et al, 2008) in 200 μL of PBS also at 24 hours after Ad-Track-PSMA or Ad-Track-Luciferase. Animals received inhalational anesthesia (isoflurane) through a nose cone attached to the imaging bed. NIR images were acquired 48 hours post-injection of conjugated J591 and imaged using the Pearl Impulse (LI-COR, Lincoln, NE), while SPECT images were acquired after 15 minutes of radiopharmaceutical uptake using the X-SPECT. For SPECT scans, mice were scanned over 180° in 5.5° , 30 second increments. SPECT images were co-registered with CT. For each modality, images for Ad-Track-PSMA or Ad-Track-Luciferase were acquired at the same parameter settings and scaled to the same maximum values.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). An unpaired, 2-tailed t test was used and P-values < 0.05 were considered significant.

Radiochemical Syntheses. Radiotracers used for PET, i.e., FHBG, [^{124}I]NaI, DCFPyL and N-[N-[(S)-1,3-dicarboxypropyl]carbonyl]-4-[^{18}F]fluorobenzyl-L-cysteine (DCFBC) (Cho et al, 2012), were synthesized at specific activities of 340 ± 14 Ci/mmol ($12.6 \pm 0.5\text{GBq}^{\wedge}\text{mol}$), with no carrier added, $1,180 \pm 665$ Ci/mmol (43.7

$\pm 24.6 \text{ GBq}/\mu\text{mol}$) and $21,233 \pm 14,651 \text{ Ci}/\text{mmol}$ ($786 \pm 542 \text{ GBq}/\mu\text{mol}$), respectively. Despite the significant difference in specific activity between DCFPyL and DCFBC, similar results were obtained on imaging (see herein below).

5 Adenoviral Reporter Comparison Model

The presently disclosed subject matter provides the development of PSMA as an imaging reporter and the comparison of PSMA to established imaging reporters. Three putative reporter genes, PSMA, mutant herpes simplex virus thymidine kinase (HSV-sr39tk), and human sodium iodide symporter (hNIS), were sub-cloned
 10 downstream of a cytomegalovirus (CMV) promoter in the E1 region of non-replicating serotype 5 adenoviruses (Table 1). HSV-sr39tk and hNIS have been applied clinically (Barton et al, 2008; Yaghoubi et al, 2001). Each viral construct also expressed GFP for fluorescence detection of viral infection and titering. Two cell
 15 line models, HCT1 6 colon cancer cells and PC3 prostate cancer cells engineered to express stably the coxsackie adenovirus receptor (PC3-CAR), were applied to viral reporter studies. The expression of each reporter was validated by western blot analysis of infected HCT1 6 cells (multiplicity of infection, MOI=1) 48 hours after infection.

Each reporter was detected at the appropriate molecular weight in infected
 20 cells and no endogenous expression was visible under mock-infected or Ad-Track (GFP) infected control conditions (FIG. 1A). In addition, specific transgene delivery and expression were detected by a cell-based fluorescent immunoassay (FIG. 1A). Specifically, PC3-CAR cells were equally infected with each reporter adenovirus (MOI=500) and fixed 48 hours after infection. Reporter protein expression was
 25 detected by primary antibody incubation followed by fluorescent secondary antibody staining and detection on a fluorescent plate reader. The results confirm homogeneous transgene expression and specificity by immunodetection.

Table 1. Description of reporter adenoviruses and respective imaging modalities used
 30 for detection of reporter genes.

Virus	Reporter Gene	Imaging Modalities
Ad-Track-PSMA	prostate-specific membrane antigen	Optical, PET, SPECT
Ad-Track-hNIS	human sodium iodide symporter	PET, SPECT

Ad-HSV-sr39tk	herpes simplex virus mutant thymidine kinase	PET
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In Vitro Functionality of Adenoviral Reporter Vectors

Functional assays for each reporter were analyzed using HCT1 16 and PC3-CAR cell lines by [³H]GCV uptake, ¹²⁵I uptake, and YC-I-27 binding. Cells were

5 infected with each reporter adenovirus. After 48 hours, the level of adenovirus infection was quantified by GFP fluorescence and the level of imaging reporter activity was quantified by functional binding and/or uptake of radiolabeled probes. Specific assay conditions were applied for each reporter gene due to the different mechanisms of reporter probe binding and/or uptake. Conditions were based on

10 previously established assay conditions from previous efforts and in the literature (De la Vieja, 2005; Yaghoubi et al, 2006). For GCV uptake, a prolonged washout period was not performed and as a result does not differentiate between phosphorylated GCV and unphosphorylated GCV that is associated with the cells at time of harvest. YC-I-27 uptake in PSMA infected cells proved more time dependent than the corresponding

15 radiotracers were for NIS or HSV-sr39tk. Without wishing to be bound to any one particular theory, it is believed that this may be due to the mechanism of sequestration, whereby [¹²⁴I]NaI and GCV were able to diffuse passively or are transported into cells where they are trapped, whereas YC-I-27 can only enter the cell via binding and internalization of PSMA at a 1:1 stoichiometry. Despite those caveats,

20 in all cases specific and significant uptake was observed in cells infected with reporter adenovirus resulting in a signal-to-noise ratio of at least 10- to 20-fold relative to control adenovirus (FIGS. 1B-1D).

In Vivo Comparison of Adenoviral Imaging Reporters

25 To compare reporter-probe systems *in vivo*, a matrigel suspension model was developed (FIG. 2). In this model, PC3-CAR cells were infected *in vitro* with equal MOI (multiplicity of infection) of a PET imaging reporter or control adenovirus (AdTrack-Luciferase) and then each population of infected cells was separately mixed with matrigel and implanted contralaterally into the upper flanks of a nude mouse (24

30 hours post-infection). Luciferase was not used for imaging but as a control gene. Twenty-four hours after implantation, *in vivo* GFP imaging was performed to ensure

that a similar level of control and reporter-infected cells remained at the sites of implantation. The contralateral cells masses were generally within 30% as measured by GFP imaging *in vivo* (FIG. 3).

The next day (48 hours after implantation), animals were subjected to a dynamic PET study whereby -7.4 MBq (200 μ Ci) of each respective radiotracer was administered intravenously and cellular uptake was quantified for a duration of 90 minutes. FHBG was applied for HSV-sr39tk imaging, [124 I]NaI for hNIS imaging, and DCFPyL was applied for imaging of PSMA (Yaghoubi et al, 2001; Chen et al, 2011; Dingli et al, 2006). This model was used for comparing the reporter-probe systems for at least three reasons: (1) *in vitro* infection allowed equal transgene delivery of reporters and direct evaluation of implantation by GFP imaging; (2) the application of a relatively small number of cells and the short duration of the experiment minimized the influence of biologic events, such as cell growth or substantial development of tumor vasculature, on imaging reporter activity; and (3) the reporter virus was compared to a control virus within the same animal, providing an additional control to obviate animal-to-animal variation.

Kinetic uptake of each imaging reporter in target and control tissue was represented as a standardized uptake value (SUV) (FIGS. 4A-4C) or SUV relative to control tissue (FIGS. 4D-4F). For the HSV- sr39tk reporter, high initial uptake was observed in all tissues followed by rapid clearance from cells (FIG. 4A). HSV-sr39tk-specific sequestration of the radiotracer was observed after 30 to 40 minutes. The initial and rapid uptake of FHBG was attributed to diffusion of the compound into the cells where the unphosphorylated radiotracer diffuses out of the cells while phosphorylated tracer was retained. A peak target-to-background ratio of 2.81 ± 0.27 relative to control cells and 4.99 ± 0.38 relative to muscle was observed (FIG. 4G). The signal accumulation in hNIS expressing cells occurred quickly and reached a plateau roughly 80 to 90 minutes after injection (FIG. 4B). No significant uptake was observed in control tissue. A peak target-to-background ratio of 3.13 ± 0.20 relative to control cells and 4.96 ± 0.65 relative to muscle was observed (FIG. 4G). Despite differences in the mechanism of reporter probe accumulation, both the maximum signal-to-background ratios and the maximum percentage injected dose per cubic centimeter of tissue (%ID/cc) values of these two imaging reporters were similar.

This supports the utility of the matrigel suspension model as a means to compare PSMA to the existing reporter-probe systems.

Kinetics of the PSMA imaging reporter using DCFPyL displayed rapid probe uptake with the signal peaking 15 minutes post-injection, followed by a plateau shortly thereafter (FIG. 4C). Clearance from control tissue occurred more slowly for DCFPyL than for the radioligands used for the hNIS and HSV-sr39tk systems. A peak target-to-background ratio of 5.19 ± 0.78 relative to control cells and 13.80 ± 3.52 relative to muscle were observed (FIG. 4G). The maximum intensity projection images, cropped above the liver and below the thyroid for each reporter-probe system, demonstrate the specific uptake in the reporter expressing cells when compared to the muscle or to control matrigel suspension (FIG. 5A). The target-to-background profiles revealed that a signal-to-noise ratio of greater than two (relative to muscle) could be detected after 45 minutes for HSV-sr39tk, 20 minutes for hNIS, and 10 minutes for PSMA (FIGS. 4D-4F).

Maximum signal-to-noise ratios were observed at 90 minutes, with the PSMA system having the highest ratio relative both to the luciferase matrigel suspension and the muscle (FIG. 4G). Uncropped images from each scan highlight the differences in efficiency of the reporter to sequester its cognate probe and demonstrate background due to natural expression of reporter genes or probe metabolism (FIG. 5B). High gastrointestinal uptake was observed in the HSV-sr39tk system, which has previously been attributed to probe metabolism (Tjuvajev et al, 2002). Background signal from the stomach and thyroid were observed in the hNIS system due to natural expression of hNIS in these tissues. For PSMA, kidney uptake was observed in part due to renal clearance as well as PSMA expression in the proximal tubules (Bacich et al, 2001). The PSMA reporter-probe system was also evaluated using DCFBC, which has been administered to patients (Cho et al, 2012; Mease et al, 2008). Results from DCFBC were similar to results obtained with DCFPyL, with the exception of a slower clearance from the control matrigel suspension (FIG. 6).

Application of the Adenoviral PSMA Reporter System

It has been previously reported in rodent models that adenoviral vectors administered via systemic injection will result in hepatocyte infection and transgene expression (Johnson et al, 2006). Optical imaging of mouse livers was performed 72

hours post-infection and 48 hours post-administration of 15 or 30 μ g of J591-IR800 (FIG. 7A). Regions of interest were drawn around the liver and a signal-to-noise ratio of approximately 3:1 was observed for the PSMA imaging reporter relative to control adenovirus. This model was also applied to PSMA-specific imaging with SPECT using YC-VI-1 (Chen et al., 2008), with similar results, where a signal-to-noise ratio of approximately 2:1 was observed 15 min post-administration of radiotracer (FIG. 7B). Similar levels of radioactivity were observed in the liver at 4 h post-injection. Collectively, these results reveal PSMA as a sensitive and specific imaging reporter that can be applied to multiple imaging modalities.

10

Non-Viral PSMA Reporter System

Three non-viral vector systems were created for systemic delivery and expression of PSMA as an imaging reporter (FIGS. 8A-8C). These vectors employed PEG-Promoter, a tumor-specific promoter from a minimal promoter region of progression elevated gene-3 (*Peg-3*), for cancer-selective expression of PSMA.

One of the vectors comprised additional features to enhance its functionality as an imaging reporter vector (FIG. 8C). This vector had the Gal4-VP16 and UAS transcriptional amplification system to enhance the expression level of PSMA. In addition, this vector used OriP, a viral origin of plasmid replication, and EBNA-1, a viral protein associated with the Epstein-Barr virus, for prolonged action in transfected cells.

A third vector was created to comply with the clinical requirement for human application. This vector featured CpG-free sequences for reduced immunity and used the FDA-approved Neomycin resistant gene as a selection marker (FIG. 8B). This vector used a CpG-free R6K origin for plasmid replication and a CpG-free S/MAR sequence for elongated expression (FIG. 11). FIG. 12 shows a representative cDNA sequence of a CpG-free PSMA gene (SEQ ID NO:5).

Various truncations of PSMA were also created in addition to the full-length gene (FIGS. 8D-8E) to test the effect on the physiological status of the transfected cells. It was found that the full-length (FL) and two N-terminally modified PSMA (FL(W2G) and N-1) were successfully localized to the cell membrane and centrosome as examined by FACS analyses (FIG. 9A) and an immunofluorescent study (FIG. 9B), whereas the C-terminally truncated PSMA (C625 and N-1/C-625)

failed to localize to the plasma membrane, indicating the importance of the C-terminal dimerization domain for its proper folding and localization.

The basic expression vectors (FIG. 8A) were tested for the ability to uptake a radio-labeled PSMA-targeted molecule (^{125}I -YC27). FIG. 10 shows an *in vitro* radio uptake assay using MDA-MB-231 cell lines transiently transfected with the basic non-viral vector with full-length and truncations of PSMA. Twenty-four hours after the transfection, cells were treated with $1\mu\text{Ci}$ of ^{125}I -YC27, washed twice and the uptake was measured. Only full-length and N-terminally truncated PSMA were able to specifically uptake the substrate (FIG. 10).

10

Discussion

The presently disclosed subject matter provides PSMA, a biomarker for prostate cancer located on the cell membrane which possesses receptor, transporter and enzymatic functions, as an imaging reporter. The results demonstrate that PSMA is able to sequester imaging ligands with high affinity and with a high target-to-background ratio. Additional studies support PSMA as an imaging reporter with PET, SPECT, and optical imaging modalities.

The PSMA probes evaluated herein, as well as other probes in clinical and preclinical development, bind to the active site of PSMA on the surface of the cell. Enzymatic and transporter based reporter genes are able to amplify the signal by accumulation of probe, whereas receptor based reporters can only yield a 1:1 stoichiometry of probe to reporter gene molecule. The presently disclosed subject matter suggests that ligand binding alone is sufficient for detection and yields a higher signal-to-noise ratio with the PSMA-based reporter-probe system than the hNIS and HSV-sr39tk reporter-probe systems. In addition, after ligand binding, PSMA can internalize and the enzymatic and/or transporter properties of PSMA can be exploited for signal retention and amplification. As advances in cell engineering and gene therapies emerge, PSMA can provide a sensitive and non-immunogenic reporter by which to follow their fate *in vivo*, non-invasively.

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REFERENCES

All publications, patent applications, patents, and other references mentioned in the specification are indicative of the level of those skilled in the art to which the

presently disclosed subject matter pertains. All publications, patent applications, patents, and other references are herein incorporated by reference to the same extent as if each individual publication, patent application, patent, and other reference was specifically and individually indicated to be incorporated by reference. It will be understood that, although a number of patent applications, patents, and other references are referred to herein, such reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

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25 Although the foregoing subject matter has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be understood by those skilled in the art that certain changes and modifications can be practiced within the scope of the appended claims.

30

THAT WHICH IS CLAIMED:

1. A method for using prostate-specific membrane antigen (PSMA) as an imaging reporter, the method comprising:
- 5 a) introducing a reporter gene construct comprising a PSMA gene operably linked to a transcriptional promoter to a cell;
- b) allowing the cell to express PSMA protein;
- c) adding an imaging probe that can detect the PSMA protein to the cell; and
- d) imaging the imaging probe, thereby detecting the PSMA protein.
- 10
2. The method of claim 1, wherein the reporter gene construct is a viral reporter gene construct.
3. The method of claim 2, wherein the viral reporter gene construct is an
- 15 adenoviral reporter gene construct.
4. The method of claim 3, wherein the transcriptional promoter is a constitutive promoter.
- 20
5. The method of claim 4, wherein the constitutive promoter is the cytomegalovirus immediate-early gene (CMV) promoter.
6. The method of claim 1, wherein the reporter gene construct is a non-viral, episomal reporter gene construct.
- 25
7. The method of claim 6, wherein the transcriptional promoter is the PEG-Promoter.
8. The method of claim 7, wherein the cell is a cancer cell.
- 30
9. The method of claim 6, wherein the non-viral, episomal reporter gene construct further comprises a Gal4-VP16 and Upstream Activation Sequence (UAS) transcriptional amplification system.

10. The method of claim 9, wherein the transcriptional amplification system comprises five copies of the UAS.
- 5 11. The method of claim 6, wherein the non-viral, episomal reporter gene construct further comprises the *EBNA-1* gene.
12. The method of claim 6, wherein the non-viral, episomal reporter gene construct further comprises OriP.
- 10 13. The method of claim 1, wherein the PSMA gene comprises the coding sequence of the full-length PSMA protein.
14. The method of claim 1, wherein the PSMA gene is truncated at the 5' end of the coding sequence of the PSMA protein.
- 15 15. The method of claim 14, wherein the truncation results in a mutated PSMA protein that is lacking the first 11 amino acids of the wild-type PSMA protein.
- 20 16. The method of claim 1, wherein the PSMA protein is mutated such that its second amino acid is glycine instead of tryptophan.
17. The method of claim 1, wherein the PSMA gene is CpG-free.
- 25 18. The method of claim 1, wherein the reporter gene construct comprising a PSMA gene is CpG-free.
19. The method of claim 1, wherein the reporter gene construct comprising a PSMA gene further comprises a neomycin or kanamycin resistant gene.
- 30 20. The method of claim 1, wherein the imaging probe detects the PSMA protein by binding to the active site of the PSMA protein.

21. The method of claim 1, further comprising the cleavage of the imaging probe by the PSMA protein.
22. The method of claim 21, wherein cleavage of the imaging probe results
5 in at least one cleavage product that is fluorescent and/or radioactive.
23. The method of claim 1, wherein the imaging probe is 2-(3-{ 1-carboxy-5-[(6-[¹⁸F]fluoro-pyridine-3-carbonyl)-amino]-pentyl}-ureido)-pentanedioic acid (DCFPyL) or N-[N-[(S)-1,3-dicarboxypropyl]carbamoyl]-4-[¹⁸F]fluorobenzyl-L-
10 cysteine (DCFBC).
24. The method of claim 1, wherein imaging the PSMA protein occurs by at least one method selected from the group consisting of near-infrared (NIR) optical tomography, positron emission tomography (PET), and single photon emission
15 computed tomography (SPECT).
25. The method of claim 1, wherein the PSMA protein is detected on the surface of the cell.
- 20 26. The method of claim 1, wherein the PSMA protein is detected inside the cell.
27. The method of claim 1, wherein the cell is found in a subject.
- 25 28. The method of claim 27, wherein the subject is human.
29. An adenoviral reporter gene construct comprising a prostate-specific membrane antigen (PSMA) gene operably linked to a cytomegalovirus immediate-early gene (CMV) promoter.
- 30 30. The construct of claim 29, wherein the PSMA gene comprises the coding sequence of the full-length PSMA protein.

31. The construct of claim 29, wherein the PSMA gene is truncated at the 5' end of the coding sequence of the PSMA protein.

32. The construct of claim 31, wherein the truncation results in a mutated PSMA protein that is lacking the first 11 amino acids of the wild-type PSMA protein.

33. The construct of claim 29, wherein the PSMA gene is mutated such that the construct expresses a mutated PSMA protein that has glycine as a second amino acid instead of tryptophan.

10

34. The construct of claim 29, wherein the PSMA gene is CpG-free.

35. The construct of claim 29, wherein the adenoviral reporter gene construct comprising a PSMA gene is CpG-free.

15

36. The construct of claim 29, wherein the adenoviral reporter gene construct comprising a PSMA gene further comprises a neomycin or kanamycin resistant gene.

20

37. A non-viral, episomal reporter gene construct comprising a prostate-specific membrane antigen (PSMA) gene operably linked to a PEG-Promoter.

38. The construct of claim 37, wherein the construct further comprises a Gal4-VP 16 and Upstream Activation Sequence (UAS) transcriptional amplification system.

25

39. The construct of claim 38, wherein the transcriptional amplification system comprises five copies of the UAS.

30

40. The construct of claim 37, wherein the construct further comprises the *EBNA-1* gene.

41. The construct of claim 37, wherein the construct further comprises OriP.
42. The construct of claim 37, wherein the PSMA gene comprises the coding sequence of the full-length PSMA protein.
43. The construct of claim 37, wherein the PSMA gene is truncated at the 5' end of the coding sequence of the PSMA protein.
44. The construct of claim 43, wherein the truncation results in a mutated PSMA protein that is lacking the first 11 amino acids of the wild-type PSMA protein.
45. The construct of claim 37, wherein the PSMA gene is mutated such that the construct expresses a mutated PSMA protein that has glycine as a second amino acid instead of tryptophan.
46. The construct of claim 37, wherein the PSMA gene is CpG-free.
47. The construct of claim 37, wherein the reporter gene construct comprising a PSMA gene is CpG-free.
48. The construct of claim 37, wherein the reporter gene construct comprising a PSMA gene further comprises a neomycin or kanamycin resistant gene.
49. A kit comprising a construct of claim 29 or claim 37.

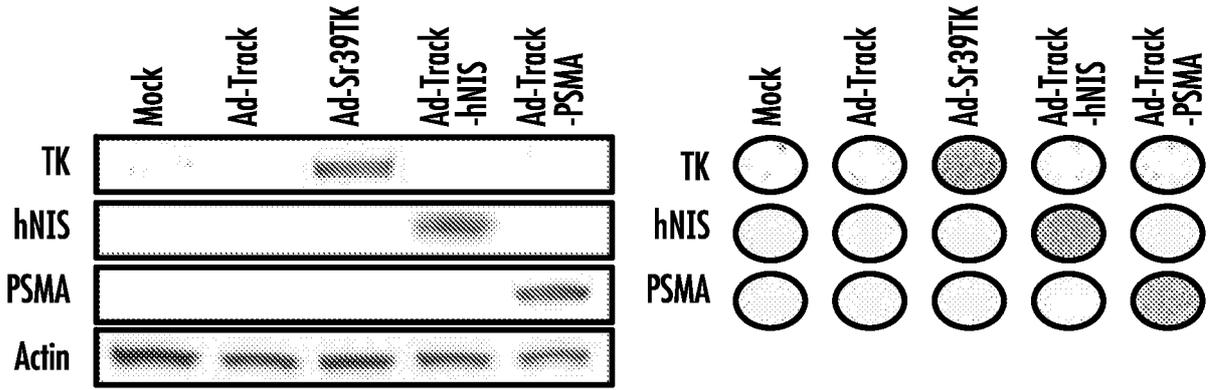


FIG. 1A

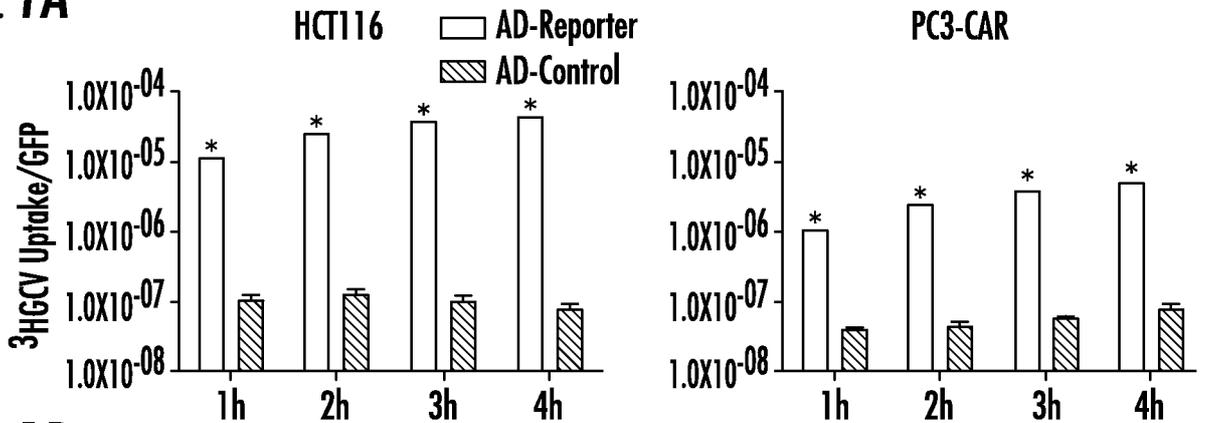


FIG. 1B

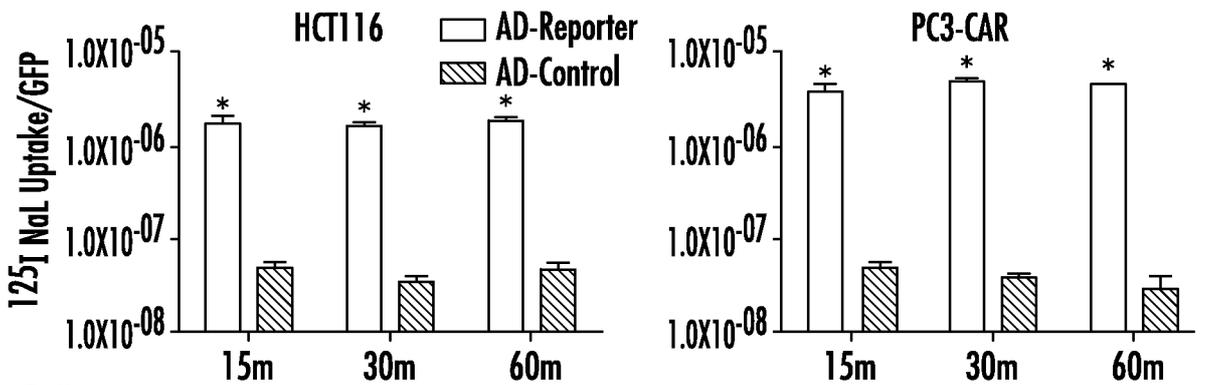


FIG. 1C

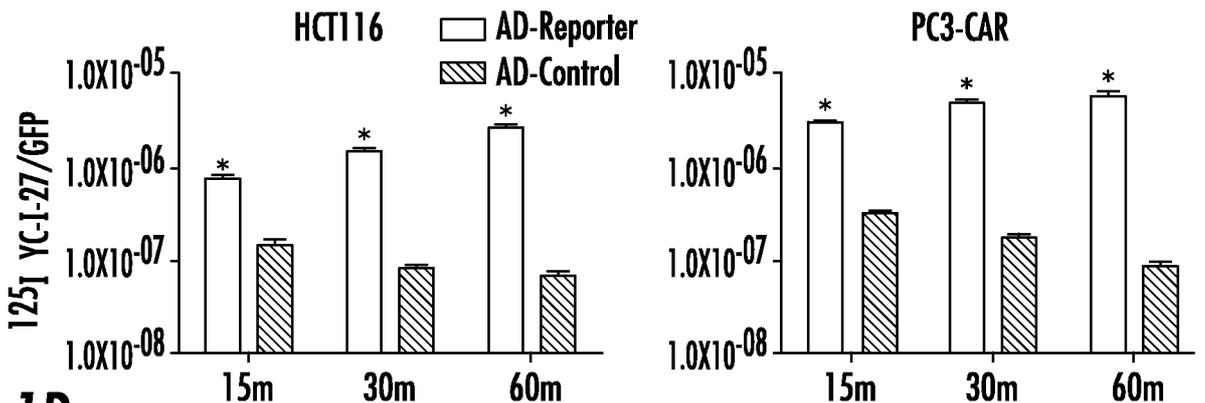


FIG. 1D

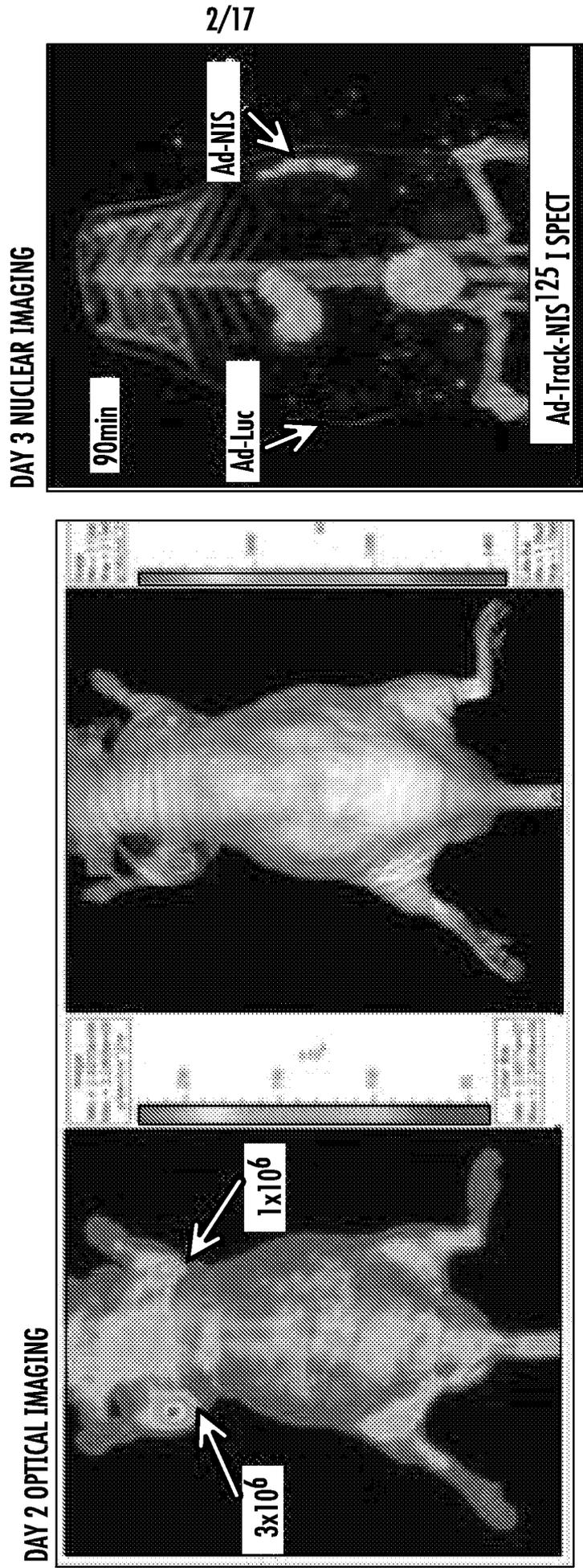
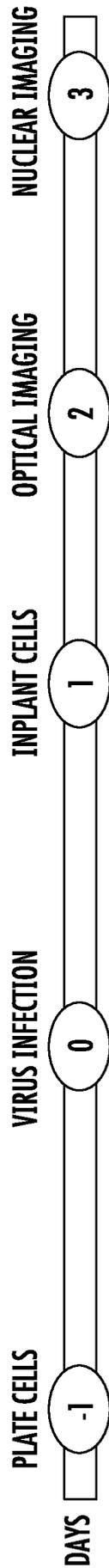


FIG. 2

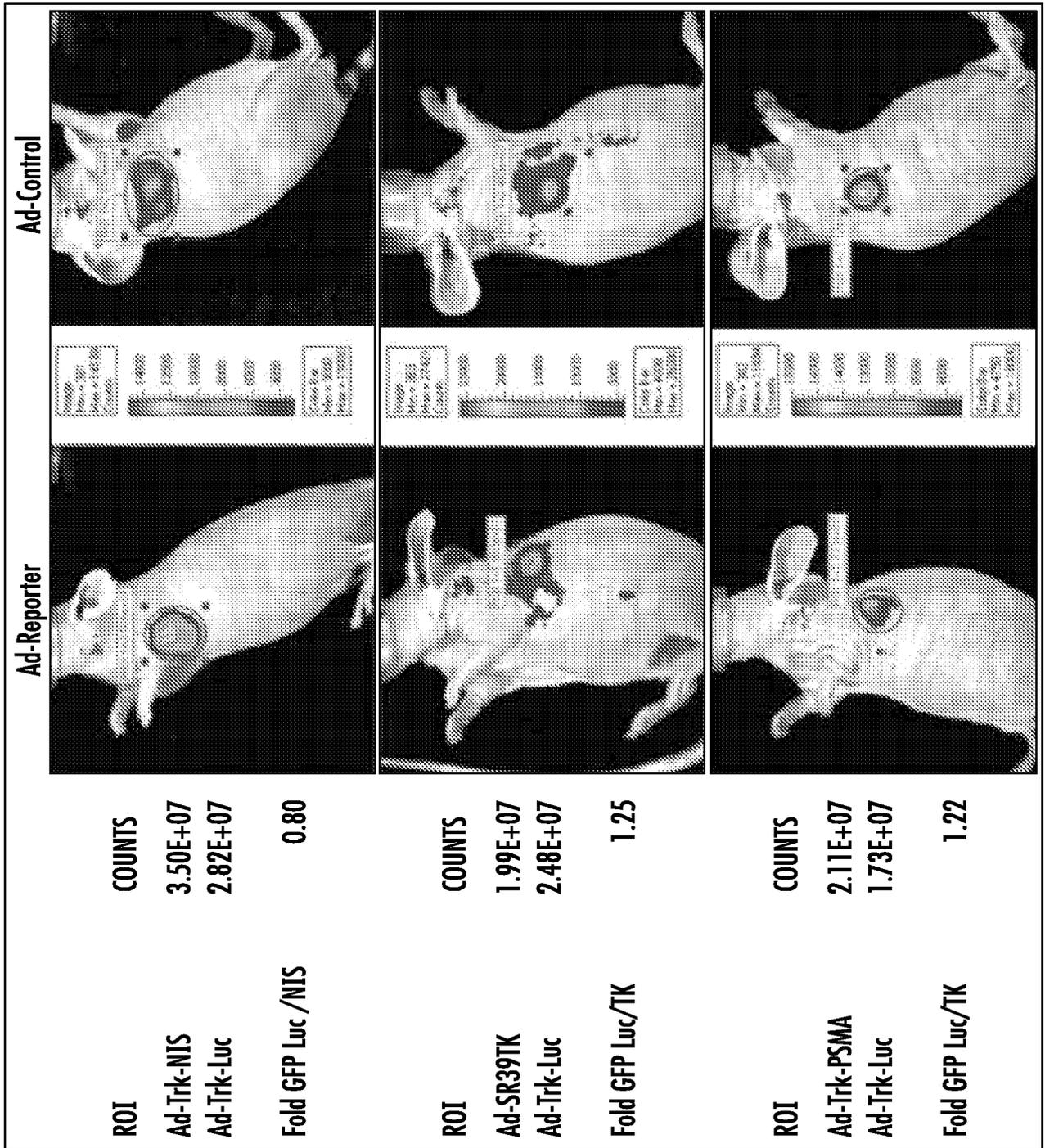


FIG. 3

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FIG. 4A

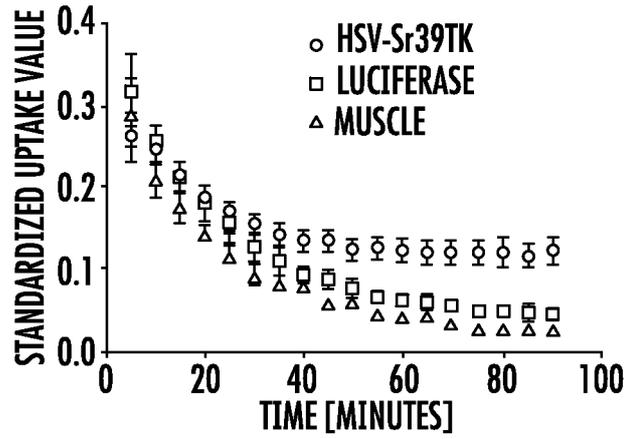


FIG. 4B

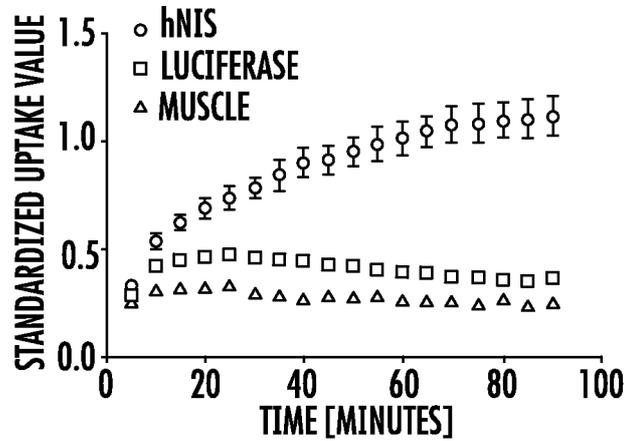


FIG. 4C

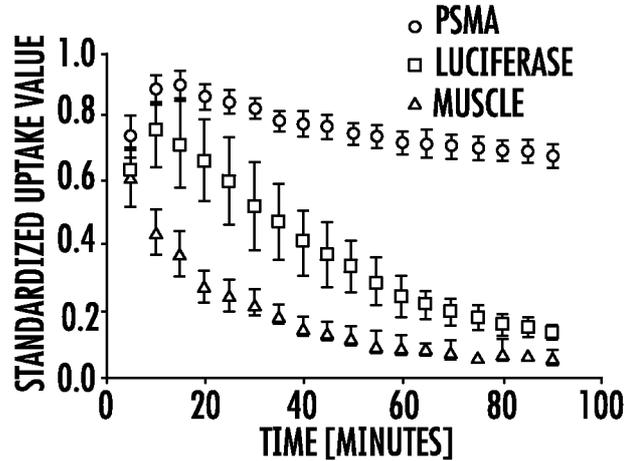
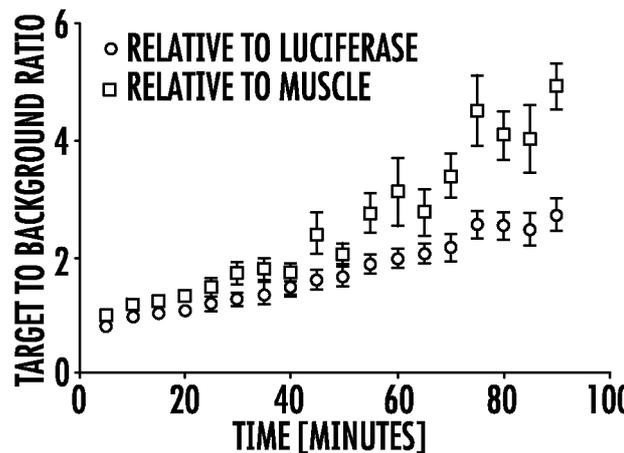


FIG. 4D



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FIG. 4E

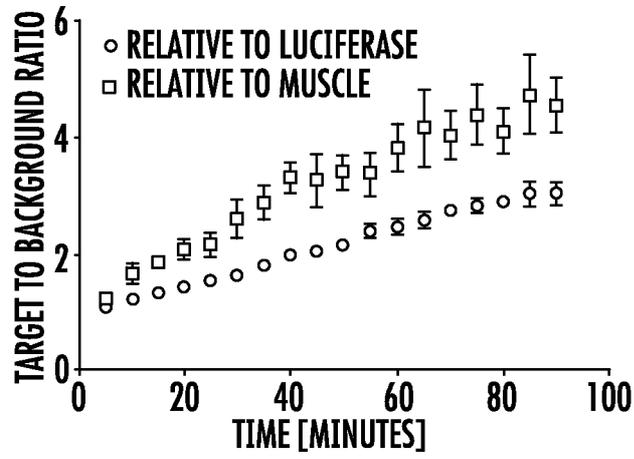


FIG. 4F

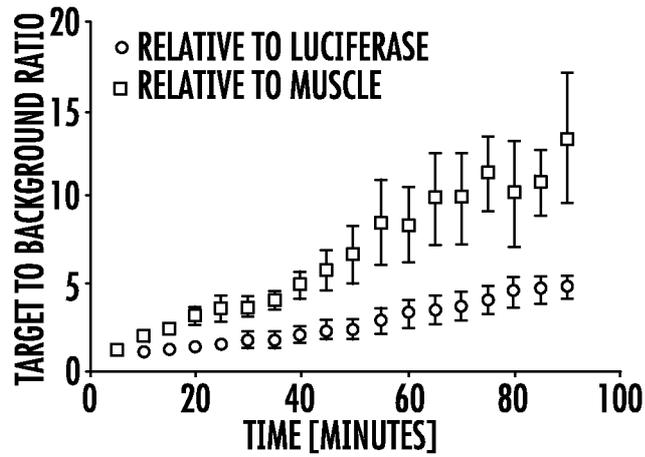
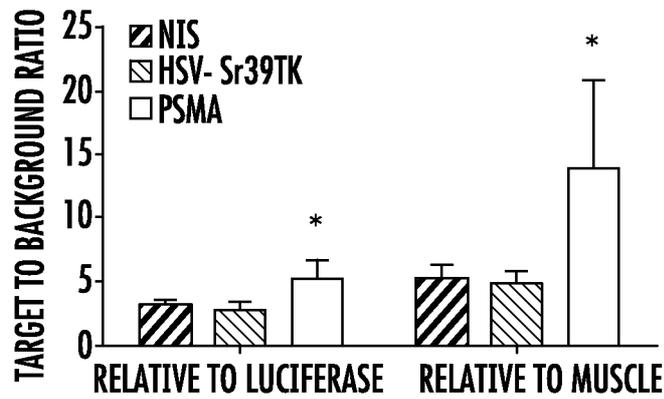


FIG. 4G



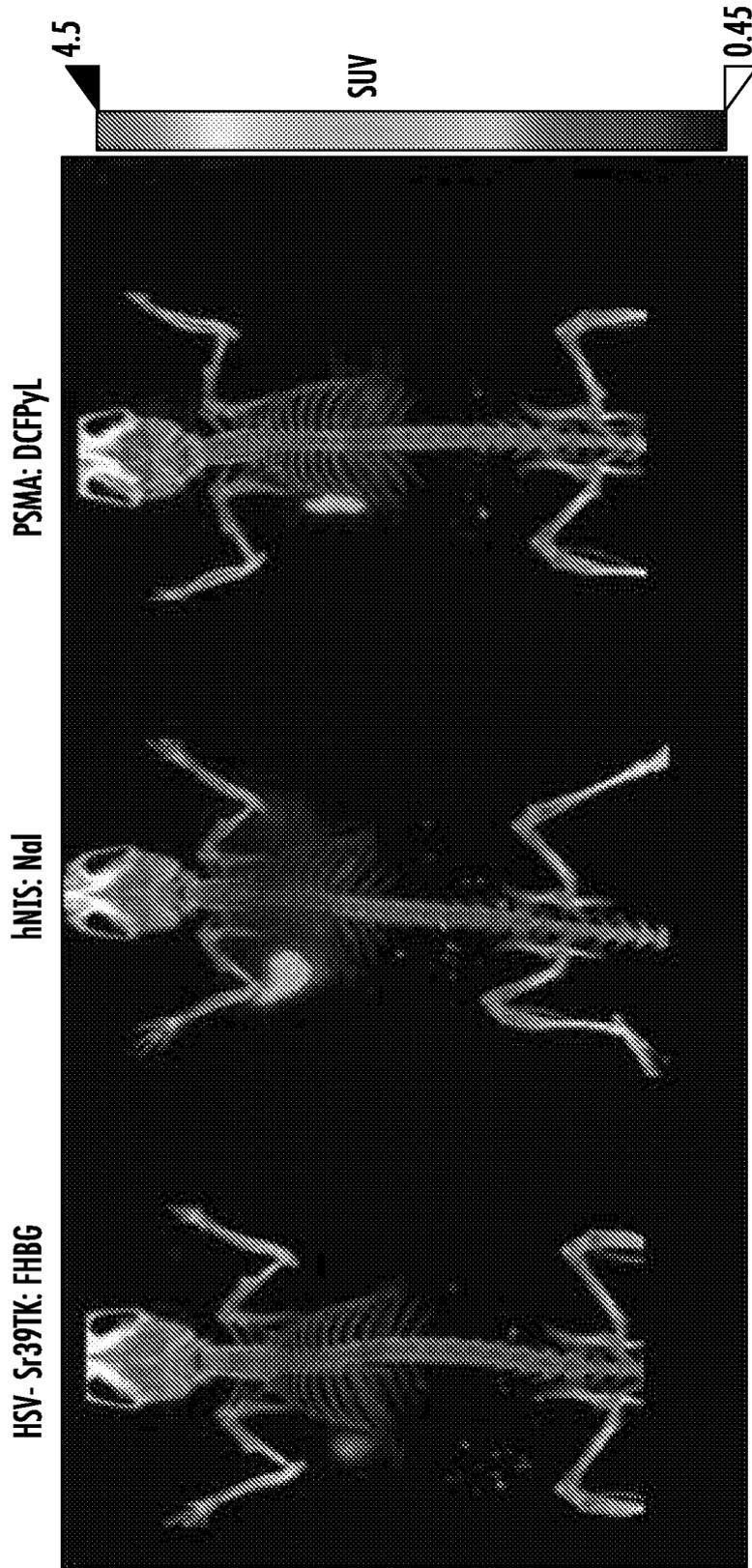


FIG. 5A

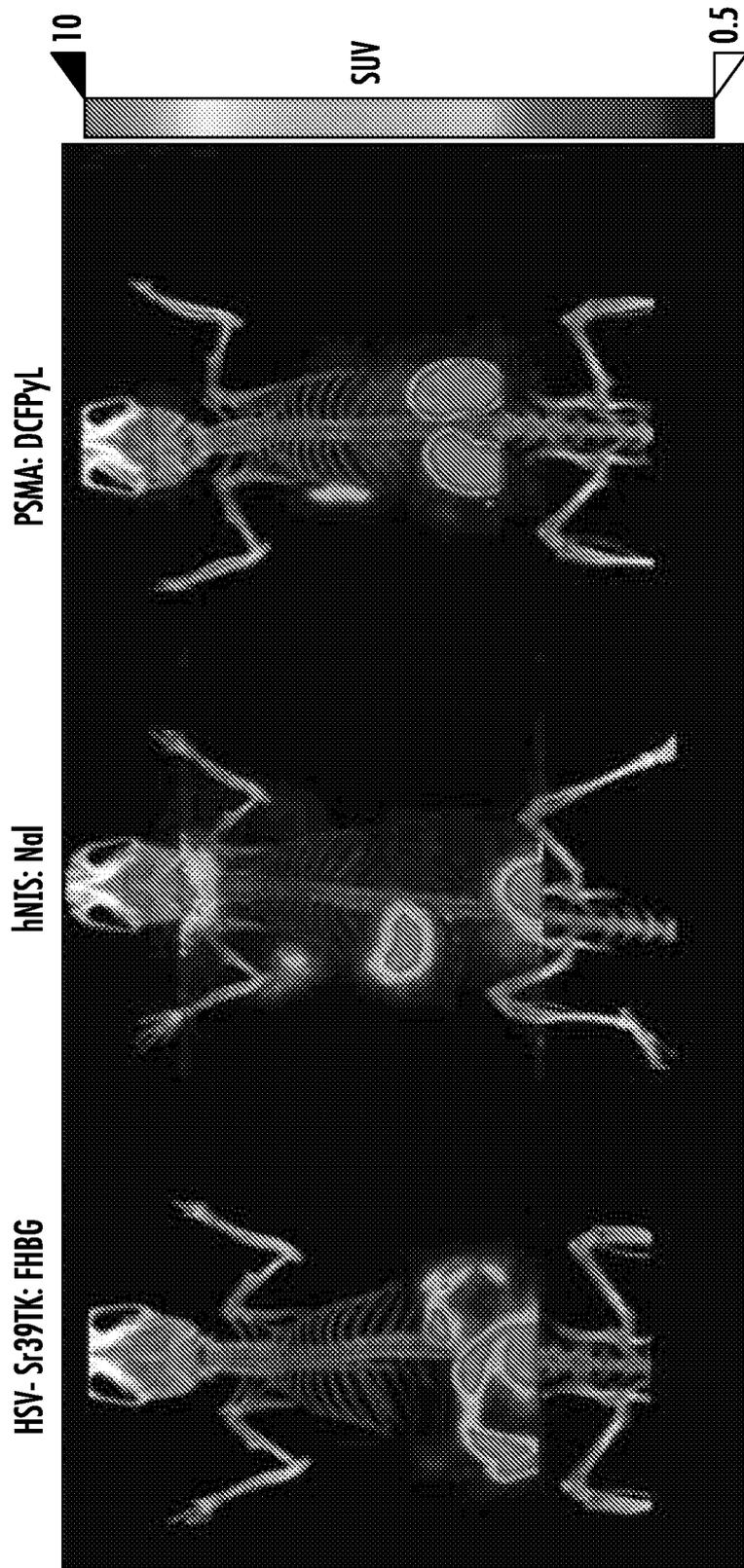


FIG. 5B

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FIG. 6B

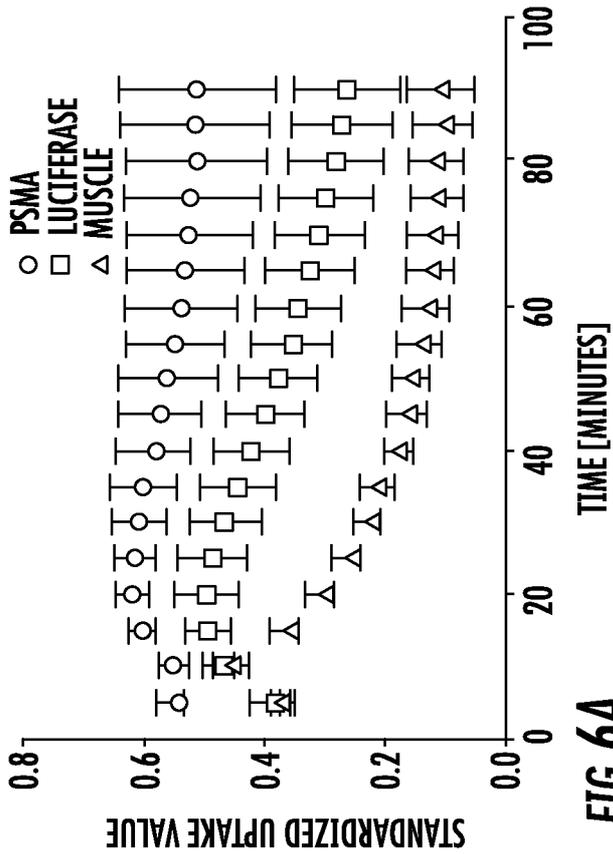


FIG. 6A

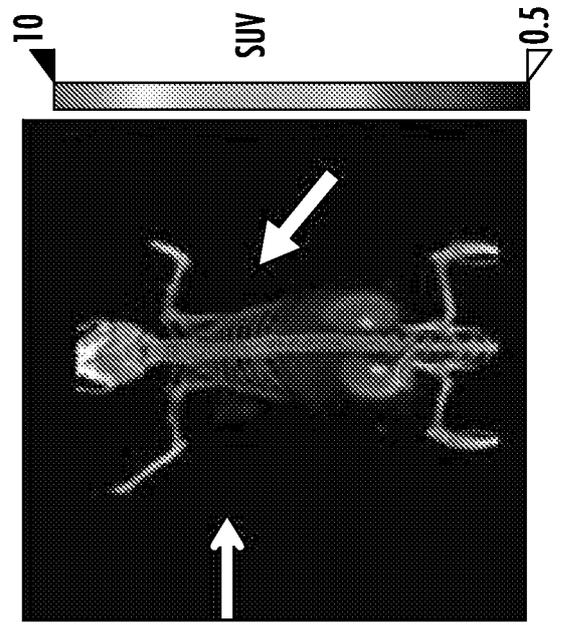


FIG. 6D

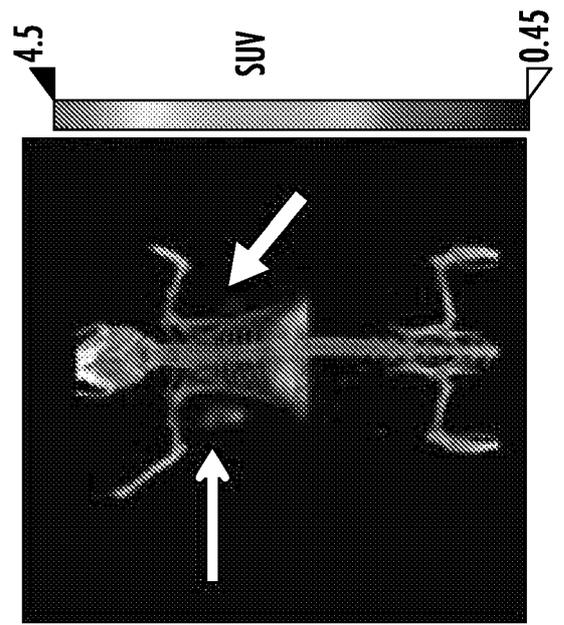


FIG. 6C

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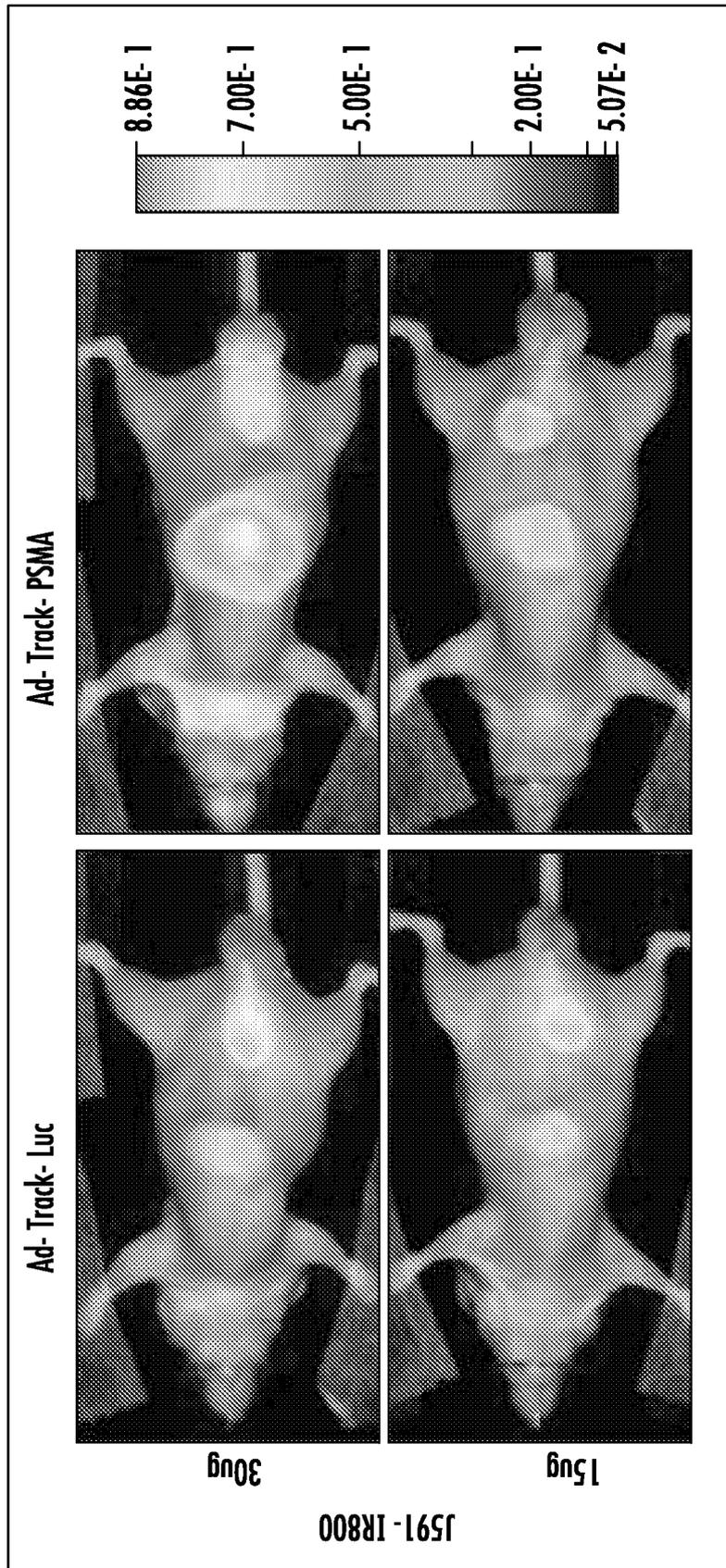


FIG. 7A

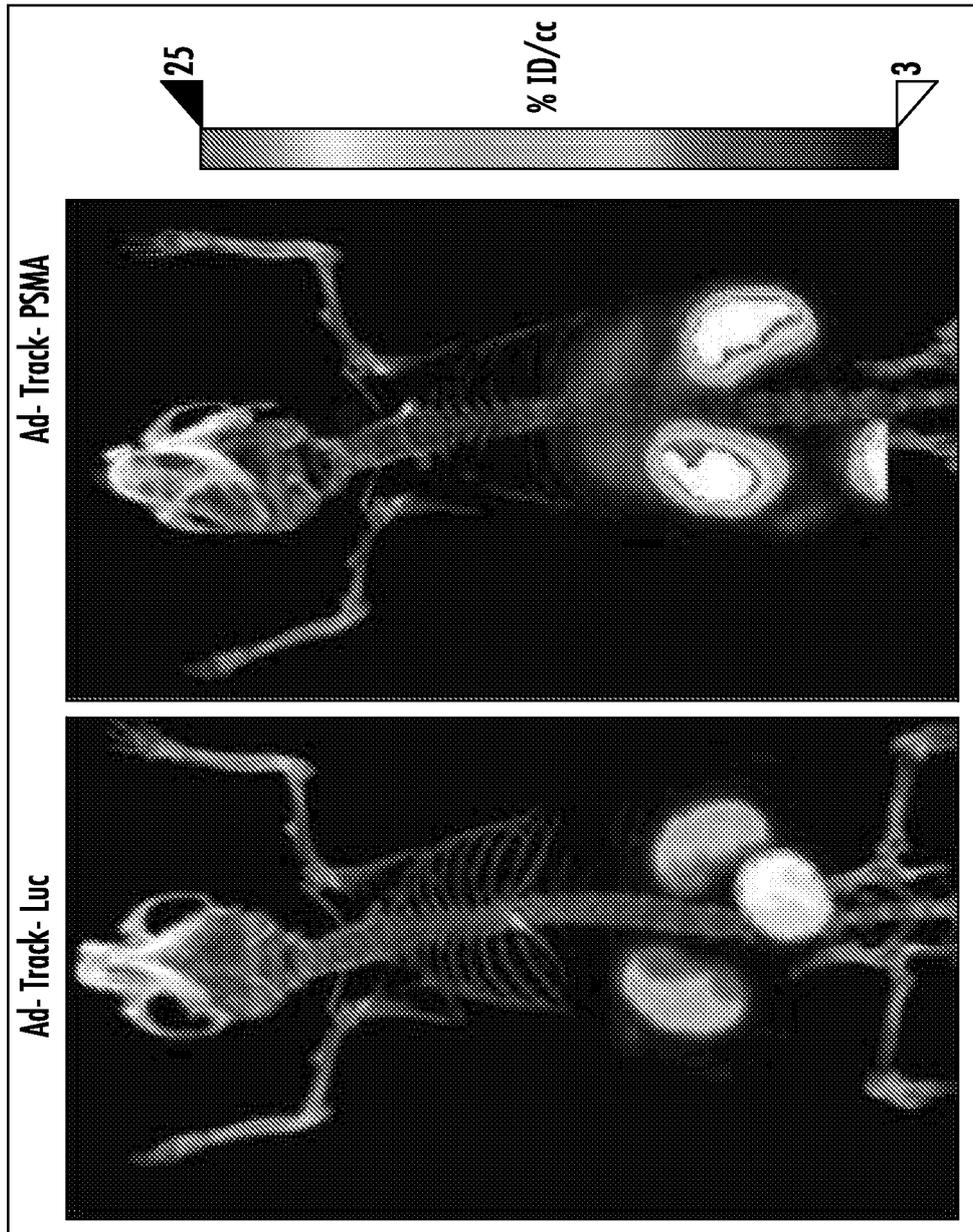


FIG. 7B

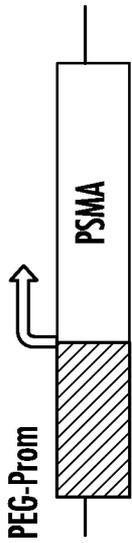


FIG. 8A

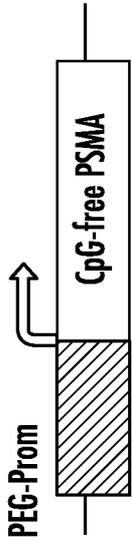


FIG. 8B

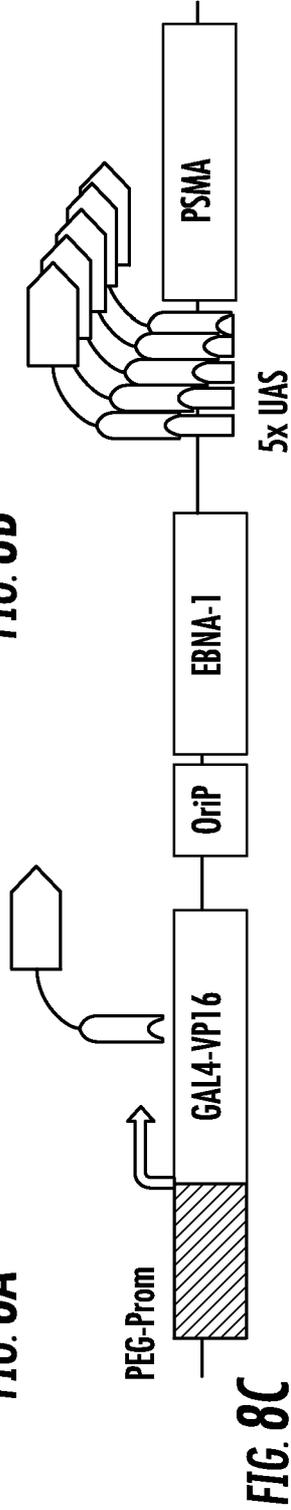


FIG. 8C

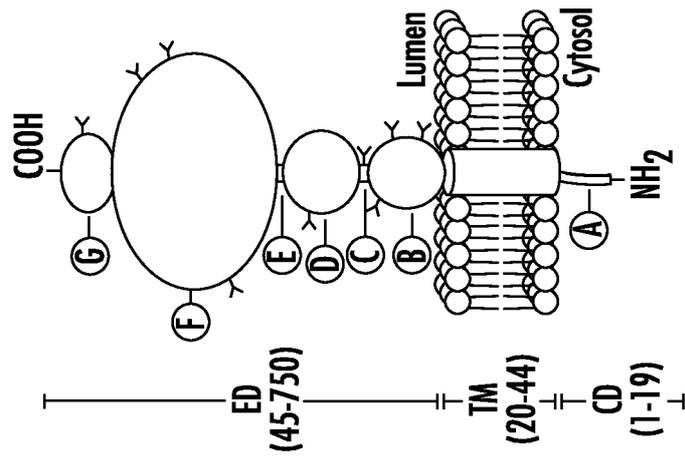


FIG. 8D

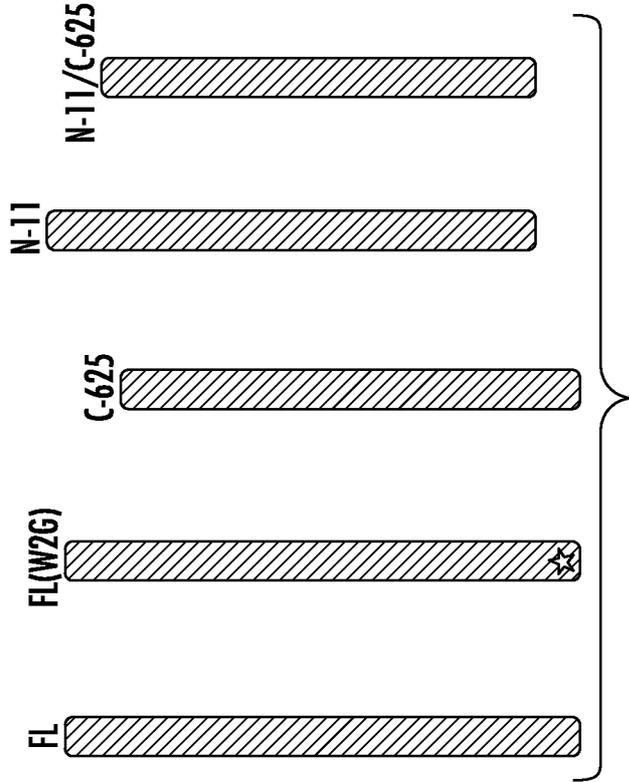


FIG. 8E

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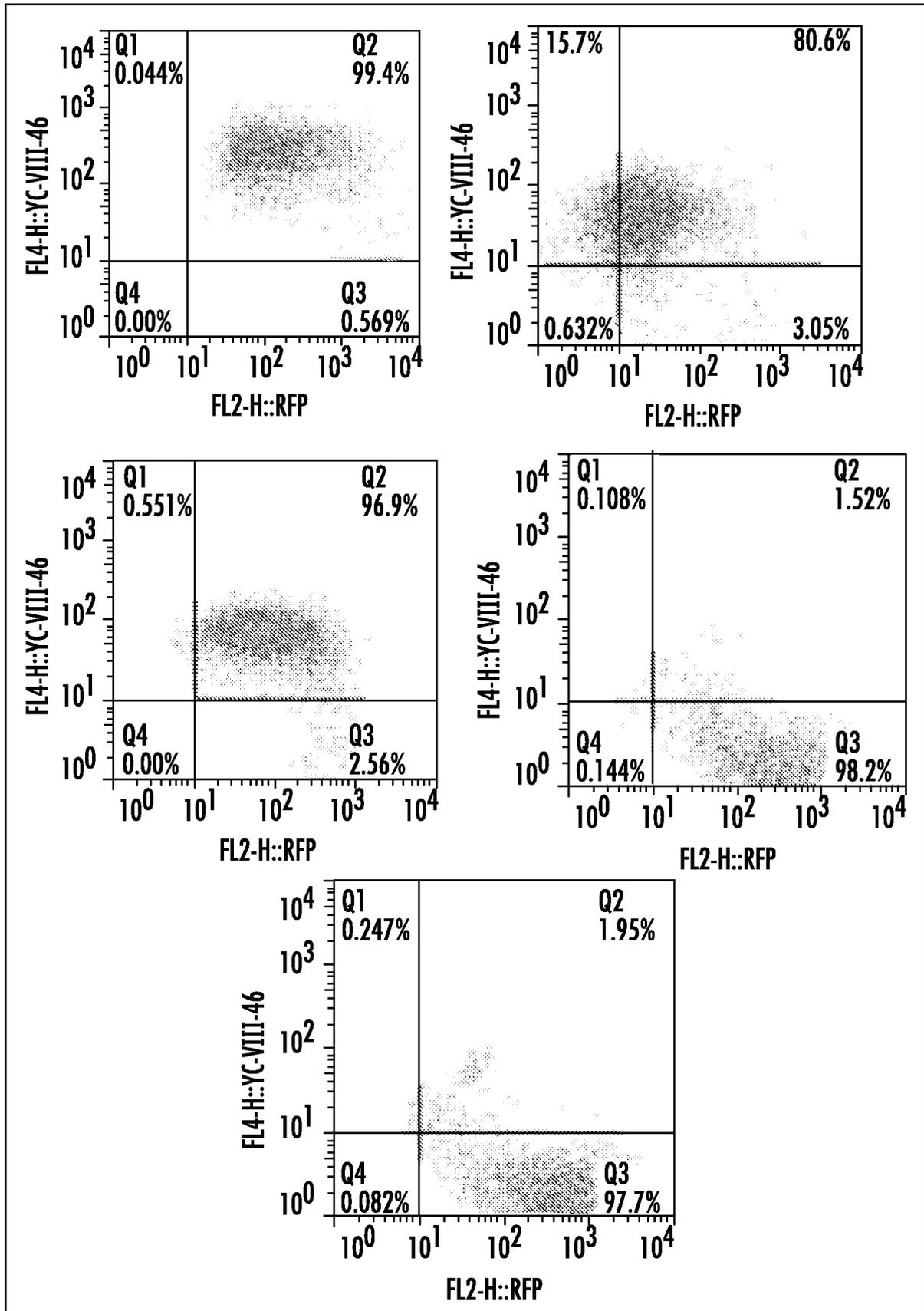


FIG. 9A

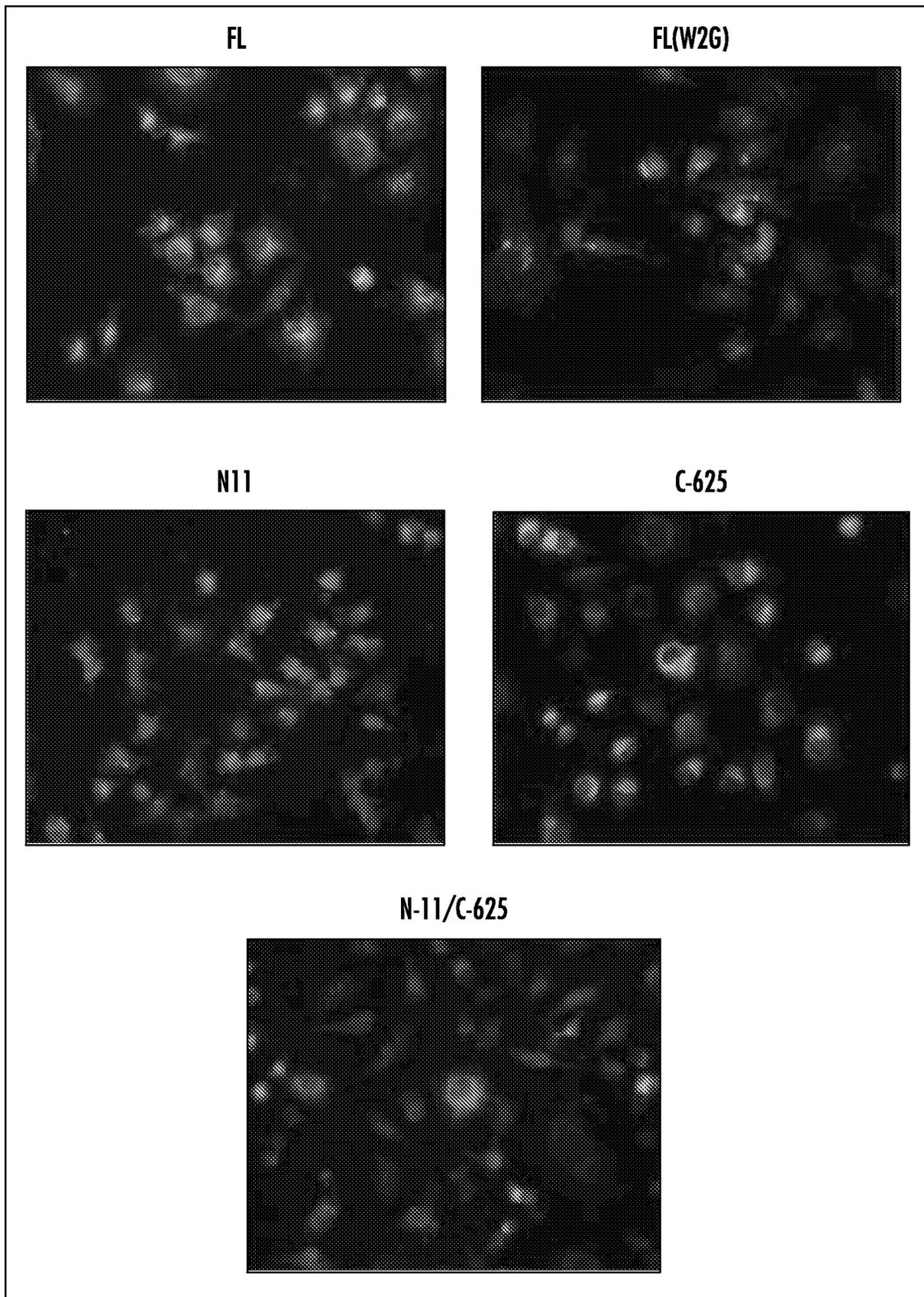


FIG. 9B

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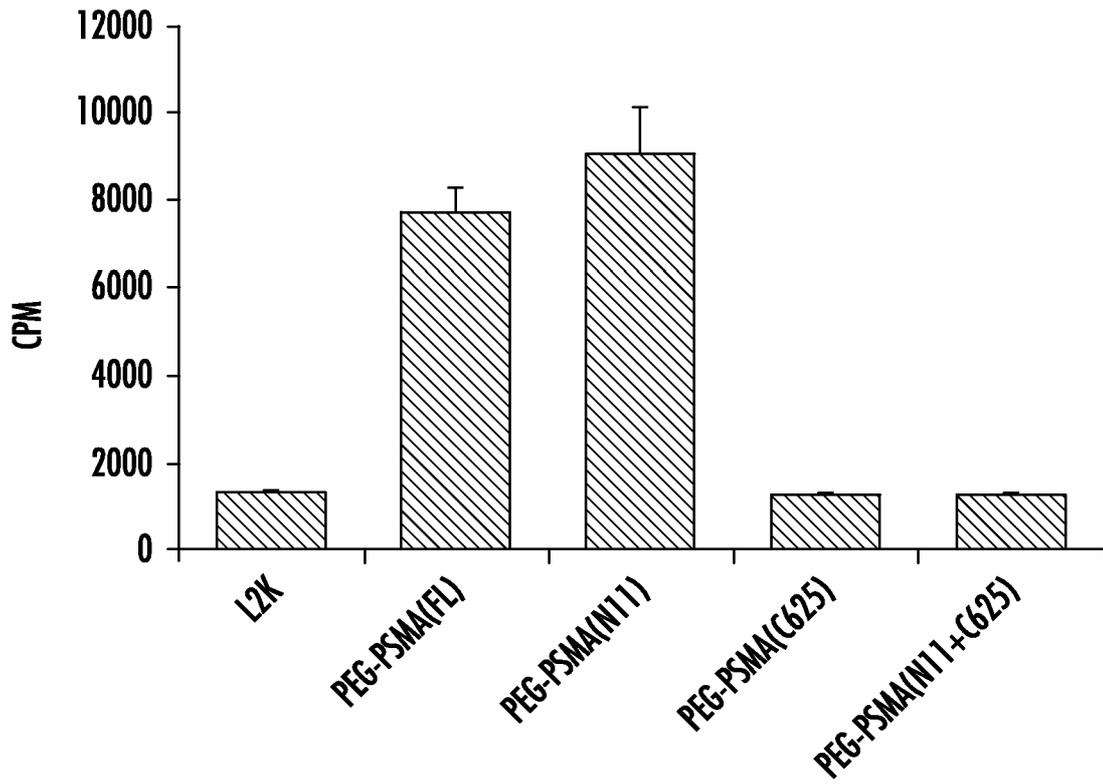


FIG. 10

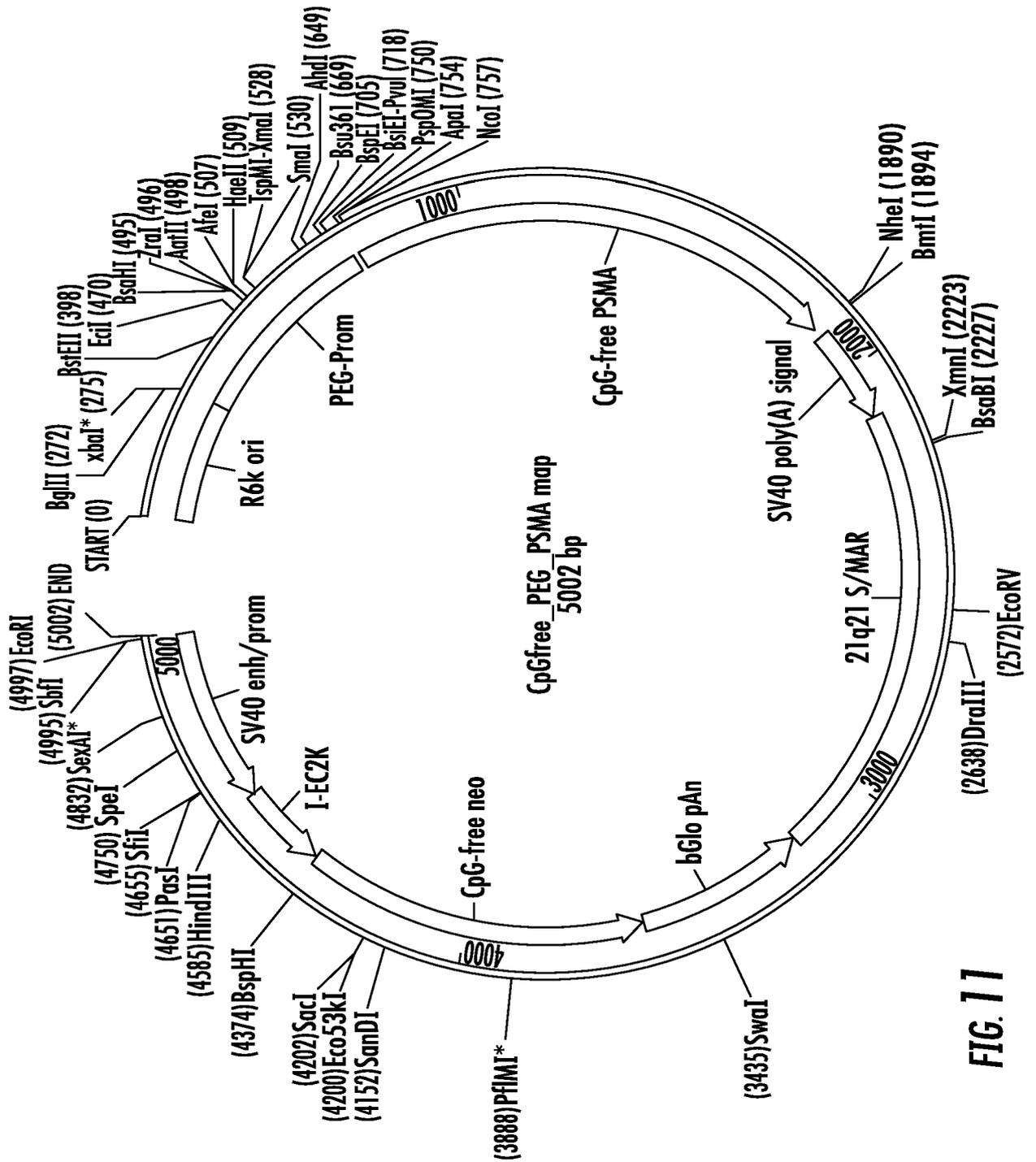


FIG. 11

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ATGTGGAATCTCCTTCATGAAACAGACTCTGCTGTGGCCACAGCCAGAAGACCCAGATGG
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TCCTATCCAGATGGTTGGAATCTTCTGGAGGTGGTGTCCAGAGAGGAAATATCCTAAAT
CTGAATGGTGCAGGAGACCCTCTCACACCAGGTTACCCAGCAAATGAATATGCTTATAGG
AGAGGAATTGCAGAGGCTGTTGGTCTTCCAAGTATTCCTGTTTCAATTC AATGGATACTAT
GATGCACAGAAGCTCCTAGAAAAAATGGGTGGCTCAGCACCACCAGATAGCAGCTGGAGA
GGAAGTCTCAAAGTGCCCTACAATGTTGGACCTGGCTTTACTGGAACTTTTCTACACAA
AAAGTCAAGATGCACATCCACTCTACCAATGAAGTGACAAGAATTTACAATGTGATAGGT
ACTCTCAGAGGAGCAGTGAACCAGACAGATATGTCATTCTGGGAGGTCACAGGGACTCA
TGGGTGTTTGGTGGTATTGACCCTCAGAGTGGAGCAGCTGTTGTTTCAATGAAATTGTGAGG
AGCTTTGGAACACTGAAAAAGGAAGGGTGGAGACCTAGAAGAACAATTTTGTGTTGCAAGC
TGGGATGCAGAAGAATTTGGTCTTCTTGGTCTACTGAGTGGGCAGAGGAGAATTCAAGA
CTCCTTCAAGAGAGGGGAGTGGCTTATATTAATGCTGACTCATCTATAGAAGGAACTAC
ACTCTGAGAGTTGATTGTACACCCTGATGTACAGCTTGGTACACAACCTAACAAAAGAG
CTGAAAAGCCCTGATGAAGGCTTTGAAGGCAAATCTCTTTATGAAAGTTGGACTAAAAAA

FIG. 12

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AGTCCTTCCCAGAGTTCAGTGGCATGCCCAGGATAAGCAAATTGGGATCTGGAAATGAT
TTTGAGGTGTTCTTCCAAAGACTTGAATTGCTTCAGGCAGAGCAAGGTATACTAAAAAT
TGGGAAACAAACAAATTCAGTGGCTATCCACTGTATCACAGTGTCTATGAAACATATGAG
TTGGTGGAAAAGTTTTATGATCCAATGTTTAAATATCACCTCACTGTGGCCCAGGTTAGA
GGAGGGATGGTGTGTTGAGCTAGCCAATTCCATAGTGCTCCCTTTTGATTGTAGAGATTAT
GCTGTAGTTTTAAGAAAGTATGCTGACAAAATCTACAGTATTTCTATGAAACATCCACAG
GAAATGAAGACATACAGTGTATCATTGATTCACTTTTTCTGCAGTAAAGAATTTTACA
GAAATTGCTTCCAAGTTCAGTGAGAGACTCCAGGACTTTGACAAAAGCAACCCAATAGTA
TTAAGAATGATGAATGATCAACTCATGTTTCTGGAAAGAGCATTTATTGATCCATTAGGG
TTACCAGACAGGCCTTTTTATAGGCATGTCATCTATGCTCCAAGCAGCCACAACAAGTAT
GCAGGGGAGTCATTCCCAGGAATTTATGATGCTCTGTTTGATATTGAAAGCAAAGTGGAC
CCTTCCAAGGCCTGGGGAGAAGTGAAGAGACAGATTTATGTTGCAGCCTTCACAGTGCAG
GCAGCTGCAGAGACTTTGAGTGAAGTAGCCTAA

FIG. 12 (cont.)

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item I.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.

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3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/021233**A. CLASSIFICATION OF SUBJECT MATTER****C12N 15/63(2006.01)i, C12N 15/86(2006.01)i, GOIN 21/17(2006.01)i, GOIN 33/68(2006.01)i, A61K 49/00(2006.01)i**

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)

C12N 15/63; C12N 9/02; C12N 15/86; A61K 49/00; C12N 15/85; C12N 5/06; C12N 15/00; G01N 21/17; G01N 33/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords reporter gene, prostate-specific membrane antigen, imaging probe, promoter

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ¹	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CASTANARES, MARK A., Thesis for Ph. D.: 'Development and application of a reporter-probe system based on the prostate specific membrane antigen' The Johns Hopkins University, 2012, pp. 1-154. See abstract; page 1, lines 19-22; page 2, lines 1-4; page 3, paragraph 2; page 12, lines 20-23; figures 3.16-3.17 and VI.5.	29-30, 36, 49
Y		1-13, 17-28, 34-35, 37-42, 46-48
A		14-16, 31-33, 43-45
Y	US 2013-0263296 AI (POMPER, MARTIN GILBERT et al.) 3 October 2013 See abstract; paragraphs [0022], [0031] and [0082]; claims 1 and 16.	1-13, 17-28, 37-42, 46-48
Y	ASAKAWA, KAZUhide et al., 'Targeted gene expression by the Gal4-UAS system in zebrafish', Development, Growth & Differentiation, August 2008, Vol. 50, No. 6, pp. 391-399. See abstract; page 392, left column, paragraph 2; figure 2.	9-10, 38-39
Y	US 2003-0059942 AI (CHO, MYUNG-SAM et al.) 27 March 2003 See abstract; paragraph [0050]; claims 1-3; figures 1-1 and 1-2.	11-12, 40-41
Y	US 8435777 B1 (REYNES, JEAN PAUL et al.) 7 May 2013 See abstract; column 5, lines 34-41; claims 1 and 13.	17-18, 34-35, 46-47

 Further documents are listed in the continuation of Box C. See patent family annex.

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Date of the actual completion of the international search

01 June 2015 (01.06.2015)

Date of mailing of the international search report

03 June 2015 (03.06.2015)

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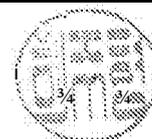


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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/021233

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category ⁴	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OSBORNE, JOSEPH R. et al., 'Prostate-specific membrane antigen-based imaging' , Urologic Oncology, February 2013, Vol. 31, No. 2 , pp. 144-154. See the whole document.	1-49
PX	CASTANARES, MARK A. et al., 'Evaluation of prostate-specific membrane antigen as an imaging reporter' , Journal of Nuclear Medicine, Epub. 3 April 2014, Vol. 55, No. 5 , pp. 805-811. See abstract; pages 806-809; figures 1-2.	1-8,13,19-30,36-37 ,42,48-49

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2015/021233

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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us 8435777 B1	07/05/2013	EP 2617813 AI EP 2617813 B1	24/07/2013 14/01/2015