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(54) Title: CRISPR-BASED METHODS FOR DETECTING SCARCE NUCLEIC ACIDS

(57) Abstract: Aspects of the present invention relate to methods for detecting a target nucleic acid sequence in a sample from a subject using a CRISPR-associated (Cas) protein, a CRISPR guide RNA (crRNA), and a signal template that is configured for detecting the target nucleic acid sequence via a detectable signal (e.g., a fluorescent signal). In some aspects, such methods are useful for diagnosing and treating a subject such as a subject having a cancer.

CRISPR-BASED METHODS FOR DETECTING SCARCE NUCLEIC ACIDS**CLAIM OF PRIORITY**

This application claims the benefit of U.S. Provisional Patent Application Nos.
5 63/518,602, filed on August 10, 2023 and 63/578,535 filed August 24, 2023, which are
incorporated by reference herein in their entireties.

SEQUENCE LISTING

This application contains a Sequence Listing that has been submitted electronically as
10 an XML file named "29539-0780WO1_SL_ST26.XML." The XML file, created on August
8, 2024, is 80,635 bytes in size. The material in the XML file is hereby incorporated by
reference in its entirety.

BACKGROUND

15 Detection of nucleic acids can be challenging due to their low cellular concentrations.
It is therefore of interest to develop new assays for detecting scarce nucleic acids that ensure
high analytical sensitivity while maintaining sequence specificity.

SUMMARY

20 Provided herein are methods for detecting nucleic acids that leverage CRISPR-
mediated recognition of a target nucleic acid to trigger target replication and signal
amplification. Such methods can achieve a sub-attomolar detection limit while maintaining
single-nucleotide resolution.

Accordingly, aspects of the present disclosure provide a method of detecting a target
25 nucleic acid sequence, the method comprising providing a sample; contacting the sample
with a RNA-guided Cas protein; a CRISPR guide RNA (crRNA), wherein the crRNA
comprises a nucleic acid sequence that is sufficiently complementary to a target nucleic acid
sequence; a polymerase; and a signal template, wherein the signal template comprises a
polymerase promoter sequence, a RNA sequence comprising a Cas protein cleavable
30 sequence, and a DNA sequence that is sufficiently complementary to the target nucleic acid
sequence, wherein the polymerase promoter sequence comprises an acceptor moiety and the
RNA sequence comprises a donor moiety, or vice versa; incubating the sample under an
isothermal amplification condition and for a time sufficient for nucleic acid amplification;
and detecting a signal from the donor moiety.

In some embodiments, the signal from the donor moiety is indicative of a level of the target nucleic acid sequence in the sample.

In some embodiments, the target nucleic acid sequence comprises a mutation. In some embodiments, the mutation is an insertion, a deletion, a substitution, or a fusion. In some
5 embodiments, the mutation is a single nucleotide polymorphism.

In some embodiments, the target nucleic acid sequence comprises messenger RNA (mRNA). In some embodiments, the target nucleic acid sequence comprises a nucleic acid sequence from a gene selected from *GAPDH*, *CD63*, *KRAS*, *IDH*, *EGFR*, and *BRAF*. In some
10 embodiments, the target nucleic acid sequence comprises a nucleic acid sequence selected from SEQ ID NOs:2-16.

In some embodiments, the Cas protein comprises Cas13a or Cas13b. In some embodiments, the Cas protein comprises an amino acid sequence having at least 80% identity to SEQ ID NO:1. In some embodiments, the Cas protein comprises SEQ ID NO:1.

In some embodiments, the crRNA comprises a nucleic acid sequence selected from
15 SEQ ID NOs:17-31.

In some embodiments, the polymerase comprises T7 RNA polymerase or SP6 RNA polymerase.

In some embodiments, the signal template comprises a nucleic acid sequence selected from SEQ ID NOs:32-46.

In some embodiments, the donor moiety and the acceptor moiety comprise a
20 fluorescence resonance energy transfer (FRET) pair or a chemiluminescence resonance energy transfer (CRET) pair. In some embodiments, the donor moiety and the acceptor moiety are positioned to undergo FRET or CRET prior to cleavage of the Cas protein cleavable sequence. In some embodiments, the donor moiety is a fluorophore donor. In some
25 embodiments, the fluorophore donor is selected from the group consisting of Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 647, Cy2, Cy3, BODIPY, GFP, fluorescein, IEDANS, EDANS, and a lanthanide metal. In some embodiments, the acceptor moiety is a fluorophore acceptor or a fluorescence quencher. In some embodiments, the fluorescence quencher is selected from the group consisting of black hole quencher (BHQ),
30 deep dark quencher (DDQ), Eclipse quencher, Dabcyl, QSY quencher, Iowa Black FQ, Iowa Black RQ, ZEN Quencher, and TAMRA.

In some embodiments, the isothermal amplification condition comprises a temperature of about 35 to 45 °C.

In some embodiments, the sample is a biological sample obtained from a subject. In some embodiments, the subject has a cancer. In some embodiments, the sample comprises blood, plasma, serum, urine, nasal secretions, or a combination thereof. In some embodiments, the sample comprises extracellular vesicles (EVs).

5 In some embodiments, methods further comprise, prior to the providing, isolating EVs and extracting RNA.

In some embodiments, the sample further comprises 3'-amino-2',3'-dideoxyribonucleotide 5'-triphosphates (nNTPs), a divalent ion, a buffer, a salt, or a combination of any of these.

10 In some embodiments, binding of the Cas protein and the crRNA forms a crRNA/Cas complex, and wherein binding of the crRNA/Cas complex to the target nucleic acid sequence activates a trans-cleavage activity of the Cas protein in the crRNA/Cas complex.

Aspects of the present disclosure provide a method for treating a subject having a cancer, the method comprising detecting a level of the target nucleic acid sequence in the
15 sample according to any one of the methods described herein; comparing the level of the target nucleic acid sequence in the sample to a reference level; and administering a treatment if the level of the target nucleic acid sequence in the sample is equal to or higher than the reference level.

In some embodiments, the cancer comprises a solid tumor, renal cell carcinoma, anal
20 cancer, bladder cancer, brain cancer, breast cancer, cervical cancer, colorectal cancer, endometrial cancer, esophageal cancer, gastric cancer, glioblastoma, glioma, kidney cancer, liver cancer, lung cancer, lymphoma, melanoma, myeloma, neuroblastoma, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma, skin cancer, thyroid cancer, urothelial cancer, uterine cancer, or a combination thereof.

25 In some embodiments, the treatment comprises a chemotherapy, an immune checkpoint inhibitor, surgery, radiation, or a combination thereof.

Aspects of the present disclosure provide a kit comprising a CRISPR guide RNA (crRNA), wherein the crRNA comprises a nucleic acid sequence that is sufficiently complementary to a target nucleic acid sequence; a signal template, wherein the signal
30 template comprises a polymerase promoter sequence, a RNA sequence comprising a Cas protein cleavable sequence, and a DNA sequence that is sufficiently complementary to the target nucleic acid sequence, wherein the polymerase promoter sequence comprises an acceptor moiety and the RNA sequence comprises a donor moiety, or vice versa; and instructions for performing any one of the methods described herein.

In some embodiments, the kit further comprises a RNA-guided Cas protein and a polymerase. In some embodiments, the crRNA comprises a nucleic acid sequence selected from SEQ ID NOs:17-31. In some embodiments, the signal template comprises a nucleic acid sequence selected from SEQ ID NOs:32-46.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGs. 1A-1B. Self-amplified and CRISPR-aided operation (SCOPE) to profile extracellular vesicles (EVs). **FIG. 1A:** SCOPE mechanism and assay flow. The assay couples Cas13a/crRNA and polymerase reactions through a signal template. The signal template contains an RNA segment with a quenched fluorescent dye and a DNA template for T7 polymerase. The fluorescent signal is initially quenched (F, fluorescent dye; Q, quencher). **Target recognition:** Cas13a/crRNA first recognizes the EV RNA target, which activates its ribonuclease function. **Trans-cleavage:** The activated enzyme cuts the RNA segment in the signal template, generating a fluorescent signal and exposing the DNA template. **Transcription:** T7 RNA polymerase binds to the promoter region of the DNA template and synthesizes target RNA replicas. **Target replicas:** The amplified target replicas bind to free Cas13a/crRNA, enforcing the overall assay process. Note that the initial CRISPR-mediated target recognition is necessary to unlock RNA replication and signal generation. **FIG. 1B:** SCOPE workflow for onsite diagnostics. EVs are isolated from clinical samples and lysed. EV lysates are then placed in surface-treated tubes for RNA extraction (10 min). Subsequently, the SCOPE reaction is performed in a compact, portable device (30 min). The assay produces molecular information within our hour, enabling same-day clinical decisions.

FIGs. 2A-2E. SCOPE device engineering. **FIG. 2A:** Single-tube system for SCOPE assay. PCR tubes coated with poly[(2-dimethylamino)ethyl acrylate] (pDMAEA) polymer were used. With EV or cell lysates added, the dissolved polymer was positively charged initially, forming polyplexes with negatively charged nucleic acids. Following brief centrifugation to collect polyplexes, then SCOPE reagents were added. The polymer underwent charge-shifting hydrolysis to release nucleic acids, and the SCOPE reaction continued in the same tube. **FIG. 2B:** Comparison of RNA extraction yield between pDMAEA tubes and standard silica-based columns. The pDMAEA method demonstrated similar extraction yields as the column devices, with an average extraction yield of 96% across different input RNA amounts (3, 30, 60, and 100 ng). Extracting nucleic acids with

pDMAEA tubes was fast (10 min) and compatible with SCOPE reactions. Data are shown as mean $\pm$ s.d. from technical triplicates. **FIG. 2C**: Integrated device for onsite SCOPE tests. This compact device accommodated 16 samples in a tray-like sample holder. The tray was wrapped with a flexible heater and docked to the mainframe that regulated the heater.

5 Fluorescent signals were detected by an optical module (top right). The system was connected to a tablet via wireless communication. A user-friendly graphical interface (lower right) in a tablet provided intuitive windows for system operation and data analysis. **FIG. 2D**: Upon completion of the reaction, the optical module quantified fluorescent signals emitted from the samples. A single module linearly scanned the sample tray, simplifying the system design. **FIG. 2E**: The SCOPE device was used to process samples containing different
10 amounts of synthetic CD63 RNA. The SCOPE results were highly consistent for a given RNA concentration, regardless of the sample location in the heating block. The coefficient of variation was $<1.5\%$. The bar represents a mean fluorescent signal from four different sites in the heating block. Color codes matched the sample location with RNA concentration. a.u.,
15 arbitrary unit.

FIGs. 3A-3C. SCOPE assay kinetics. **FIG. 3A**: SCOPE integrates two enzymatic reactions via the signal template. First, Cas13a/crRNA generates fluorescence by degrading the RNA segment in the signal template, and then T7 polymerase replicates RNA targets. SCOPE reactions commence upon initial recognition of mRNA targets by Cas13a/crRNA.

20 **FIG. 3B**: When performed independently, Cas13a/crRNA and T7 reactions exhibited a linear increase in end product over time (left panel and middle panel). Coupling these two reactions enabled SCOPE to emulate first-order rate kinetics. The analytical signals amplified exponentially, reaching a steady state within 30 min (right panel). Data are shown as mean $\pm$ s.d. from technical triplicates. **FIG. 3C**: Assay validation. Signal intensity was maximized
25 when all SCOPE assay components were present. Removing T7 polymerase diminished the signal due to the absence of target RNA amplification. Assay conditions that hindered the initial mRNA recognition produced negligible signals. The initial target RNA (*KRAS*^{G12D}) concentration was fixed at 1 nM. Data are shown as mean $\pm$ s.d. from technical triplicates. a.u., arbitrary unit.

30 **FIGs. 4A-4D**. SCOPE assay characterization. **FIG. 4A**: SCOPE (30 min) and conventional RT-PCR (80 min) were employed to analyze samples containing varying concentrations of synthetic *KRAS*^{G12D} RNA. SCOPE exhibited a detection limit (LOD) of 8.5 copies/ μ L (14.2 zM), outperforming RT-PCR (LOD of 1.1×10^{10} copies/ μ L, 18.6 pM). Data are displayed as mean $\pm$ s.d. from technical triplicates. Some error bars are too small to be

visible. The net intensity (Δ Intensity) was obtained by removing background fluorescent signals (no-target controls). a.u., arbitrary unit. **FIG. 4B**: EV samples were prepared from KP1.9 cell culture and analyzed for *KRAS*^{G12D} mRNA. SCOPE's LOD was approximately 10⁵ EVs/mL, whereas RT-PCR's LOD was about 10⁸ EVs/mL. In addition, SCOPE displayed a broader dynamic range than RT-PCR. Data are shown as mean $\pm$ s.d. from technical triplicates. **FIG. 4C**: The specificity of SCOPE probes targeting *KRAS* wild type (WT) and its mutated subtypes (G12C, G12D, G12S, G12V) was evaluated. SCOPE displayed high signal contrast between on-target and off-target samples. At a target concentration of 1 nM, the contrast ratio exceeded 30. The heatmap shows mean values from technical triplicate measurements. **FIG. 4D**: A mixture of *KRAS*^{WT} and *KRAS*^{G12D} synthetic RNA samples was prepared, varying the variant allele fractions (VAFs). SCOPE detected *KRAS*^{G12D} down to 0.01% VAF, with a signal significantly different from the background ($p = 0.011$; unpaired, two-sided t-test). The bars represent mean values from technical triplicate measurements.

FIGs. 5A-5E. Early cancer detection with SCOPE. **FIG. 5A**: Experimental design. A genetically engineered mouse model was used to mimic the development of non-small cell lung cancer. The animal had a Cre-activatable *Kras*^{LSL-G12D/+}; *Trp53*^{flax/flax} genetic background (KP model). Tumor growth was initiated through intranasal administration of adenoviruses expressing Cre recombinase (Ad-Cre). Serial blood samples were collected from the animals before tumor induction and at weekly intervals after that. **FIG. 5B**: *In vitro* validation of SCOPE for *KRAS* genotyping. Cell lines with distinct *KRAS* genetic profiles were used: H2228 (WT/WT), KP1.9 (G12D/WT), and A549 (G12S/G12S). EVs were isolated from cell culture media. SCOPE *KRAS* assays on these EV samples yielded results consistent with the cellular *KRAS* status. Data are presented as mean $\pm$ s.d. from biological triplicates. a.u., arbitrary unit. **FIG. 5C**: Tumor development in the Cre-treated mice was corroborated via immunohistochemistry on lung tissue specimens. Lesions positive for *Kras*^{G12D} protein were detectable one week after tumor induction, and the lesions enlarged in subsequent weeks. **FIG. 5D**: SCOPE analyses on EV *Kras*^{G12D} mRNA during tumor development. Serial blood samples were collected over eight weeks from the same individual mice. In the non-cancer cohort ($n = 3$), the SCOPE signal remained at background values throughout the duration. In the tumor-bearing cohort ($n = 10$), the SCOPE signal increased from background values one week after tumor induction and reached saturation approximately three weeks after the onset of tumor growth. The heatmap shows mean values from technical triplicate measurements. **FIG. 5E**: Estimation of *Kras*^{G12D} mRNA copy numbers. SCOPE results in FIG. 5D were converted based on the titration curve (FIG. 4A). In the tumor-bearing cohort, the copy

number was below the baseline (< 1) initially and then significantly increased ($p = 0.042$, paired two-sided t-test) one week after tumor induction. Each data point is a mean value from technical triplicate measurements.

FIGs. 6A-6F. Monitoring colorectal cancer patients using SCOPE. **FIG. 6A:** Study design. Plasma samples were collected from colorectal cancer (CRC) patients before and after curative surgery, as well as during standard care. Tissue samples were obtained during surgery and analyzed for the *KRAS* gene. **FIG. 6B:** Plasma EVs were analyzed via SCOPE for a panel of *KRAS* mRNA mutations. Samples were from non-CRC controls ($n = 15$) and pre-surgery CRC patients ($n = 107$). SCOPE results reflected *KRAS* mutation status from tumor tissue analysis, identifying CRC patients ($n = 17$) with *KRAS* mutations. *KRAS* signals from EVs were normalized against that of GAPDH (loading control). The heatmap shows mean values from technical triplicate measurements. **FIG. 6C:** Representative images of tissue staining (immunohistochemistry, $200\times$ magnification). For a patient positive with *KRAS*^{G12D} EV mRNA, the tumor tissue was also positively stained for *KRAS*^{G12D} protein. FIG. 25 shows full images. **FIG. 6D:** Results in FIG. 6B were converted into *KRAS* variant allele fraction (VAF_{KRAS}) by calculating the signal ratio between *KRAS* mutation (*KRAS*^{MT}) and *KRAS* wild type (*KRAS*^{WT}). The profiling data from non-CRC controls and *KRAS*^{WT} CRC patients were used to establish the threshold ($VAF_{KRAS} = 5.5\%$) for *KRAS*^{MT} positivity. For CRC patients with *KRAS* mutation, their VAF_{KRAS} values for the corresponding mutant target were above the threshold. Half-filled circles (◐) denote patients whose *KRAS* mutation type was confirmed by clinical tissue analysis. Each data point is a mean value from technical triplicate measurements. **FIG. 6E:** Longitudinal EV monitoring. The seventeen CRC patients with *KRAS* mutations were followed up for one year. For all patients, VAF_{KRAS} values immediately after surgery were lower than their initial values. Among the non-recurrent patients ($n = 14$), VAF_{KRAS} continued to decrease and fell below the threshold (5.5%), reflecting favorable clinical outcomes (tumor-free). In the recurrent patients ($n = 3$), VAF_{KRAS} values rebounded and eventually returned to pre-surgery levels. Each data point is a mean value from technical triplicate measurements. See FIG. 27 for individual patient data. **FIG. 6F:** Kinetics of VAF_{KRAS} decline. In the non-recurrent CRC patients, the VAF_{KRAS} values would fall below the threshold approximately ten days following surgery, suggesting a potentially optimal window for EV analysis in post-surgery prognosis. The dotted line is the optimal fit for the post-surgery data, $VAF_{KRAS} \sim d^{0.3}$, where d is the number of days after surgery. The shaded region represents the 95% confidence interval. Data are shown as mean $\pm$ s.d. ($n = 14$ patients).

FIGs. 7A-7E. Stratifying glioma patients. **FIG. 7A:** SCOPE analyzed plasma samples collected from radiologically confirmed glioma patients. Tissue samples were used for clinical pathology. **FIG. 7B:** Target RNA sequences for glioma-SCOPE analyses. *IDH1* probes were designed to detect wild-type (WT)

(CCCATCATCATAGGTCGTCATGCTTAT; SEQ ID NO:53) and single nucleotide mutation (R132H) (CCCATCATCATAGGTCATCATGCTTAT (SEQ ID NO:54), and *EGFR* probes to detect WT (GAAAAGAAAGGTAAGGGCGTGTCTCGCC; SEQ ID NO:55) as well as the variant III (*EGFRvIII*)

(AAGAAAGGTAATTATGTGGTGACAGATC; SEQ ID NO:56) resulting from genomic

deletion of exons 2–7 in the *EGFR* gene. **FIG. 7C:** The specificity of glioma-SCOPE probes was evaluated. The designed probes achieved a high signal contrast (>30) between on-target and off-target samples. Synthetic RNA samples (1 nM) were used. The heatmap displays mean values from technical triplicate measurements. a.u., arbitrary unit. **FIG. 7D:**

Application of SCOPE to profile EVs for *IDH1* (WT, R132H) and *EGFR* (WT, vIII). EVs

were harvested from glioma cell lines. The results confirmed that EVs reflected the genotype of parent glioma cells. Data are shown as mean $\pm$ s.d. from biological triplicates. a.u.,

arbitrary unit. (e) Plasma EVs were analyzed via SCOPE for *IDH1* (WT, R132H) and *EGFR* (WT, vIII). Control samples were from healthy donors (n = 15). Glioma samples were from

patients with different genotypes: *EGFR* amplification (GBM-WT; n = 20), *IDH1-R132H*

mutation (n = 20), and *EGFRvIII* mutation (n = 20). The SCOPE assay identified glioma patients and correctly stratified them according to their molecular genotypes. The heatmap shows mean values from technical triplicate measurements.

FIGs. 8A-8B. Designing a signal template. **FIG. 8A:** The signal template consists of a fluorescent RNA segment (UUTUU; SEQ ID NO:57), a T7-promotor sequence

(TAATACGACTCA; SEQ ID NO:58 and CCCTATAGTGAGTCGTATTA; SEQ ID NO:59), and a single-stranded DNA sequence

(GAGCTTGACAAAGTGGTCGTTGAGGGCA; SEQ ID NO:60) that is complementary to the target mRNA. The fluorescence of the dye is initially quenched by the Black Hole quencher. **FIG. 8B:** For a given mRNA marker, we downloaded its sequence from the

National Center for Biotechnology Information (NCBI). We then used Snapgene software to

select a target region with a 40-60% GC content and a melting temperature (T_m) of 65-75 °C. The target candidate was further checked for a possible secondary structure (IDT oligo

analyzer) and for a binding efficiency (>80%; Nupack). These steps produced the final target

sequence. We then used its complementary sequence in the signal template.

FIG. 9. Sequence-based illustration of the SCOPE assay. The detection target is *KRAS*^{G12D} mRNA. **Top panel:** Cas13a/crRNA recognizes the initial target RNA (top strand). **Top middle panel:** Activated Cas13a/crRNA (scissors) cleaves the RNA region in the signal template. This process releases the fluorescent dye in the signal template and exposes the template region complementary to the RNA target. **Bottom middle panel:** T7 RNA polymerase docks into the promotor region and replicates target RNA strands. **Bottom panel:** Unreacted Cas13a/crRNA binds a target replica to get activated, and the processes (cleavage, synthesis, and recognition) are repeated. Target: UAU AAA CUU GUG GUA GUU GGA GCU GAU G (SEQ ID NO:6); crRNA: GAU UUA GAC UAC CCC AAA AAC GAA GGG GAC UAA AAC ACA ACC UCC AAC UAC CAC AAG UUU AUA (SEQ ID NO:66); Signal template: TAA TAC GAC TCA UUT UUC ATC AGC TCC AAC TAC CAC AAG TTT ATA CC CTA TAG TGA GTC GTA TTA (SEQ ID NO:67); T7 promoter: TAA TAC GAC TCA (SEQ ID NO:68); RNA replicas: G GG UAU AAA CUU GUG GUA GUU GGA GCU GAU G (SEQ ID NO:69); Cleaved signal template: C ATC AGC TCC AAC TAC CAC AAG TTT ATA CC CTA TAG TGA GTC GTA TTA (SEQ ID NO:70);

FIGs. 10A-10B. Characterization of the pDMAEA coating. **FIG. 10A:** Fourier transform infrared (FTIR) spectra of DMAEA monomer (bottom) and pDMAEA (top). pDMAEA was deposited on a PCR tube via the initiated chemical vapor deposition (iCVD) process. After polymerization, the C=C vinyl stretching peak (at 1635 cm⁻¹) of the DMAEA monomer disappeared, indicating that the vinyl group was consumed during the free radical addition reaction. In contrast, the peaks reflecting the tertiary aminomethyl moiety (2800 - 3000 cm⁻¹) remained unchanged, supporting the retention of the tertiary amine functional group in pDMAEA during the iCVD process. **FIG. 10B:** SCOPE reactions (target: *KRAS*^{G12D} RNA) were performed in both pristine and pDMAEA-coated tubes. Across varying RNA concentrations, the measured fluorescent intensities were statistically non-different (nd) between the two tube types (p > 0.05; unpaired t-test). The results underscore that pDMAEA is compatible with SCOPE reactions. Data are displayed as mean ± s.d. from technical triplicates. a.u., arbitrary unit.

FIG. 11. Self-catalyzed hydrolysis of pDMAEA. **Top left panel:** Chemical reaction. pDMAEA degrades into poly(acrylic acid) and dimethylaminoethanol through the hydrolysis of the ester groups located on the polymer side chains. The polymer's charge shifts from positive to negative. **Top right panel:** Zetapotential changed from positive to negative after pDMAEA was subjected to the hydrolysis condition (40 °C, 30 min). Data are displayed as mean ± s.d. from technical triplicates. **Bottom left panel:** ¹H nuclear magnetic resonance

(NMR) spectra of pDMAEA in CDCl_3 solution (no hydrolysis). The inset shows pDMAEA with hydrogen positions annotated. **Bottom right panel:** ^1H NMR spectra of pDMAEA in buffered D_2O solution after 30 min of hydrolysis at 40°C . The presence of three peaks (f, g, h) was consistent with the production of dimethylaminoethanol through the hydrolysis of ester groups in pDMAEA. The inset shows pDMAEA and dimethylaminoethanol with hydrogen positions annotated. NMR spectra were obtained at 600 MHz.

FIGs. 12A-12E. Portable SCOPE device. **FIG. 12A:** Photo of the prototype system. **FIG. 12B:** Schematic of the device inside. Sixteen PCR tubes can be loaded in a linear array format. After a heating process is completed, a two-channel optical module scans the tube array to measure fluorescent signals. **FIG. 12C:** System diagram. A microcontroller coordinates the operation of heating blocks, an optical detection module, and a linear scanner. The system connects with an external terminal (*e.g.*, a tablet, a smartphone, or a computer) to present a graphical user interface. LED, light emitting diode; PWM, pulse-width modulation; I2C, inter-integrated circuit. **FIG. 12D:** The sample holder was wrapped with flexible heating tape, except for the sample loading side. (Inset) Cross-sectional schematic of the sample holder. **FIG. 12E:** The device was set to heat samples at 40°C (red dotted line). The measured temperature was highly uniform across all 16 samples, with well-to-well variations of less than 0.5°C . Data is displayed as mean $\pm$ standard deviation from 100-second measurements.

FIG. 13. Polyacrylamide gel electrophoresis (PAGE) analysis for SCOPE mechanism. (Lane1) The target mRNA (*CD63*) only. (Lane 2) The signal template only. (Lane 3) When the target mRNA and *CD63*-specific Cas13a/crRNA were mixed, the Cas13a enzyme was activated to cleave the signal template. (Lane 4) Cas13a with scrambled crRNA was used. No cleaving activity was observed. (Lane 5) The target mRNA was missing. No cleaving activity was observed. (Lane 6) No Cas13a enzyme was added. No cleaving activity was observed. (Lane 7) Subsequent RNA replication by T7 polymerase was successful when Cas13a/crRNA was able to cleave the signal template. (Lanes 8-9) Without active Cas13a/crRNA machinery, no RNA was produced. (Lane 10) *CD63*-specific Cas13a/crRNA was mixed with a scrambled control RNA (random sequence). No RNA was produced. (Lane 11) *CD63*-specific Cas13a/crRNA was mixed with a non-target RNA (*GAPDH*). No RNA was produced. bp, base pair.

FIGs. 14A-14B. T7 RNA polymerase binding to the signal template. **FIG. 14A:** When the signal template has an intact RNA segment, the RNA polymerase fails to replicate RNA targets (left). This is likely due to steric hindrance, which obstructs the binding of the

polymerase to the T7 promoter region. When Cas13a degrades the RNA segment in the signal template (right), the polymerase can effectively synthesize RNA targets complementary to the template strand. **FIG. 14B:** Signal templates with and without the RNA segment were used in the T7 RNA polymerase reaction. Molecular beacons were used to detect transcribed RNAs. When the signal template with an intact RNA segment was used, no detectable signal was observed, indicating an inhibited T7 polymerase reaction. A detectable fluorescent signal, indicative of RNA synthesis, was observed when the signal template lacking the RNA segment was used. Data are displayed as mean $\pm$ s.d. from technical triplicates. a.u., arbitrary unit.

FIG. 15. SCOPE conditions. Assay components and concentrations were tested to maximize the SCOPE fluorescent signal. For all experiments, the target RNA concentration was fixed at 1 nM. Dark gray bars indicate the selected condition. **Top panel:** Testing of signal template design. The lengths of three parts were varied: the double-stranded region of the T7 promoter, complementary RNA sequence, and a fluorescent RNA segment. The final lengths for each region are indicated (Left). The best T7 promoter length was 12 base pairs (bp) for the double-stranded region. For the complementary RNA sequence, the best length was 28 nucleotides (nt). The fluorescent RNA segment showed optimal performance with four uracils (Us), resulting in a total length of five nucleotides. **Bottom panel:** SCOPE reactions were performed under varying reagent concentrations. Data are displayed as mean $\pm$ s.d. from technical triplicates. a.u., arbitrary unit.

FIGs. 16A-16B. SCOPE test for CD63 mRNA. **FIG. 16A:** SCOPE probe for *CD63* was designed and tested. The signal was the highest when all reagents were present. The signal was lower in the absence of RNA synthesis. The initial [*CD63*] was 1 nM. **FIG. 16B:** SCOPE and RT-PCR assays were compared for *CD63* RNA detection. SCOPE showed a lower limit of detection (LOD) and a broader dynamic range in comparison to RT-PCR. The estimated LODs were 0.8 zM (SCOPE) and 2.1 fM (RT-PCR). All experiments are from technical triplicates, and data are displayed as mean $\pm$ s.d. a.u., arbitrary unit.

FIG. 17. SCOPE specificity assessment. SCOPE probes were designed to detect *KRAS* wild type (WT) and its point mutations (G12C, G12D, G12V, G12S) in the codon 12. These probes were tested with samples, each containing a distinct *KRAS* target at a concentration of 1 nM. SCOPE assays demonstrated high specificity with negligible crosstalk. Data are displayed as mean $\pm$ s.d. from technical triplicates. a.u., arbitrary unit.

FIGs. 18A-18C. *KRAS* mRNA detection with RT-PCR. **FIG. 18A:** Target RNA sequences used in the RT-PCR assay include WT: GAGCUGGUGGCGUAGGCAA (SEQ

ID NO:61); G12C: GAGCUUGUGGCGUAGGCAA (SEQ ID NO:62); G12D: GAGCUGAUGGCGUAGGCAA (SEQ ID NO:63); G12S: GAGCUAGUGGCGUAGGCAA (SEQ ID NO:64); and G12V GAGCUGUUGGCGUAGGCAA (SEQ ID NO:65). **FIG. 18B:** RNA samples, each containing 1 nM of a distinct KRAS target, were analyzed. The procedure involved initial reverse transcription, followed by PCR amplification using a commercial detection kit (THERASCREEN® KRAS RGQ PCR Kit; Qiagen 870021). The graphs show real-time amplification curves. The dotted lines indicate no target controls (NTCs). Off-target amplifications were observed in WT, G12C, G12S, and G12V samples. **FIG. 18C:** Data in FIG. 18B are summarized. The cycle threshold (Ct) was determined at the intensity value of 800, and ΔC_t was calculated as the Ct difference between a complete RNA assay and its NTC. Note the crosstalk of the probe among different KRAS samples. a.u., arbitrary unit.

FIGs. 19A-19B. SCOPE analysis of *Kras*^{G12D} in mouse plasma. **FIG. 19A:** Serial plasma samples were collected weekly from control KP mice (n = 3) that did not receive tumor induction. Plasma EVs were then analyzed via SCOPE for *Kras*^{G12D}. The expression was at the background level for all eight weeks. **FIG. 19B:** Plasma samples were collected from KP mice (n = 10) before tumor induction (week 0) and then on a weekly basis. SCOPE detected an increase in *Kras*^{G12D} levels in plasma EVs, starting as early as week 1. All data are displayed as mean $\pm$ s.d. from technical triplicates. Mean values were used for the heatmap (FIG. 5D) in the main text. a.u., arbitrary unit.

FIG. 20. Computerized tomography (CT) imaging of tumor-bearing mice. Tumor-bearing KP mice (n = 9) underwent weekly CT imaging before and after tumor induction, and tumor volumes were estimated. Tumor growth was observed over time, although lesions became detectable approximately two weeks after tumor induction. (Inset) A representative CT image of a mouse lung at week 3 is shown, with the lung rendered in dark gray and the tumor in light gray for visualization purposes.

FIG. 21. *Kras*^{G12D} copy numbers in non-cancer KP mice. SCOPE data (FIG. 19A) were converted to *Kras*^{G12D} mRNA copy numbers using the calibration curve (FIG. 4A). The estimated copy number was below a single copy in all samples.

FIG. 22. Profiling cell-line derived EVs for KRAS mutations. SCOPE was used to detect *KRAS* wild-type (WT) and codon 12 point mutations in EV samples. EVs were collected from a panel of cell lines, including normal human colon cells (CCD18Co) and colorectal cancer cells (Colo205, HT29, LS123, LS174T, SW480, and SW620). The EV

profiling results matched the known *KRAS* genotypes of these cells. Data are displayed as mean $\pm$ s.d. from technical triplicates. a.u., arbitrary unit.

FIG. 23A-23C. Characterization of EV samples. **FIG. 23A:** A plasma sample (3 mL) from a colorectal cancer patient was processed via size exclusion chromatography to isolate EVs. Western blot analysis revealed high expression of established EV markers (CD63, Alix) and the lack of a non-EV marker (histone H2B). The whole blot image is shown. **FIG. 23B:** A plasma sample from a CRC patient was processed via SEC. Both the isolated EV fraction (light gray dots) and the non-EV fraction (dark gray dots) were analyzed using the SCOPE assay to detect mRNA targets (*GAPDH*, *CD63*, *KRAS*^{G12D}). Only the EV fraction exhibited high SCOPE signals, strongly suggesting an association between these mRNA biomarkers and EVs in plasma. Data are displayed as mean $\pm$ s.d. from technical triplicates. **FIG. 23C:** SCOPE signals were compared between samples with (+) and without (–) RNase A treatment (before EV lysis). The SCOPE signal showed no significant difference ($P = 0.288$, non-paired two-sided t-test). Data are displayed as mean $\pm$ s.d. from technical triplicates.

FIG. 24. SCOPE profiling of EVs in clinical samples. Plasma EVs from non-cancer subjects ($n = 15$) and CRC patients ($n = 107$) were subjected to SCOPE analysis for *GAPDH*, *KRAS* wild-type (WT) mRNA, and *KRAS* point mutations (G12C, G12D, G12S, G12V). *GAPDH* expression was used to normalize other detection targets. No mutation targets were detected in samples from noncancer subjects. Among the samples from CRC patients, seventeen of them exhibited *KRAS* point mutations, which were consistent with the *KRAS* typing in the corresponding tumor tissue specimens. The heatmap was generated using mean values from technical triplicate measurements.

FIG. 25. Full images of stained tissues in FIG. 6C. The stained tissue was imaged at 200 $\times$ magnification. (Left) Tissue sample from *KRAS*^{G12D}-negative CRC patient. (Right) Tissue sample from *KRAS*^{G12D}-positive CRC patient.

FIG. 26. Setting the threshold for *KRAS* mutation negativity. The *KRAS* variant allele fraction (VAF_{KRAS}) was calculated as the ratio between *KRAS* mutation (*KRAS*^{MT}) and *KRAS* wildtype (*KRAS*^{WT}) signals from SCOPE assays. VAF_{KRAS} values from non-cancer controls ($n = 15$) and *KRAS*^{WT} CRC patients ($n = 90$) were used to construct the VAF_{KRAS} distribution, which was then fitted to a normal distribution. The VAF_{KRAS} threshold for *KRAS*^{MT} negativity was set as the 99th percentile of this distribution ($VAF_{KRAS} = 5.5\%$). Samples with VAF_{KRAS} values above this threshold were classified as *KRAS*^{MT} positive.

FIG. 27. Raw EV profiling data. Serial blood samples from CRC patients with *KRAS* mutations ($n = 17$) were analyzed using *KRAS* SCOPE assay. Each data point is displayed as mean $\pm$ s.d. from technical triplicate measurements.

FIGs. 28A-28D. SCOPE probes for glioma EVs. RNA probes for wild-type *IDH1* (*IDH1^{WT}*) (FIG. 28A), *IDH1^{R132H}* (FIG. 28B), wild-type EGFR (*EGFR^{WT}*) (FIG. 28C), and EGFR variant III (*EGFRvIII*) (FIG. 28D) were designed and tested. Each probe generated the highest signal only when all SCOPE reagents were present. The estimated detection limit was in the attomolar range. Data are displayed as mean $\pm$ s.d. from technical triplicate measurements. a.u., arbitrary unit.

FIG. 29. SCOPE assay for glioma detection and subtyping. SCOPE was used to profile plasma EVs for *GAPDH*, *CD63*, *IDH1^{WT}*, *IDH1^{R132H}*, *EGFR^{WT}*, and *EGFRvIII* mRNA expressions. Plasma samples were obtained from non-GBM controls ($n = 15$) and glioma patients ($n = 60$). Patients were molecularly classified as GBM wild type ($n = 20$), IDH1-R132H mutant ($n = 20$), and EGFRvIII mutant ($n = 20$) based on independent tissue analysis. *GAPDH*-normalized expressions of *CD63* and *IDH1^{WT}* were similar among non-GBM controls and glioma patients. Other markers were differentially expressed in glioma patients, which matched classification results from tissue analyses. Each data point is a mean value from technical triplicate measurements. The threshold value was determined from non-GBM controls – *IDH1^{R132H}*, *EGFR*, and *EGFRvIII* data were pooled and fitted to a normal distribution. The 99th percentile of this distribution was set as the threshold.

FIGs. 30A-30C. Pilot evaluation of SCOPE's applicability with other sample types and pre-processing methods. **FIG. 30A:** SCOPE with other biofluids. **Left panel:** EVs were isolated via SEC from a urine sample of a healthy donor. The SCOPE assay was used to detect *CD63* mRNA, a known EV marker. **Right panel:** SCOPE was used to detect the presence of SARS-CoV-2 viral RNA (ORF1 region) in a nasopharyngeal swab sample collected from a COVID-19 patient. In both cases, signal intensity was maximal when all SCOPE assay components were present (dark gray bars). Removing T7 polymerase diminished the signal due to the absence of target RNA amplification (grey bars). **FIG. 30B:** EVs were isolated from the supernatant of A549 lung adenocarcinoma cells using either SEC or ultracentrifugation (UC). Isolated EVs from both methods were then subjected to SCOPE for the detection of *GAPDH*, *CD63*, and *KRAS^{G12S}* mRNA targets. The marker expression profiles exhibited high similarity between SEC and UC-isolated EV preparations. **FIG. 30C:** The SCOPE assay was used to detect *KRAS^{G12D}* mRNA in a plasma sample from a colorectal cancer patient. Direct use of the plasma sample produced negligible signal, whereas the

signal was recovered in an SEC-isolated EV sample. Data are displayed as mean $\pm$ s.d. from technical triplicate measurements. a.u., arbitrary unit.

FIGs. 31A-31B. SCOPE assay for BRAF detection. **FIG. 31A:** Probe validation. RNA probes for wild-type (WT) *BRAF* and its point mutations (V600E, V600K, V600R) were designed and tested with synthetic RNA targets – this process was completed within a two-week timeframe. SCOPE assays demonstrated high specificity with negligible crosstalk. The target RNA concentration was 1 nM. Data are displayed as mean $\pm$ s.d. from technical triplicates. a.u., arbitrary unit. **FIG. 31B:** In a pilot test, SCOPE was used to profile plasma EVs for *BRAF* mutations. Samples were from pre-surgery CRC patients ($n = 11$). SCOPE results matched BRAF mutation status from tumor tissue analysis. *BRAF* signals from EVs were normalized against that of *GAPDH* (loading control). The heatmap shows mean values from technical triplicate measurements.

FIG. 32. The purification of LwaCas13a was confirmed by SDS-PAGE, followed by silver staining and comparison with Commercial LwaCas13a. Ni-NTA, nickel-nitrilotriacetic acid; SUMO, small ubiquitin-like modifier.

DETAILED DESCRIPTION

Provided herein are methods for detecting a target nucleic acid sequence using a Cas-crRNA complex and a signal template. Such methods involve recognition of the target nucleic acid sequence by the Cas-crRNA complex via hybridization of the crRNA to the target nucleic acid sequence, and amplification of the target nucleic acid sequence via hybridization of the signal template to the target nucleic acid sequence.

FIG. 1A provides a non-limiting schematic depiction of components and methods for detecting a target nucleic acid sequence as described herein. As shown in **FIG. 1A**, the Cas13a-crRNA complex recognizes and binds to a target RNA sequence, which can be isolated from extracellular vesicles (EVs). The Cas13a-crRNA complex becomes an active ribonuclease and degrades the RNA segment in the signal template. This trans-cleavage turns on fluorescent signals by releasing dye molecules from quenchers, and exposes the portion of the signal template that includes the DNA template complementary to the target RNA sequence. The T7 RNA polymerase attaches to the promoter region of the signal template and replicates RNA targets. Unreacted Cas13a-crRNA complex can recognize RNA replicas, thereby repeating the reaction cycle.

Accordingly, described herein are improved methods for rapid detection of a target nucleic acid sequence using a Cas-crRNA complex and a signal template. The methods

described herein can discriminate between target nucleic acid sequences, even down to single-nucleotide variations. The methods described herein can also achieve high sensitivity, with a sub-attomolar detection limit. For example and without limitation, this high sensitivity can be achieved through dual amplifications in which activated Cas cleaves RNA segments to boost a detectable signal (*e.g.*, a fluorescent signal) and polymerases synthesize multiple copies of the target nucleic acid sequence. The methods described herein are fast and can be carried out in a single tube at a constant temperature, which renders such methods readily accessible across laboratory settings.

I. Assay Components

Methods described herein involve the use of an RNA-guided Cas protein, a CRISPR guide RNA (crRNA), a polymerase, and a signal template, to detect target nucleic acid sequences.

RNA-Guided Cas Proteins

An RNA-guided Cas protein refers to a Cas protein comprising a crRNA binding domain and an RNA cleavage domain. Non-limiting examples of RNA-guided Cas proteins for use in methods described herein include Cas13a, Cas13b, Cas13c, and Cas13d.

The RNA-guided Cas protein (*e.g.*, Cas13) for use in methods described herein can be from any organism including, but not limited to, *Azospirillum*, *Bacteroides*, *Campylobacter*, *Corynebacter*, *Eubacterium*, *Flaviivola*, *Flavobacterium*, *Filifactor*, *Gluconacetobacter*, *Lachnospira*, *Lactobacillus*, *Legionella*, *Leptotrichia*, *Listeria*, *Mycoplasma*, *Neisseria*, *Nitratifactor*, *Parvibaculum*, *Roseburia*, *Staphylococcus*, *Streptococcus*, *Sphaerochaeta*, *Sutterella*, and *Treponema*.

The RNA-guided Cas protein (*e.g.*, Cas13) can comprise a full-length protein or a fragment thereof. In some embodiments, the RNA-guided Cas protein comprises the full-length amino acid sequence of Cas13a from *Leptotrichia wadei* (LwaCas13a), which is provided below as SEQ ID NO:1. In some embodiments, the RNA-guided Cas protein comprises a fragment of the full-length amino acid sequence of LwaCas13a, *e.g.*, the RNA-guided Cas protein comprises a fragment of SEQ ID NO:1.

Cas13a from *Leptotrichia wadei* (LwaCas13a)

(NCBI Reference Sequence: WP_021746774.1)

MKVTKVDGISHKKYIEEGKLVKSTSEENRTSERLSELLSIRLDIYIKNPDNASEEENRIRRENLKKFFSN

KVLHLKDSVLYLKNRKEKNAVQDKNYSEEDISEYDLKNKNSFSLKKILLNEDVNSEELEIFRKDVEAKL
 NKINSLKYSFEENKANYQKINENNVEKVGKSKRNIIYDYRESAKRNDYINNVQEAQFDKLYKKEDIEKL
 FFLIENSKKHEKYKIREYYHKII GRKNDKENFAKIIYEEIQNVNNIKELIEKIPDMSELKKSQVFYKYLL
 DKEELNDKNIKYAFCHFVEIEMSQLLKNYVYKRLSNISNDKIKRIFEYQNLKKLIENKLLNKLDITYVRNC
 5 GKYYNYLQVGEIATSDFIARNRQNEAFLRNIIGVSSVAYFSLRNILETENENDITGRMRGKTVKNNKGEE
 KYVSGEVDKIYNENKQNEVKENLKMFIYSYDFNMDNKNEIEDFFANIDEAIISSIRHGIVHFNLELEGKDI
 AFKNIAPSEISKMFQNEINEKKLKLKIFKQLNSANVFNYEYKDVIIKYLKNTKFNFNKNI PFVPSFTK
 LYNKIEDLRNTLKFVSWPKDKEEKDAQIYLLKNIYYGEFLNKFKVNSKVFFKITNEVIKINKQRNQKTG
 10 HYKYQKFENIEKTVPEYLAIIQSREMINNQDKEEKNTYIDFIQQIFLKGFDYLNKNNLKYIESNNND
 ELTNLKGSLKEYQSANKEETFSDELELINLLNLDNNRVTEDFELEANEIGKFLDFNENKIKDRKELKKFD
 TNKIYFDGENIIKHRAFYNIKKYGMNLNLEKIADKAKYKISLKEKKEYSNKKNEIEKNYTMQQNLHRKYA
 RPKKDEKFNDYKEYEKAIGNIQKYTHLKNKVEFNELNLLQGLLLKILHRLVGYTSIWERDLRFRKGE
 15 FPENHYIEEIFNFDNSKNVYKSGQIVEKYINFYKELYKDNVEKRSIYSDKKVKKLKQEKDLYIRNYIA
 HFNYIPHAELSLLEVLNLRKLLSYDRKLKNAIMKSIVDILKEYGFVATFKIGADKKIEIQTLESEKIVH
 LKNLKKKKLMTDRNSEELCELVKVMFEYKALE (SEQ ID NO:1)

The RNA-guided Cas protein (*e.g.*, Cas13) can comprise a mutation, which can be an
 20 insertion, a deletion, a substitution, a fusion (*e.g.*, a gene fusion), or a combination thereof.
 For example, the RNA-guided Cas protein can comprise a mutant Cas13a comprising an
 amino acid sequence that is at least 70%, at least 80%, at least 85%, at least 90%, at least
 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least
 98%, or at least 99% identical to SEQ ID NO:71.

25 The RNA-guided Cas protein (*e.g.*, Cas13) to be used in methods described herein can
 be prepared by any known method. The RNA-guided Cas protein (*e.g.*, Cas13) can be added
 to a sample as a recombinant protein and/or as a cell lysate from cells that express the RNA-
 guided Cas protein (*e.g.*, Cas13). In some embodiments, the RNA-guided Cas protein (*e.g.*,
 Cas13) can comprise a fusion protein or a tagged protein (*e.g.*, a His₆-tagged RNA-guided
 30 Cas protein (*e.g.*, His₆-tagged Cas13)).

CRISPR Guide RNAs (crRNAs)

A crRNA refers to a polynucleotide comprising a nucleic acid sequence having
 sufficient complementarity with a target nucleic acid sequence to hybridize to the target
 35 nucleic acid sequence and direct sequence-specific binding of a crRNA-Cas protein complex
 to the target nucleic acid sequence.

A crRNA for use in methods described herein can include any nucleic acid sequence.
 For example, a crRNA for use in methods described herein can comprise or consist of a
 nucleic acid sequence selected from SEQ ID NOs:17-31. In another example, a crRNA for
 40 use in methods described herein can comprise or consist of a nucleic acid sequence selected
 from SEQ ID NOs:17-31 with one or more mutations (*e.g.*, 1, 2, 3, 4 or more mutations).

A crRNA for use in methods described herein can be completely complementary or substantially complementary to a target nucleic acid sequence. In some embodiments, the crRNA can be at least 50%, at least 60%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97.5%, at least 99%, or more complementary to a target nucleic acid sequence over any useful region (*e.g.*, over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides).

Any length crRNA can be used in methods described herein. In some embodiments, the crRNA is 40 to 80 nucleotides in length, *e.g.*, the crRNA is 45 to 80 nucleotides, 50 to 80 nucleotides, 55 to 80 nucleotides, 60 to 80 nucleotides, 65 to 80 nucleotides, 70 to 80 nucleotides, 75 to 80 nucleotides, 40 to 75 nucleotides, 40 to 70 nucleotides, 40 to 65 nucleotides, 40 to 60 nucleotides, 40 to 55 nucleotides, 40 to 50 nucleotides, or 40 to 45 nucleotides in length.

A crRNA for use in methods described herein can comprise any modification such as those known in the art. Non-limiting examples of a modification include a backbone modification, a base modification, a sugar modification, or a combination of any of these. Non-limiting examples of backbone modifications include phosphorothioate modifications, methylphosphonate modification, phosphoramidate modifications, and locked nucleic acid (LNA) backbone modifications. Non-limiting examples of base modifications include substituted purines and pyrimidines. Non-limiting examples of sugar modifications include 2'-O-alkylated or 2'-fluorinated ribose and arabinose. Other such modifications are well known to those of skill in the art.

Polymerases

A polymerase refers to any enzyme that synthesizes polymers of nucleic acids. Polymerases include, but are not limited to, DNA polymerases and RNA polymerases. DNA polymerases include, but are not limited to, DNA-dependent DNA polymerases and RNA-dependent DNA polymerases including reverse transcriptases. RNA polymerases include, but are not limited to, DNA-dependent RNA polymerases and RNA-dependent RNA polymerases. Polymerases for use in methods described herein can be naturally occurring or genetically modified. In some embodiments, the polymerase is a T7 RNA polymerase or a SP6 RNA polymerase.

Signal Template

A signal template refers to an RNA-DNA hybrid that couples target recognition using CRISPR and signal amplification using a polymerase. A signal template can comprise a

polymerase promoter sequence, an RNA sequence comprising a Cas protein cleavable sequence, and a DNA sequence that is sufficiently complementary to the target nucleic acid sequence.

Any polymerase promoter sequence can be included in a signal template for use in methods described herein. Non-limiting examples of polymerase promoter sequences include a T7 promoter sequence and an SP6 promoter sequence. It should be understood that the polymerase promoter sequence can include any sequence that can be recognized by a polymerase to initiate synthesis. For example, when the signal template includes a T7 promoter sequence, the T7 promoter sequence can be any T7 promoter sequence that can be recognized by a T7 polymerase to initiate synthesis.

Any RNA sequence comprising a Cas protein cleavable sequence can be included in a signal template for use in methods described herein. A non-limiting example of an RNA sequence comprising a Cas protein cleavable sequences includes UUTUU (SEQ ID NO:57). Other Cas protein cleavable sequences can be used. For example, the signal template can include an RNA sequence comprising a Cas protein cleavable sequences provided as UUTUU (SEQ ID NO:57). In another example, the signal template can include an RNA sequence comprising a Cas protein cleavable sequences provided as UUTUU (SEQ ID NO:57) with one or more mutations.

Any DNA sequence that is completely complementary or substantially complementary to a target nucleic acid sequence can be included in a signal template for use in methods described herein. In some embodiments, the signal template can include a DNA sequence that can be at least 50%, at least 60%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97.5%, at least 99%, or more complementary to a target nucleic acid sequence over any useful region (*e.g.*, over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides).

Non-limiting examples of a signal template for use in methods described herein include a nucleic acid sequence selected from SEQ ID NOs:32-46. In some embodiments, a signal template for use in methods described herein can comprise or consist of a nucleic acid sequence selected from SEQ ID NOs:32-46. In another example, a signal template for use in methods described herein can comprise or consist of a nucleic acid sequence selected from SEQ ID NOs:32-46 with one or more mutations (*e.g.*, 1, 2, 3, 4 or more mutations).

A signal template can include at least one modified nucleotide. Any modified nucleotide known in the art can be included in signal templates for use in methods described herein. Non-limiting examples of a modified nucleotide include a locked nucleic acid (LNA),

a peptide nucleic acid (PNA), a bridged nucleic acid (BNA), an unlocked nucleic acid (UNA), and a self-avoiding molecular recognition system (SAMRS).

A signal template can include one or more donor and acceptor moieties, *e.g.*, donor and acceptor moieties known in the art or described herein. The one or more donor and acceptor moieties can be included anywhere in the signal template, *e.g.*, 3' end of the signal template, 5' end of the signal template, internally, or a combination thereof.

Donor and Acceptor Moieties

The signal template for use in methods described herein can be labeled with a donor moiety and an acceptor moiety. For example and without limitation, a signal template can include both a donor moiety and an acceptor moiety that are each attached to differing positions on the signal template.

In some embodiments, the donor moiety comprises a fluorophore donor and the acceptor moiety comprises a fluorophore acceptor or a fluorescence quencher. Any fluorophore donor and acceptor moiety suitable for fluorescence resonance energy transfer (FRET) can be used in methods disclosed herein. Fluorophore donor and acceptor pairs for use in methods described herein are known in the art and commercially available, *e.g.*, from Life Technologies (Carlsbad, CA), GE Healthcare (Piscataway, NJ), Integrated DNA Technologies (Coralville, IA), and Roche Applied Science (Indianapolis, IN).

As used herein, a "fluorophore donor" refers to a fluorophore that, upon absorbing light, can transfer excitation energy to a fluorophore acceptor or a fluorescence quencher. Non-limiting examples of a fluorophore donor include Alexa Fluor[®] dyes (*e.g.*, Alexa Fluor[®] 488, Alexa Fluor[®] 568, Alexa Fluor[®] 594, Alexa Fluor[®] 647), Cy[™] 2, Cy[™] 3, BODIPY[™], GFP, fluorescein, IEDANS (5-naphthalene-1-sulfonic acid), EDANS (5-((2-Aminoethyl)aminonaphthalene-1-sulfonic acid,)), ATTO dyes (*e.g.*, ATTO 488, ATTO 550, ATTO 647N), DYELIGHT[™] dyes (*e.g.*, DYELIGHT[™] 488, DYELIGHT[™] 549, DYELIGHT[™] 633), TRITC (tetramethylrhodamine), TAMRA (tetramethylrhodamine), DAPI (4',6-diamidino-2-phenylindole), or a lanthanide metal (*e.g.*, europium, terbium, and samarium).

As used herein, a "fluorophore acceptor" refers to a fluorophore that can accept excitation energy transferred by a fluorophore donor and use the transferred energy to emit light at its own characteristic emission wavelength spectrum. Non-limiting examples of a fluorophore acceptor include Cy[™] 3, Cy[™] 5, R-Phycoerythrin (R-PE), allophycocyanin

(APC), Alexa Fluor[®] 532, Alexa Fluor[®] 546, Alexa Fluor[®] 610, Alexa Fluor[®] 647, BODIPY[™], fluorescein, and YFP.

As used herein, a “fluorescence quencher” refers to a non-fluorescent molecule that can accept energy from an excited fluorophore, thereby reducing the fluorescence signal of the fluorophore. Non-limiting examples of a fluorescence quencher include Dabcyl, Tamra, Black Hole Quenchers, FAM, HEX, ROX, VIC, Cy[™] 5.5, Texas Red[®], Iowa Black[®], Deep Dark Quenchers, Eclipse[®], QSY[®], nanoparticles (*e.g.*, gold nanoparticles), DNP (dinitrophenyl), DABSYL (dimethylaminophenylazobenzene sulfonyl), BODIPY[™] Quencher, QXL[®] Quencher Series, IR Quenchers (*e.g.*, IR Quencher 700, IR Quencher 800, or ATTO Quenchers (*e.g.*, ATTO 540Q, ATTO 590Q).

It should be understood that a fluorophore can be a fluorophore donor when paired with one fluorophore, and it can be a fluorophore acceptor when paired with another fluorophore. For example, Cy[™] 3 is a fluorophore donor when paired with Cy[™] 5, and Cy[™] 3 is a fluorophore acceptor when paired with Cy[™] 2.

In some embodiments, the donor moiety comprises a chemiluminescence donor and the acceptor moiety comprises a chemiluminescence acceptor or a chemiluminescence quencher. Any chemiluminescence donor and acceptor moiety suitable for chemiluminescence resonance energy transfer (CRET) can be used in methods disclosed herein. Chemiluminescence donor and acceptor pairs for use in methods described herein are known in the art. In some embodiments, methods involve use of CRET and one or more of DNAzyme, RNAzyme, horseradish peroxidase (HRP)-conjugated gold nanoparticles, and HRP-conjugated antibodies.

Target Nucleic Acid Sequences

Methods described herein detect a target nucleic acid sequence or a mutation in a target nucleic acid sequence. The term “target nucleic acid” refers to a nucleic acid whose presence or absence in a sample is to be detected.

A target nucleic acid sequence can include any sequence, *e.g.*, a wild-type sequence or a mutant sequence. Any target nucleic acid sequence can be analyzed by methods described herein. In some embodiments, the target nucleic acid sequence comprises a nucleic acid sequence from a gene associated with cancer, *e.g.*, *CD63*, *KRAS*, *BRAF*, *EGFR*, and *IDH1*. In some embodiments, the target nucleic acid sequence comprises a nucleic acid sequence from a gene associated with cancer, *e.g.*, *BRAF*, *CD63*, *CTNNB*, *DICER1*, *EGFR*, *ERBB2*, *FBXW7*, *FGFR2*, *GNAI1*, *GNAQ*, *HRAS*, *IDH1*, *IDH2*, *KIT*, *KRAS*, *MAP2K1*, *MTOR*, *NRAS*, *PIK3CA*, *NFE2L2*, *PIK3R1*, *PIK3CA*, *PPP2R1A*, *PTPN11*, *RHOA*, *SF3B1*, *SMAD4*, *TP53*, and *VHL*.

The target nucleic acid sequence can include one or more mutations, *e.g.*, 1, 2, 3, 4, or more mutations, *e.g.*, *KRAS*^{G12C}, *KRAS*^{G12D}, *KRAS*^{G12V}, *KRAS*^{G12S}, *IDH1*^{R132H}, *BRAF*^{V600E}, *BRAF*^{V600K}, and *BRAF*^{V600R}. See Examples below.

The target nucleic acid sequence can comprise DNA, RNA, or a combination of DNA and RNA. In some embodiments, the target nucleic acid sequence can comprise messenger RNA (mRNA), pre-mRNA, ribosomal RNA (rRNA), transfer RNA (tRNA), micro-RNA (miRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), double stranded RNA (dsRNA), non-coding RNA (ncRNA), long non-coding RNA (lncRNA), or small cytoplasmic RNA (scRNA). In some embodiments, the target nucleic acid sequence can be a sequence within an RNA molecule (*e.g.*, mRNA or pre-mRNA).

Non-limiting examples of a target nucleic acid sequence for use in methods described herein include a nucleic acid sequence selected from SEQ ID NOs:2-16. In some embodiments, a target nucleic acid sequence for use in methods described herein can comprise or consist of a nucleic acid sequence selected from SEQ ID NOs:2-16. In another example, a target nucleic acid sequence for use in methods described herein can comprise or consist of a nucleic acid sequence selected from SEQ ID NOs: 2-16 with one or more mutations (*e.g.*, 1, 2, 3, 4 or more mutations).

II. Assays for Detecting a Target Nucleic Acid Sequence

Aspects of the present disclosure provide methods for detecting a target nucleic acid sequence. To perform the assay method described herein, a sample comprising a RNA-guided Cas protein, a crRNA, a polymerase, and a signal template, is incubated under conditions sufficient for nucleic acid amplification.

Methods described herein encompass incubating a sample for any period of time sufficient for nucleic acid amplification. In some embodiments, the sample is incubated for about 1 to about 60 minutes, *e.g.*, about 10 to about 60 minutes, about 20 to about 60 minutes, about 30 to about 60 minutes, about 40 to about 60 minutes, about 50 to about 60 minutes, about 1 to about 50 minutes, about 1 to about 40 minutes, about 1 to about 30 minutes, about 1 to about 20 minutes, about 1 to about 10 minutes, or about 1 to about 5 minutes.

Methods described herein encompass incubating a sample at any temperature sufficient for nucleic acid amplification. In some embodiments, the sample is incubated at a temperature of about 20 to 55 °C, *e.g.*, about 25 to 55 °C, about 30 to 55 °C, about 35 to 55

°C, about 40 to 55 °C, about 45 to 55 °C, about 50 to 55 °C, about 20 to 50 °C, about 20 to 45 °C, about 20 to 40 °C, about 20 to 35 °C, about 20 to 30 °C, or about 20 to 25 °C.

Methods provided herein encompass detecting a target nucleic acid sequence, or lack thereof, in a sample. In some embodiments, the sample is a biological sample obtained from a subject. In some embodiments, the biological sample is obtained from a subject having (or at risk of having) a cancer. In some embodiments, the biological sample comprises blood, serum, plasma, urine, saliva, cerebrospinal fluid (CSF), sweat, nasal secretions (*e.g.*, nasal secretions collected via a nasopharyngeal swab), breast milk, amniotic fluid, synovial fluid, seminal fluid, pleural fluid, ascitic fluid, bile, bronchoalveolar lavage fluid (BALF), tears, or a combination thereof. In some embodiments, the biological sample comprises extracellular vesicles (EVs).

Methods described herein can further comprise subjecting a sample to one or more processing steps. In some embodiments, the sample can be processed prior to performing any of the methods described herein to isolate EVs and/or RNA from the sample. Alternatively, or in addition to, the sample can be processed prior to performing any of the methods described herein to remove unwanted molecules, cells, cell debris, or other contaminants present in the sample.

Samples can include additional components including, but not limited to, deoxyribonucleotide triphosphates (dNTPs, including ATP), buffers, water, salts, divalent ions (*e.g.*, divalent cations, such as Mg^{++}), detergents, denaturants, crowding agents or combinations thereof.

Methods described herein encompass detecting a signal from a detectable label (*e.g.*, a donor moiety and an acceptor moiety) using any method known in the art or described herein. In some embodiments, methods comprise detecting a colorimetric signal, a fluorescent signal, a chemiluminescent signal, or a combination thereof. For example, the signal template can be labeled with a fluorophore (*e.g.*, FAM) and a quencher (*e.g.*, BHQ), and detection can be performed using optical detection.

III. Applications

Methods described herein can be applied to evaluating a disease, *e.g.*, diagnosis and/or prognosis of a disease. Evaluation can include identifying a subject as being at risk for or having a disease, *e.g.*, a cancer. Evaluation can also include monitoring treatment of a disease (*e.g.*, a cancer) such as evaluating the effectiveness of a treatment for a disease (*e.g.*, a cancer).

Examples of a cancer include, but are not limited to, a solid tumor, renal cell carcinoma, anal cancer, bladder cancer, brain cancer, breast cancer, cervical cancer, colorectal cancer, endometrial cancer, esophageal cancer, gastric cancer, glioblastoma, glioma, kidney cancer, liver cancer, lung cancer, lymphoma, melanoma, myeloma, neuroblastoma, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma, skin cancer, thyroid cancer, urothelial cancer, uterine cancer, or a combination thereof.

(a) *Diagnosis*

Methods described herein can be used to determine the level of the target nucleic acid sequence in a sample (*e.g.*, a blood sample, a serum sample, a plasma sample, a urine sample, a saliva sample, a cerebrospinal fluid sample, a sweat sample, a nasal secretions sample) collected from a subject, and the level of the target nucleic acid sequence is then compared to a reference level to determine whether the subject has or is at risk for a disease, *e.g.*, a cancer. In some examples, the disease (*e.g.*, prior to onset of symptoms) is diagnosed if the level of target nucleic acid sequence in the sample from the subject is lower than a control level. In other examples, the disease (*e.g.*, prior to onset of symptoms) is diagnosed if the level of target nucleic acid sequence in the sample from the subject is higher than a control level.

By comparing the level of target nucleic acid sequence in a sample obtained from a subject to the reference level as described herein, it can be determined as to whether the subject has or is at risk for a disease (*e.g.*, a cancer). For example, if the level of target nucleic acid sequence in the sample from the subject is elevated as compared to the reference value, the subject is identified as having or at risk for a cancer. The assay disclosed herein can be used to predetermine a cutoff value representing target nucleic acid sequence levels in normal subjects. Such a cutoff value can be used for determining whether a subject has or is at risk for a disease (*e.g.*, a cancer). In some instances, the level of target nucleic acid sequence in a subject is greater than the cutoff value may indicate disease risk or occurrence.

As used herein, “an elevated level or a level above a reference value” means that the level of target nucleic acid sequence is higher than a reference value, such as a pre-determined threshold or a level of target nucleic acid sequence in a control sample.

An elevated level of target nucleic acid sequence includes a target nucleic acid sequence level that is, for example, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 300%, 400%, 500% or more above than a reference value. In some embodiments, an elevated level of target nucleic acid sequence includes a target nucleic acid sequence level that is at least 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 25, 50, 75, 100, 150, 200, 300, 400, 500, 1000-fold or more higher than the level of the reference level. An

elevated level of target nucleic acid sequence also includes increasing a phenomenon from a zero state (*e.g.*, no or undetectable target nucleic acid sequence in a sample) to a non-zero state (*e.g.*, some or detectable target nucleic acid sequence in a sample).

(b) *Treatment Effectiveness*

5 Methods described herein can also be applied to evaluate the effectiveness of a treatment for a cancer. For example, multiple biological samples (*e.g.*, blood, serum, plasma, urine, saliva, cerebrospinal fluid, sweat, nasal secretions, or a combination of any of these) can be collected from a subject to whom a treatment is performed either before and after the treatment or during the course of the treatment. The levels of target nucleic acid sequence can
10 be measured by any method described herein. For example, if the level of the target nucleic acid sequence decreases after the treatment or over the course of the treatment (the level of target nucleic acid sequence in a later collected sample as compared to that in an earlier collected sample), remains the same or decreases, it indicates that the treatment is effective.

 If the subject is identified as not responsive to the treatment, a higher dose and/or
15 frequency of dosage of the therapeutic agent are administered to the subject identified. In some embodiments, the dosage or frequency of dosage of the therapeutic agent is maintained, lowered, or ceased in a subject identified as responsive to the treatment or not in need of further treatment. Alternatively, a different treatment can be applied to the subject who is found as not responsive to the first treatment.

20 If the level of the target nucleic acid sequence in the sample from the subject deviates from the reference level (*e.g.*, is lower than the reference level or is higher than the reference level), the subject can be administered a treatment (*e.g.*, chemotherapy, an immune checkpoint inhibitor, surgery, radiation, or a combination thereof). For example, when the reference level is the level of target nucleic acid sequence in a sample from a healthy subject,
25 if the level of the target nucleic acid sequence in the sample from a subject having a cancer deviates from the reference level, the subject can be administered a treatment.

 In some examples, if the level of the target nucleic acid sequence in the sample from the subject is equal to the reference level, the subject can be administered a treatment. For example, when the reference level is the level of target nucleic acid sequence in a sample
30 from a first subject having a cancer, if the level of the target nucleic acid sequence in the sample from a second subject having a cancer is equal to the reference level, the second subject can be administered a treatment.

 Any of the methods described herein can further comprise administering a treatment to a subject. Methods for evaluating treatment effectiveness described herein can be applied

to any treatment suitable for use in a subject (*e.g.*, a subject having a cancer described herein).

Treatments for a cancer can vary depending on the condition. Non-limiting examples of treatments for a cancer include chemotherapy, an immune checkpoint inhibitor, surgery, radiation, or a combination thereof.

(c) *Controls*

Methods described herein involve determining the level of a target nucleic acid sequence in a sample from a subject, wherein an elevated level of target nucleic acid sequence in the sample compared to a reference level predicts whether a subject is likely to develop a cancer. In some embodiments, the control level is a level of target nucleic acid sequence in a control sample. In some embodiments, a control sample is obtained from a healthy subject or population of healthy subjects. As used herein, a healthy subject is a subject that is apparently free of a cancer at the time the level of target nucleic acid sequence is measure or a subject that has no history of the disorder. The control level as described herein can be determined by methods described herein or by methods known in the art.

The control level can also be a predetermined level. The predetermined level or score can be a single cut-off (threshold) value, such as a median or mean, or a level or score that defines the boundaries of an upper or lower quartile, tertile, or other segment of a population that is determined to be statistically different from the other segments. It can be a range of cut-off (or threshold) values, such as a confidence interval. It can be established based upon comparative groups, such as where association with risk of developing disease or presence of disease in one defined group is a fold higher, or lower, (*e.g.*, approximately 2-fold, 4-fold, 8-fold, 16-fold or more) than the risk or presence of disease in another defined group. It can be a range, for example, where a population of subjects (*e.g.*, control subjects) is divided equally (or unequally) into groups, such as a low-risk group, a medium-risk group and a high-risk group, or into quartiles, the lowest quartile being subjects with the lowest risk and the highest quartile being subjects with the highest risk, or into n-quantiles (*i.e.*, n regularly spaced intervals) the lowest of the n-quantiles being subjects with the lowest risk and the highest of the n-quantiles being subjects with the highest risk.

The predetermined value can depend upon the particular population of subjects (*e.g.*, human subjects) selected. For example, an apparently healthy population will have a different 'normal' range of levels or scores than will a population of subjects which have, are likely to have, or are at greater risk to have, a disorder described herein. Accordingly, the predetermined values selected may take into account the category (*e.g.*, sex, age, health, risk,

presence of other diseases) in which a subject (*e.g.*, human subject) falls. Appropriate ranges and categories can be selected with no more than routine experimentation by those of ordinary skill in the art.

In some embodiments, the predetermined level or score is a level or score determined in the same subject, *e.g.*, at a different time point, *e.g.*, an earlier time point. In characterizing likelihood, or risk, numerous predetermined values can be established.

Accordingly, method described herein include determining if the level of target nucleic acid sequence falls above or below a predetermined cut-off value.

A cut-off value is typically a target nucleic acid sequence above or below which is considered predictive of something, *e.g.*, likely to develop a cancer or responsiveness of a subject to a therapy. Thus, in accordance with the methods described herein, a reference level of target nucleic acid sequence is identified as a cut-off value, above or below of which is predictive of a subject being likely to develop a cancer. Some cut-off values are not absolute in that clinical correlations can still remain significant over a range of values on either side of the cutoff; however, it is possible to select an optimal cut-off value (*e.g.*, varying H-scores) of the level of target nucleic acid sequence for a particular sample type. Cut-off values determined for use in the methods described herein can be compared with, *e.g.*, published ranges of levels of target nucleic acid sequence, but can be individualized to the methodology used and patient population. It is understood that improvements in optimal cut-off values could be determined depending on the sophistication of statistical methods used and on the number and source of samples used to determine reference level values for the different target nucleic acid sequences and sample types. Therefore, established cut-off values can be adjusted up or down, on the basis of periodic re-evaluations or changes in methodology or population distribution.

IV. Kits

The present disclosure also provides kits for detecting a target nucleic acid sequence. Such kits can include a crRNA and a signal template. The signal template can include a donor moiety and an acceptor moiety. The kit can also include an RNA-guided Cas protein, a polymerase, or both. The kit can also include instructions for practicing any of the methods described herein. Instructions supplied in the kits of the present disclosure are typically written instructions on a label or a package insert.

The kits provided herein are in suitable packaging. Suitable packaging includes, but is not limited to, containers, bottles, vials, and flexible packaging. Kits can include additional components such as buffers and interpretive information.

Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are incorporated by reference for the purposes or subject matter referenced herein.

EXAMPLES

In order that the invention described may be more fully understood, the following examples are set forth. The examples described in this application are offered to illustrate the methods and compositions provided herein and are not to be construed in any way as limiting their scope.

MATERIALS AND METHODS

Materials

All oligonucleotides used in this study were purchased from Integrated DNA Technologies, Inc. (USA). The specific sequences of the oligonucleotides are listed in **Table 1** (SCOPE) and **Table 2** (RT-PCR). MONARCH® RNA Lysis Buffer, MONARCH® RNase A, RNase inhibitor (Murine), Proteinase K, DNase I, HISCRIBE™ T7 High Yield T7 RNA synthesis kit, LUNA® Universal One-Step RT-qPCR kit, and QUICK-LOAD® Purple Low Molecular Weight DNA Ladder were obtained from New England BioLabs (USA). QUBIT™ RNA HS Assay kit and DEPC-DW were purchased from Thermo Fisher Scientific (USA). The RNeasy Micro kit was purchased from Qiagen (Germany), and the Total Exosome RNA & Protein Isolation kit was from Invitrogen (USA). GelRed nucleic acid stain (41003) was sourced from Biotium (USA). All other chemicals used in the experiments were of analytical grade and used without further purification.

Table 1. SCOPE probe sequences.

mRNA	Name	Sequence (5' to 3')
GAPDH	Target	UGC CCU CAA CGA CCA CUU UGU CAA GCU C (SEQ ID NO:2)

	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac GAG CUU GAC AAA GUG GUC GUU GAG GGC A (SEQ ID NO:17)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU GAG CTT GAC AAA GTG GTC GTT GAG GGC ACC CTA TAG TGA GTC GTA TTA (SEQ ID NO:32)
CD63	Target	GCU GUG GGG CUG CUA ACU ACA CAG AUU G (SEQ ID NO:3)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac CAA UCU GUG UAG UUA GCA GCC CCA CAG C (SEQ ID NO:18)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU CAA TCT GTG TAG TTA GCA GCC CCA CAG CCC CTA TAG TGA GTC GTA TTA (SEQ ID NO:33)
KRAS WT	Target	AUA UAA ACU UGU GGU AGU UGG AGC UGG U (SEQ ID NO:4)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac ACC ACC UCC AAC UAC CAC AAG UUU AUA U (SEQ ID NO:19)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU ACC AGC TCC AAC TAC CAC AAG TTT ATA TCC CTA TAG TGA GTC GTA TTA (SEQ ID NO:34)
KRAS G12C	Target	AUA UAA ACU UGU GGU AGU UGG AGC UUG U (SEQ ID NO:5)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac ACA ACC UCC AAC UAC CAC AAG UUU AUA U (SEQ ID NO:20)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU ACA AGC TCC AAC TAC CAC AAG TTT ATA TCC CTA TAG TGA GTC GTA TTA (SEQ ID NO:35)
KRAS G12D	Target	UAU AAA CUU GUG GUA GUU GGA GCU GAU G (SEQ ID NO:6)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac CAU CUG CUC CAA CUA CCA CAA GUU UAU A (SEQ ID NO:21)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU CAT CAG CTC CAA CTA CCA CAA GTT TAT ACC CTA TAG TGA GTC GTA TTA (SEQ ID NO:36)
KRAS G12V	Target	UAU AAA CUU GUG GUA GUU GGA GCU GUU G (SEQ ID NO:7)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac CAA CUG CUC CAA CUA CCA CAA GUU UAU A (SEQ ID NO:22)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU CAA CAG CTC CAA CTA CCA CAA GTT TAT ACC CTA TAG TGA GTC GTA TTA (SEQ ID NO:37)
KRAS G12S	Target	AUA UAA ACU UGU GGU AGU UGG AGC UAG U (SEQ ID NO:8)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac ACU ACC UCC AAC UAC CAC AAG UUU AUA U (SEQ ID NO:23)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU ACT AGC TCC AAC TAC CAC AAG TTT ATA TCC CTA TAG TGA GTC GTA TTA (SEQ ID NO:38)
IDH WT	Target	UGG GUA AAA CCU AUC AUC AUA GGU CGU C (SEQ ID NO:9)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac GAC GUC CUA UGA UGA UAG GUU UUA CCC A (SEQ ID NO:24)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU GAC GAC CTA TGA TGA TAG GTT TTA CCC ACC CTA TAG TGA GTC GTA TTA (SEQ ID NO:39)
IDH R132H	Target	UGG GUA AAA CCU AUC AUC AUA GGU CAU C (SEQ ID NO:10)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac GAU GUC CUA UGA UGA UAG GUU UUA CCC A (SEQ ID NO:25)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU GAT GAC CTA TGA TGA TAG GTT TTA CCC ACC CTA TAG TGA GTC GTA TTA (SEQ ID NO:40)
EGFR WT	Target	UCU GGA GGA AAA GAA AGG UAA GGG CGU G (SEQ ID NO:11)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac CAC GCC CUU ACC UUU CUU UUC CUC CAG A (SEQ ID NO:26)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU CAC GCC CTT ACC TTT CTT TTC CTC CAG ACC CTA TAG TGA GTC GTA TTA (SEQ ID NO:41)
EGFRvIII	Target	GAA AAG AAA GGU AAU UAU GUG GUG ACA G (SEQ ID NO:12)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac CUG UCA CCA CAU AAU UAC CUU UCU UUU C (SEQ ID NO:27)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU CTG TCA CCA CAT AAT TAC CTT TCT TTT CCC CTA TAG TGA GTC GTA TTA (SEQ ID NO:42)
BRAF WT	Target	AUA GGU GAU UUU GGU CUA GCU ACA GUG A (SEQ ID NO:13)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac UCA CAG UAG CUA GAC CAA AAU CAC CUA U (SEQ ID NO:28)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU TCA CTG TAG CTA GAC CAA AAT CAC CTA TCC CTA TAG TGA GTC GTA TTA (SEQ ID NO:43)
BRAF V600E	Target	AUA GGU GAU UUU GGU CUA GCU ACA GAG A (SEQ ID NO:14)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac UCU CAG UAG CUA GAC CAA AAU CAC CUA U (SEQ ID NO:29)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU TCT CTG TAG CTA GAC CAA AAT CAC CTA TCC CTA TAG TGA GTC GTA TTA (SEQ ID NO:44)
BRAF V600K	Target	AUA GGU GAU UUU GGU CUA GCU ACA AAG A (SEQ ID NO:15)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac UCU UAG UAG CUA GAC CAA AAU CAC CUA U (SEQ ID NO:30)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU TCT TTG TAG CTA GAC CAA AAT CAC CTA TCC CTA TAG TGA GTC GTA TTA (SEQ ID NO:45)
BRAF V600R	Target	AUA GGU GAU UUU GGU CUA GCU ACA AGG A (SEQ ID NO:16)
	crRNA	gau uua gac uac ccc aaa aac gaa ggg gac uaa aac UCC UAG UAG CUA GAC CAA AAU CAC CUA U (SEQ ID NO:31)
	Signal template	[BHQ1] TAA TAC GAC TCA UU [FAM-T] UU TCC TTG TAG CTA GAC CAA AAT CAC CTA TCC CTA TAG TGA GTC GTA TTA (SEQ ID NO:46)

BHQ1: black hole quencher 1; FAM-T: thymine (T) labelled with fluorescein.

Table 2. RT-PCR probe sequences.

mRNA	Name	Sequence (5' to 3')
<i>GAPDH</i>	Target	UGC CCU CAA CGA CCA CUU UGU CAA GCU C (SEQ ID NO:2)
	Forward primer	GTC TCC TCT GAC TTC AAC AGC G (SEQ ID NO:47)
	Reverse primer	ACC ACC CTG TTG CTG TAG CCA A (SEQ ID NO:50)
<i>CD63</i>	Target	GCU GUG GGG CUG CUA ACU ACA CAG AUU G (SEQ ID NO:3)
	Forward primer	CAA CCA CAC TGC TTC GAT CCT G (SEQ ID NO:48)
	Reverse primer	GAC TCG GTT CTT CGA CAT GGA AG (SEQ ID NO:51)
<i>KRAS WT</i>	Target	AUA UAA ACU UGU GGU AGU UGG AGC UGG U (SEQ ID NO:4)
	Forward primer	TAA GGC CTG CTG AAA ATG AC (SEQ ID NO:49)
	Reverse primer	GTC CTG CAC CAG TAA TAT GC (SEQ ID NO:52)

5 Expression and purification of LwaCas13a

LwaCas13a protein was prepared through bacterial expression and subsequent purification. A plasmid DNA carrying the LwaCas13a encoding sequence (plasmid #90097; Addgene, USA) was introduced into the *E. coli* strain Rosetta 2 (DE3) pLysS (Millipore Sigma, Burlington, MA, USA). The transformation process relied on the well-established heat shock transformation method, specifically optimized for bacterial expression. Post-transformation, protein expression was induced using isopropylthio- β -galactoside (0.5 mM; Millipore Sigma). LwaCas13a proteins were then isolated through a systematic multi-step process, commencing with cell lysis and initial nickel-nitrilotriacetic acid (Ni-NTA; Thermo Fisher Scientific) purification. The solution underwent the first buffer exchange to a small ubiquitin-like modifier (SUMO) cleavage buffer (30 mM Tris-HCl at pH 8.0, 500 mM NaCl, and 1 mM dithiothreitol/ DTT). This was followed by treatment with SUMO protease (Thermo Fisher Scientific), a second round of Ni-NTA purification, and a final buffer exchange to the storage buffer (50 mM Tris-HCl at pH 7.5, 600 mM NaCl, and 2 mM DTT). The purified LwaCas13a proteins were stored with 5% glycerol (Millipore Sigma) and a protease inhibitor (Roche, Switzerland) at -80 °C until use. To verify the successful purification of LwaCas13a proteins, the products obtained from each purification step were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Ag staining (FIGs. 31A-31B).

25 Preparation of pDMAEA-coated PCR tubes

PCR tubes (diameter 6 mm) were used purchased from Bio-Rad (USA). Tert-butyl peroxide (TBPO, 98%) and dimethylaminoethyl acrylate (DMAEA, 98%) were purchased

from Merck (Germany). The DMAEA monomer and TBPO were vaporized through heat exposure at 35 °C and 25 °C, respectively. Subsequently, the vaporized substances were introduced into an initiated chemical vapor deposition (iCVD) chamber (160 mTorr). The filament temperature of the iCVD was set at 140 °C to generate TBPO radicals. The tubes
5 were held at 30 °C to facilitate vapor absorption. The pDMAEA thin film was produced as a result of the vapor-phase free radical polymerization, facilitated by a filament temperature of 140 °C.

Characterization of pDMAEA hydrolysis

10 For nuclear magnetic resonance (NMR) analysis, we dissolved pDMAEA in CDCl₃ or phosphate D₂O buffer at pH 8.0. Subsequently, ¹H NMR spectra were collected using a Bruker Avance Neo 600 MHz spectrometer (Bruker BioSpin, Germany). The degree of hydrolysis was determined by comparing the integrated signals for the methylene of the ester in pDMAEA (~4.41 ppm) and the alcohol of dimethylaminoethanol (DMAE) (~4.00 ppm).
15 For Fourier transform infrared (FTIR) analysis, we collected the spectra in the wavenumber range of 400 – 4000 cm⁻¹ and at a resolution of 1.4 cm⁻¹, using an Alpha instrument from Bruker Optics (USA). The zeta-potential values were determined using a Nano Zetasizer 3600 (Malvern, UK) equipped with a He-Ne laser source operating at 633 nm.

20 Characterization of pDMAEA tube

To evaluate the RNA extraction efficiency of the pDMAEA-coated tube, we added 100 µL of initial RNA (3, 30, 60, or 100 ng) solution to the tube and let the solution react for 10 min. We then centrifuged the tube for 5 min (123 × g), collected the supernatant, and quantified the RNA concentration (supernatant) using the QUBIT™ RNA HS Assay kit. For
25 the comparison, the same RNA samples (100 µL with initial RNA amounts of 3, 30, 60, and 100 ng) were processed using the RNeasy Micro Qiagen kit based on silica columns. Following the RNeasy protocol, the concentration of the extracted RNA was measured using the QUBIT™ RNA HS Assay kit.

30 Construction of SCOPE device

The device was designed using design software (Inventor; Autodesk, USA). The device's primary components were fabricated via computer numerical control (CNC) machining on Al alloy (AL6061) and injection molding with polycarbonate.

Heating block. The block was machined on an Al block to hold 16 standard PCR tubes (diameter, 6 mm). We used two polyimide heaters to heat the block: a high-powered 144-watt main heater and a 24-watt top heater. These heaters were driven by field-effect transistors connected to a 24 V power supply. The temperature of each heater was continually monitored using a 10-k Ω thermistor (NTCG103JF103F; TDK, Japan) and was maintained within a 0.3 °C tolerance through feedback control.

Fluorescent optics. The device had an optical module consisting of two independent units, each configurable for a different fluorescent channel. For the green fluorescent channel (excitation 495 nm; emission 520 nm) used in this study, the components in the unit were a blue light-emitting diode (C503B-BAN-CZ0A0451; Cree LED, USA), a collimating lens (LC12; Roithner Lasertechnik, Austria), an excitation band-pass filter (FF01-474/27; Semrock, USA), a dichroic mirror (Di02-R488, Semrock), an emission band-pass filter (FF01-515/30, Semrock), and two focusing lenses (LC7, GS7020-2; Roithner Lasertechnik). The fluorescent emission from the sample was detected by a photodiode that had a broad spectral response range from 320 to 1100 nm (S2386-44K; Hamamatsu, Japan). The current from the photodiode was converted into a voltage signal by a trans-impedance amplifier (OPA320; Texas Instruments, USA) and further boosted by a non-inverting amplifier (AD8605; Analog Devices, USA). The resultant analog signal was converted into a digital format by a 16-bit analog-to-digital converter (ADS1115, Texas Instruments) that collected data at a 62.5 samples/sec sampling rate.

Linear actuator. The optical module was mounted on a linear actuator to scan the module across 16 samples for fluorescent detection. The actuator was powered by a stepping motor (28BYJ-48; Kiatronics, New Zealand). The position of the optical module was initialized by moving the module until it contacted a limit switch. Microcontroller unit (MCU). An ESP32 MCU (Espressif, China) was programmed to operate the device. The MCU processed various data streams, including fluorescent measurement results, scanning locations, and heater temperatures. It also communicated with an external tablet through a Bluetooth connection. The MCU firmware was written in C++.

SCOPE App

The software was an Android App with an intuitive graphical user interface. We wrote the App using JAVA and the Android software development kit. The App saved all fluorescent measurements and test settings in a standardized comma-separated-value (CSV) format to ensure compatibility across various operating systems.

Cell culture

A panel of cell lines was purchased from the American Type Culture Collection (ATCC, USA) and grew them in the vendor-recommended media: H2228 (RPMI-1640, Cellgro); A549 (F-12K, ATCC); CCD-18 (DMEM, Corning); Colo205 (RPMI-1640);
5 HCT116 (MacCoy's 5a, Cellgro); HT29 (MacCoy's 5a modified with 2% NaHCO₃, Cellgro); LS174T and LS123 (Eagle's Minimum Essential Medium; Cellgro); SW480 and SW620 (DMEM, Corning). The KP1.9 cell line was derived from tumors of the KP mouse model on a C57BL/6 congenic background and was a generous gift from Dr. A. Zippelius, University Hospital Basel, Switzerland. KP1.9 cells were cultured in an Iscove's DMEM medium
10 (Corning). For GBM cell lines, we purchased GLI36 cells from ATCC and generated GLI36vIII and GLI36-R132H cell lines through lentivirus transduction. Both GLI36 and its variants were cultured in a DMEM (Corning). All media were supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Corning). All cell lines were tested and determined to be free of mycoplasma contamination (MycoAlert Mycoplasma Detection Kit,
15 LT07-418; Lonza, Switzerland).

EV isolation from cell culture media

We cultured cells at passages 1–15 in a vesicle-depleted medium (with 5% depleted FBS) for 48 h. We then collected culture media from approximately 10⁷ cells and removed
20 cellular debris through two successive rounds of centrifugation (10,000 × g, 3 min). To isolate EVs, we first concentrated the conditioned culture medium by processing it with a centrifugal filter (Centricon Plus-70; Millipore Sigma). Subsequently, we loaded the concentrated medium (0.5 mL) to a size-exclusion column (SP1; Izon, New Zealand) and collected EV fractions (F7-F9; 1.5 mL) in low-protein-binding microcentrifuge tubes (90410;
25 Thermofisher Scientific). Collected EVs were resuspended in a 1 × phosphate-buffered saline (PBS) buffer and stored at -80 °C. We estimated EV concentrations in these stock solutions via nanoparticle tracking analysis (NTA). For each sample, we acquired three 30-second video clips (NanoSight LM10; Malvern) and analyzed them using NTA software (version 3.2).

SCOPE assay

We placed an EV sample (100 µL) and a lysis buffer (10 µL) in a pDMAEA-coated tube. The lysis buffer consisted of proteinase K (0.8 U/reaction), RNase inhibitor (1 U/reaction), and DNase I (2 U/reaction) in Tris-EDTA (TE; pH 8.0). After incubating the

mixture at 25 °C for 10 min, we centrifuged the sample ($123 \times g$, 5 min) and discarded the supernatant, leaving about 1 μ L of the lysate in the tube. We next added the SCOPE master mix (20 μ L) to the same tube and let the mixture react (40 °C, 30 min) inside the SCOPE device. The master mix had the following components: 5 μ M LwaCas13a, 10 μ M crRNA, 50 μ M Signal template, 16 U/ μ L RNase inhibitor, 10 mM deoxyribonucleotide triphosphate mixture (ATP, GTP, UTP, and CTP), 0.1 μ M RNA polymerase, 0.5 $\times$ Cas13a cleavage buffer (10 mM HEPES-Na at pH 6.8, 25 mM KCl, 2.5 mM MgCl₂, and 2.5% glycerol), and 0.5 $\times$ RNAPol buffer (20 mM Tris-HCl at pH 7.9, 3 mM MgCl₂, 0.5 mM DTT, and 1 mM spermidine). Throughout the SCOPE reaction, we monitored the fluorescent signals using the device.

Quantitative PCR

We used quantitative reverse transcription-PCR (qRT-PCR) for comparison with SCOPE. We used either synthetic RNA samples (1 μ L) or EV RNA samples (1 μ L) as prepared in the SCOPE assay. A sample was mixed with the master mix (20 μ L) containing 1 $\times$ Luna Universal One-Step Reaction Mix, 1 $\times$ Luna WARMSTART® RT Enzyme Mix, and 400 nM primers. We then carried out reactions on a CFX Connect 96 Real-Time PCR System (Bio-Rad) using the following thermal cycling schedule: 55 °C, 10 min (reverse transcription) and 95 °C, 1 min (RTase inactivation), followed by 40 cycles of 95 °C (10 sec), 60 °C (30 sec), and fluorescent measurements.

Gel electrophoresis

We resolved 20 μ L of the reaction mixture on a gel composed of 15% polyacrylamide. We used a 1 $\times$ TE solution as a running buffer and applied a constant voltage of 120 V throughout the electrophoresis process (2 hours). Upon the completion of this phase, the polyacrylamide gels were subjected to staining with GelRed. Subsequently, we scanned the stained gels using a Geldoc Go imaging system (Bio-Rad).

EV profiling from mouse samples

We conducted the animal study following the guidelines provided by the Institutional Animal Care and Use Committee (IACUC) of Massachusetts General Hospital (Protocol 2019N000133). We used 129S1 mice, which carried the Cre-activatable conditional *Kras*^{LSL-G12D/+}, *Trp53*^{flox/flox} (KP mice) as an autochthonous model of lung adenocarcinoma^{43,44}. To initiate the tumor growth, we intranasally instilled adenoviruses (3×10^7 particles) expressing

Cre recombinase (Ad-Cre, University of Iowa Viral Vector Core). We collected blood samples (~50 µL) from the mice before the virus instillation and weekly thereafter, using the retro-orbital bleeding method. Mice were monitored for signs of disease progression and distress, including according to their body condition score and body weight. To collect plasma, we centrifuged blood samples (2,000 ×g, 15 min) at 4 °C and recovered supernatant. Plasma samples were stored at -80 °C until use. For EV isolation, we diluted plasma samples in PBS to 100 µL and processed them using size-exclusion columns (SP2; Izon) as described above.

EV profiling with clinical samples

The study was approved by the Institutional Review Board (IRB) of Massachusetts General Hospital (Protocol 2017P001581, 2019P003441, 2019P003472) and the IRB of Kyungpook National University Chilgok Hospital (Protocol KNUCH 2021-10-025). The procedures followed were per the respective institutional guidelines. Informed consent was obtained from all subjects. We drew peripheral blood samples (~10 mL) from patients into blood collection tubes (Vacutainer 367525; BD). Samples were then centrifuged (2,000 ×g, 3 min) to separate plasma from red blood cells and buffy coat and stored at -80 °C until use. For EV isolation, stored plasma samples (40 µL) were diluted in PBS (60 µL) and processed with size-exclusion columns (SP1; Izon).

Tissue analysis

Colorectal cancer patients. Pathologic evaluation and KRAS analysis of colorectal cancer tissues were performed by the Department of Pathology at the Kyungpook National University Chilgok Hospital. Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were used for DNA extraction. The PNA-CLAMP™ KRAS Mutation Detection kits Ver. 4 (Panagene, Korea), capable of detecting 40 KRAS mutation variants across codons 12, 13, 59, 61, 117, and 146, were employed for assays following the manufacturer's instructions. Glioma patients. The Massachusetts General Hospital Pathology Service analyzed pathologic tissues from patients. The analysis involved the use of various methods, including immunohistochemistry for EGFR, Snapshot NGS for IDH1, Genexus NGS for IDH1, and the Solid Fusion assay for EGFRvIII.

Immunohistochemistry

Lung tissue from KP mice. We anesthetized mice with isofluorane and perfused them with 4% paraformaldehyde (PFA) through the trachea and by the tail vein. Lungs were further fixed for 24 h, washed with PBS, and soaked in 30% sucrose in PBS for 24 h. The lung tissue was then embedded in the optimum cutting temperature (OCT) compound (Sakura Finetek) and snap-frozen in an isopentane bath on dry ice. Once the tissue was frozen, we prepared serial 10- μ m thick sections using a cryostat and placed them on glass slides. Samples were then sent to the Histopathology Research Core at Massachusetts General Hospital for staining: hematoxylin and eosin (H&E), and RAS G12D (GTX635362, GeneTex; 25 $\times$ dilution).

Tissue from colorectal cancer patients. IHC was performed on representative FFPE blocks using an automatic IHC staining instrument (BENCHMARK[®]XT, Ventana Medical Systems) according to the manufacturer's protocols. Briefly, 4 μ m FFPE sections were transferred to poly-L-lysine-coated slides and dried in an oven at 65 °C for 2 h. After deparaffinization in xylene and rehydration through a series of graded alcohols, we retrieved antigens by boiling samples via microwave in 10 mM sodium citrate buffer (pH 6.0). Sections were then incubated with a primary antibody against RAS G12D (GTX635362, GeneTex; 200 $\times$ dilution) overnight at 4 °C. Slides were then visualized using the UltraView Universal DAB kit (Ventana Medical Systems) and counterstained with Harris hematoxylin for nucleus. RAS G12D protein was considered positive when IHC staining was positive in the cytoplasm of tumor cells and was not expressed in normal non-neoplastic colonic epithelium cells.

Statistical analysis

We used GraphPad Prism version 9.5 (GraphPad Software Inc.) or R version 4.2.2 for statistical analyses. For two-group comparisons, we used an unpaired, two-sided t-test. For all statistical tests, p-values less than 0.05 were considered significant. Details on data presentation and the sample size are included in figure legends.

Western blotting

EVs were enriched from a plasma sample (3 mL) obtained from a colon cancer patient. The plasma was initially centrifuged at 2,000 $\times$ g for 3 min to remove cellular debris, and the resulting supernatant was processed using size exclusion chromatography (qEV, Izon Science). The isolated EVs were concentrated to 100 μ L using Amicon Ultra-2 10K filters

(Millipore Sigma). For western blot analysis, the concentrated EVs were lysed with ice-cold 10× RIPA buffer (Abcam) for 30 min, followed by centrifugation at $14,000 \times g$ for 10 min at 4 °C. Protein concentration was determined via a BCA assay, and the lysates were analyzed via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The following primary antibodies were used: Alix (1:1000, JM85-31, Invitrogen), biotinylated CD63 (1:1000, AHN16.1/46-4-5, Ancell), and Histone H2B (1:1000, D2H6, Cell Signaling Technology). Prior to use, the Alix and Histone H2B antibodies were biotinylated using sulfo-NHS-Biotin (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. Secondary detection was subsequently conducted using HRP-conjugated streptavidin (1:2000, R&D Systems).

EV preparation via ultracentrifugation

Cells were cultured until they reached approximately 70% confluency. At this stage, the supernatant was removed and replaced with F-12K Medium (ATCC, 30-2004) containing EV-depleted fetal bovine serum (FBS). After a 24-hour cell culture, the culture media supernatant was processed through sequential centrifugation. Initially, the supernatant underwent centrifugation at $300 \times g$ for 10 minutes and then at $2000 \times g$ for 10 minutes using a centrifuge to eliminate cells and debris. Subsequently, the supernatant was subjected to ultracentrifugation (Beckman, Optima L-100K) at 4 °C and $10,000 \times g$ for 30 minutes to remove proteins. The supernatant was ultracentrifuged at 4 °C and $100,000 \times g$ for 70 minutes to pellet EVs. The pellets were resuspended in cold PBS and ultracentrifuged again at $100,000 \times g$ for 70 minutes at 4 °C to eliminate residual impurities. Finally, the EV pellets were collected, resuspended in PBS (1.5 mL), and stored at -80 °C. For SCOPE analysis, we used 100 µL of this sample.

Comparison of SEC-processed and direct plasma samples for the SCOPE assay

A plasma sample from a colorectal cancer (CRC) patient was divided into two aliquots of 40 µL each. One aliquot underwent size exclusion chromatography (SEC) to yield a final volume of 600 µL of EV isolate, from which RNA was extracted using the Total Exosome RNA & Protein Isolation Kit (Invitrogen, 4478545). The remaining plasma aliquot was processed for RNA extraction using QIAamp RNA Blood Mini Kit (Qiagen, 52304) in accordance with the manufacturer's instructions. The extracted RNA was then quantified, and the final concentration was adjusted to 1 ng/µL. The extracted RNA samples (50 µL) were then used for the SCOPE assay.

Comparative experiments with RNase treatment

We utilized 40 μ L of plasma samples from CRC patients and isolated EVs via SEC. The final sample volume was 600 μ L after SEC operation. We mixed 100 μ L of this EV isolate with 3 μ L RNase A (New England Biolabs, T3018L). The mixture was incubated at 25 °C for 5 minutes. Subsequently, the mixture was filtered through a 30 kDa Amicon Ultra centrifugal filter (Millipore, UFC503008) to remove RNase A. A control sample underwent the same processes, with RNase A replaced by PBS. All prepared samples were then lysed and subjected to the SCOPE assay.

INTRODUCTION

Liquid biopsies present unprecedented opportunities in precision oncology by providing dynamic molecular information of the entire tumor through minimally invasive and repeatable tests¹⁻³. As such, both researchers and clinicians are empowered to monitor tumor evolution and heterogeneity⁴. Accessing real-time insights can profoundly impact patient care through early lesion detection, minimal residual disease tracking, personalized therapeutic decision-making informed by resistance profiles, and tumor recurrence monitoring^{5,6}.

Extracellular vesicles (EVs) are impactful analytical targets within the liquid biopsy arena⁷⁻¹⁰. These tiny particles (<1 μ m in diameter) carry diverse molecular cargo, including nucleic acids, proteins, and metabolites, thus serving as cellular surrogates¹¹⁻¹⁴. Detecting EV mRNA can generate rich and actionable clinical information: i) EV mRNA reflects somatic driver mutations (e.g., *KRAS*^{G12D}, *BRAF*^{V600E}) crucial for tumor initiation and growth that inform treatments^{15,16}; ii) while EVs rarely contain the nuclear proteins that confer drug resistance, they harbor corresponding mRNA counterparts to inform resistance status¹⁷⁻¹⁹; and iii) EV mRNAs, enclosed within vesicles, are shielded from nucleases in biofluids, allowing for the isolation of intact, high-quality nucleic acids^{20,21}. These collective advantages position EVs as a promising source of nucleic acids, complementing the benefits of circulating tumor DNA.

However, EVs' clinical potential remains underutilized, primarily due to technical drawbacks. The majority of EV RNA is non-coding, and mRNA copy numbers in EV samples can be exceedingly low. For instance, even abundant mRNA species, such as *GAPDH*, are only detected at a frequency of one copy per $10^4 - 10^6$ EVs, compared to microRNAs at one copy per 10^2 EVs²². This disparity has led most proof-of-concept assay development to conveniently focus on microRNA detection²³⁻²⁵. Moreover, tumor-derived EVs constitute a minor fraction (<5%) of total circulating EVs²⁶. The combined scarcity of

EV mRNAs and tumor-derived EVs necessitates large sample volumes (>2 mL plasma) and sophisticated resources (*e.g.*, droplet-digital PCR and next-generation sequencing), which diminishes the competitive advantages of EV tests and complicates their integration into routine preclinical and clinical assays.

5 We set out to develop a streamlined, rapid EV-mRNA test, drawing inspiration from the clustered regularly interspaced short palindromic repeats (CRISPR) method. CRISPR technologies are increasingly adopted in molecular diagnostics for their sequence-specific nuclease activity^{24,27-31}. CRISPR-associated (Cas) proteins become active endonucleases upon recognizing target nucleic acids³². This property has been exploited to amplify signals
10 through the promiscuous cleavage of reporter probes (*e.g.*, single-stranded DNAs tagged with a fluorescent dye and quencher pair). However, applying CRISPR assays to EV mRNA proves challenging due to the low abundance of targets. Separate pre-amplification is usually needed to replicate mRNA targets and improve assay kinetics²⁸. Replication errors and biases inherent in such amplifications can propagate^{33,34}, leading to confounding results. To address
15 this fundamental challenge, we rationalized repurposing the Cas activity where the high-precision Cas system both recognizes target mRNA and then triggers its replication *in situ*, bypassing the need for pre-amplification and associated errors. This robust and efficient strategy could ensure high analytical sensitivity while maintaining sequence specificity, a crucial capability for identifying scarce mRNA targets.

20 Here, we present the SCOPE (Self-amplified and CRISPR-aided Operation to Profile EVs) platform, an integrated assay for accurate EV mRNA detection and monitoring. SCOPE leverages the synergy between the Cas13a machinery and signaling templates; Cas13a first recognizes its target RNA, then both RNA targets and fluorescent signals are amplified. The high selectivity afforded by Cas13a allows SCOPE to resolve single-nucleotide
25 polymorphism. The assay further achieves a sensitivity down to a sub-attomolar detection limit, owing to its built-in dual amplification mechanism. We optimized SCOPE to advance its clinical applicability: i) a library of SCOPE probes was designed to detect clinically actionable cancer mutations in *KRAS*, *BRAF*, *EGFR*, and *IDH1* genes; ii) a streamlined protocol was established, enabling EV RNA extraction and detection in a rapid (40 min),
30 single-pot assay; and iii) a compact SCOPE device was developed for automated, multi-sample (16 tubes) detection. We applied SCOPE for various use cases: detecting early-stage lung cancer in animal models, tracking cancer mutational burden in patients with colorectal cancer undergoing standard of care, and identifying mutations in glioblastoma multiforme (GBM) for patient stratification. The developed assay can promote preclinical and clinical

opportunities to dissect cancer growth processes, identify the emergence of resistance, and gauge tumor response to treatments, including clinical trials. Ultimately, SCOPE promises to expedite standard clinical and drug trial decisions (same-day turnaround) and enhance EVs' utility in the liquid biopsy space.

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Example 1: Overall SCOPE Flow

The SCOPE strategy combines CRISPR recognition with RNA replication within a single assay format. The assay is initiated by introducing samples to an all-in-one SCOPE mixture containing the complex of Cas13a and CRISPR RNA (Cas13a/crRNA), T7 polymerase, signal template, and deoxyribonucleotide triphosphate (**FIG. 1A**). The signal template is crucial for coupling Cas13a/crRNA and T7 polymerase reactions. It is designed explicitly as an RNA-DNA hybrid comprising i) a fluorescent RNA segment cleavable by Cas13a/crRNA, ii) a fluorescent quencher, and iii) a DNA template for target RNA transcription by T7 polymerase (**FIGs. 8A-8B**).

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The major steps of the SCOPE reaction unfold as follows (**FIG. 1B** and **FIG. 9**). (i) Cas13a/crRNA recognizes and binds to RNA targets. (ii) The complex becomes an active ribonuclease and promiscuously degrades the RNA segment in the signal template. This trans-cleavage turns on fluorescent signals by releasing dye molecules from quenchers and, importantly, exposes the DNA template complementary to the target RNA. (iii) T7 RNA polymerase attaches to the promotor region of the template and replicates RNA targets. (iv) Unreacted Cas13a/crRNA further recognizes RNA target replicas, reinforcing the overall reaction rather robustly.

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The SCOPE mechanism offers significant technical advantages for RNA detection. First, it capitalizes on the high selectivity of Cas13a/crRNA complexes^{28,35}. The crRNA design incorporates a strategically placed synthetic mismatch, which empowers Cas13a to discriminate between RNA sequences, even down to single-nucleotide variations³⁵. In SCOPE, this highly specific Cas13a acts as a powerful filter – Cas13a activates target amplification only in the presence of a bona fide target RNA. This strategy enriches the desired target RNA signal within a complex background of other RNAs. Second, SCOPE achieves high sensitivity (low detection limit) through dual amplifications: activated Cas enzymes keep cleaving RNA segments to boost fluorescent signals, and RNA polymerases make multiple copies of the target RNA. Third, the reaction is fast and carried out in a single tube at a constant temperature (40 °C). These features render SCOPE simple and readily

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applicable across routine laboratory settings (*e.g.*, academia, hospitals, primary care clinics, industry).

These advantages inspired us to streamline the workflow of detecting RNA targets from EV lysates. Specifically, SCOPE simplified RNA extraction and detection steps for clinical EV analysis (**FIG. 1C**). In this procedure, RNA is swiftly (10 min) extracted from EV lysates in a polymer-coated tube. Subsequently, isothermal SCOPE reactions occur in that same tube, producing analytical results within 30 min. Starting from sample loading, the entire process is completed within 1 hour, facilitating same-day preclinical and clinical molecular diagnostics. Moreover, the assay uses standard laboratory equipment (*e.g.*, size exclusion column for EV isolation, thermal cycler) and only requires small volumes of samples (*e.g.*, EV isolates from <100 μ L of plasma).

Example 2: SCOPE Instrumentation

We implemented compact, cost-effective modules to streamline the assay process and enhance end-user friendliness. The first module was a polymer-coated tube for nucleic acid extraction (**FIG. 2A**). Specifically, we prepared PCR tubes coated with a charge-shifting polymer, poly[(2-dimethylamino)ethyl acrylate] (pDMAEA), through initiated chemical vapor deposition (**FIG. 10A**). Upon addition of an aqueous sample, the polymer inside the tube dissolved and became positively charged, allowing for electrostatic interactions between cationic polymers and negatively charged nucleic acids. This reaction resulted in the formation of polyplexes, which could be collected via centrifugation (5 min, 3000 g). Subsequently, the SCOPE mixture was added to the precipitates, and the temperature was raised to 40 °C. Under this condition, the polymer underwent self-catalyzed hydrolysis of the ester groups located in its side chains. This process induced negative charges on the polymer, resulting in the disassembly of polyplexes and the release of nucleic acids. The SCOPE reaction then continued within the same tube. We observed no significant difference ($P > 0.05$; unpaired two-sided t-test) in SCOPE signals when using pDMAEA-coated tubes compared to standard PCR tubes (**FIG. 10B**). In addition, self-catalyzed hydrolysis of pDMAEA was confirmed through nuclear magnetic resonance spectroscopy (**FIG. 11**).

FIG. 2B compares the RNA extraction efficiency between pDMAEA tubes and standard silica-based columns (see Methods)³⁶. The pDMAEA approach achieved similar extraction yields as the column devices, with an average extraction yield of 96% across different input RNA amounts (3, 30, 60, and 100 ng). Using pDMAEA tubes, however, streamlined the overall assay workflow. It facilitated rapid nucleic acid extraction (10 min)

and enabled the execution of SCOPE reactions within a single closed-tube format, eliminating the need for sample transfer in between steps.

The second assay module was a compact device for parallel SCOPE measurements (**FIG. 2C** and **FIGs. 12A-12E**). It integrated a tray-type heating block, a fluorescent-optical detector, and a line scanner. The heating block could hold up to 16 PCR tubes and docked to the main frame for an electrical connection. The system then generated pre-programmed temperature profiles (*e.g.*, isothermal, thermal cycling). Once the reaction was complete, the optical detector measured fluorescent signals (**FIG. 2D**). The detector comprised two independent fluorescent excitation-detection headers, with each header performing independent measurements. Two identical headers could be installed for redundant one-color measurements, or two different modules could be utilized for two distinct fluorescent dyes. The optical detector executed a linear motion to scan all 16 samples. System operation was controlled through a graphical user interface on an external terminal device.

The developed SCOPE system demonstrated high uniformity and reproducibility in parallel detection. The sample holder heated all 16 samples to 40 °C, with well-to-well temperature variations of less than 0.5 °C (**FIGs. 12A-12E**). We also observed highly uniform SCOPE signals for samples containing the same amount of mRNA, regardless of their location in the heating block (**FIG. 2E**).

Example 3: Validating SCOPE

We systematically examined SCOPE assay kinetics through analytical modeling. SCOPE couples two distinct catalytic reactions through the signal template (**FIG. 3A**): i) target-bound Cas13a/crRNA turns on the fluorescent signal by cleaving the RNA segment in the signal template and ii) RNA polymerase replicates target mRNAs using the DNA sequence in the signal template. When executed separately, each type of catalytic activity generated the end product (*i.e.*, fluorescent signal or mRNA replica) that increased linearly with time (**FIG. 3B**, left); these observations matched the zeroth-order nature of Cas13a and polymerase reactions in our assay conditions³⁷⁻⁴⁰. In contrast, coupling these two processes approximated a first-order rate kinetics, speeding up the overall reaction. Indeed, SCOPE signals rose exponentially and reached a plateau within 30 min (**FIG. 3B**, right).

Notably, off-target RNA amplification was suppressed. For RNA amplification to proceed, SCOPE first required Cas13a/crRNA to recognize its initial RNA target. The activated Cas enzyme then degraded the RNA segment in the signal template, which triggered downstream polymerase activities (**FIG. 13**). Conversely, the polymerase failed to replicate

RNA targets when signal templates had an intact RNA segment (**FIGs. 14A-14B**); the overall loop structure presumably hindered the polymerase from docking the promoter region of the signal template.

We further optimized the SCOPE template design and reaction conditions to maximize its signal intensity (**FIG. 15**). The optimal assay was then validated by controlling the reactant composition (**FIG. 3C**). The analytical signal was highest when all assay components (*e.g.*, target RNA, Cas13a, crRNA, T7 RNA polymerase) were present. The signal intensity decreased when RNA polymerase was absent; therefore, no additional targets were synthesized. The signals were negligible in those cases wherein initial target recognition by Cas13a/crRNA was missing.

Example 4: SCOPE Assay Characterization

SCOPE achieved high sensitivity through its dual amplification scheme (fluorescent signal and mRNA target). In RNA-titration experiments, SCOPE's limit of detection (LOD) reached sub-attomolar ranges, which was $>10^4$ -fold lower than RT-PCR's LOD (**FIG. 4A** for *KRAS*^{G12D}; **FIGs. 16A-16B** for CD63). Moreover, SCOPE displayed a broader dynamic range than RT-PCR. We observed a similar enhancement in sensitivity when analyzing EVs as mRNA sources (**FIG. 4B**); the LOD by SCOPE was about 10^5 EV/mL, which was $>10^3$ -fold lower than the LOD by RT-PCR.

SCOPE also exhibited superb sequence-specificity, allowing for precise differentiation of point mutations. We chose KRAS, the most frequently mutated oncogene, as a representative target and prepared SCOPE mRNA probes for wild-type KRAS (*KRAS*^{WT}) and its mutated subtypes, *KRAS*^{G12C}, *KRAS*^{G12D}, *KRAS*^{G12S}, and *KRAS*^{G12V} (see **Table 1**). SCOPE consistently demonstrated high signal-to-background ratios between on-target and off-target samples (**FIG. 4C** and **FIG. 17**); for 1 nM target concentrations, the ratio was >30 across different KRAS targets. SCOPE's high selectivity enabled reliable analyses of samples with low variant allele fractions (VAFs). For instance, we could detect KRAS mutant alleles down to 0.01% VAF (**FIG. 4D**), a performance on par with digital PCR and sequencing^{41,42}. In contrast, RT-PCR assays displayed poor specificity due to off-target crosstalk, even when a commercial KRAS kit was used (**FIGs. 18A-18C**).

Example 5: Early Detection of Lung Cancer in an Animal Model

SCOPE's high sensitivity could facilitate EV-based early cancer detection, which was tested in a genetically engineered mouse model of lung adenocarcinoma (KP model),

characterized by Cre-induced *Kras*^{G12D+/-} *p53*^{-/-} mutation^{43,44}. This autochthonous model allowed us to regulate tumor initiation and collect blood samples at different stages of tumor development (**FIG. 5A**), an approach that effectively replicated the challenge of detecting EVs originating from early lesions.

We first validated whether EV mRNA profiling can infer cellular genotypes. For this validation, we used cell lines with different KRAS genetic backgrounds, specifically H2228 (*KRAS*^{WT/WT}), A549 (*KRAS*^{G12S/G12S}), and a well-characterized cell line derived from the KP mouse model (KP1.9; *Kras*^{G12D/WT})⁴⁵. EVs were collected from cell culture media and subjected to the SCOPE *KRAS* mRNA assay. We observed that EV profiling results consistently matched cellular KRAS status (**FIG. 5B**). EVs from KP1.9 cells exhibited high SCOPE signals for *Kras*^{WT} and *Kras*^{G12D}, reflecting the cell line's *Kras* heterozygosity. In contrast, EVs from homozygous cell lines were exclusively positive for one of the *KRAS* targets.

We next applied SCOPE to analyze EVs in mouse plasma samples. We prepared two cohorts using KP mice with identical genetic backgrounds (*Kras*^{G12D+/-}). The tumor-bearing group received an intranasal inoculation of Cre adenovirus to induce oncogene expression and initiate tumor growth, whereas the non-cancer control group did not undergo the procedure. Immunohistochemistry performed on lung tissue specimens confirmed tumor growth in the Cre-treated mice (**FIG. 5C**). Following oncogene induction, multifocal disease develops that heterogeneously progresses from atypical adenomatous hyperplasias and small adenomas into larger adenomas and eventually invasive and disseminated adenocarcinoma⁴³. Notably, *Kras*^{G12D} protein expression in lung epithelium was observed within one week after Cre induction, followed by tumor formation in the subsequent weeks (**FIG. 5C**).

For both cancer (n = 10) and control (n = 3) cohorts, we collected longitudinal blood samples and analyzed plasma EVs for *Kras*^{G12D} mRNA; such serial EV profiling was feasible because SCOPE required only 40 μ L of plasma samples. For tumor-bearing animals, we also obtained computerized tomography (CT) scans on a weekly basis. **FIG. 5D** and **FIGs. 19A-19B** display *Kras*^{G12D} SCOPE signals tracked over eight weeks. In the non-cancer mice, the signal remained at background values throughout the duration. Conversely, in tumor-bearing mice, the signal increased from the background once oncogene expression was induced and reached saturation at about five weeks of tumor growth. CT analysis performed on the same animals demonstrated tumor growth over time, although the lesions became detectable approximately two weeks after tumor induction (**FIG. 20**).

We further estimated mRNA copy numbers (**FIG. 5E** and **FIG. 21**) by referencing the SCOPE results to the titration curve (**FIG. 4A**). In cases without tumors, we observed a consistent baseline (copy number <1). However, the copy number became significantly different from the baseline as early as one week after oncogene induction ($p = 0.042$, unpaired t-test). These findings support SCOPE's potential to detect early tumors, minimal residual disease, and/or recurrences, especially in cases involving known mutations.

Example 6: Monitoring Colorectal Cancer Patients During Clinical Care

RAS is frequently mutated in colorectal cancer (CRC), with about 40% of CRC cases showing these mutations, primarily in codon 12 (65%)^{17,46}. Assessing and monitoring KRAS mutations remains crucial for prognosis evaluation and treatment decisions. SCOPE could streamline this process by enabling minimally invasive, accessible, and highly accurate KRAS profiling. To test this approach, we applied SCOPE to analyze EVs for KRAS^{WT} and major mutations in codon 12 (*KRAS*^{G12C}, *KRAS*^{G12D}, *KRAS*^{G12S}, and *KRAS*^{G12V}). The KRAS SCOPE probes were first validated with EVs derived from a panel of CRC cell lines, each representing a different KRAS codon 12 mutation status (**FIG. 22**).

Following probe validation, we proceeded to a clinical study as outlined in **FIG. 6A** (see **Table 3** for the patient information). We collected pre-surgical plasma samples from CRC patients who had not undergone any treatment. Concurrently, tissue samples were acquired and analyzed for KRAS mutations. For those patients with codon 12 KRAS mutations confirmed in tissue samples, we further examined longitudinal plasma samples collected after surgery and during standard adjuvant chemotherapy (one year). For EV isolation, we processed plasma samples through size exclusion columns. The collected EV fraction showed positive signals for established EV markers (CD63, Alix) while lacking a non-EV marker (histone H2B), indicating EV enrichment in our samples (**FIG. 23A**)⁴⁷. Furthermore, when we applied the SCOPE assay to both EV and non-EV fractions, only the EV fraction reported high SCOPE signals for mRNA targets (**FIG. 23B**). Interestingly, treating the EV isolates with RNase did not elicit significant changes to the SCOPE signal (**FIG. 23C**). Collectively, these findings strongly suggested that EV-associated mRNA was the primary source of signals detected by the SCOPE assay.

Table 3. Clinical information of cohorts involved in CRC detection.

	Non-cancer	CRC patients with KRAS mutation	CRC patients without KRAS mutation	Total

Number	15	17	90	122
Age				
Median	39	63	62	59
Range	27–47	47–72	41–73	27–73
Sex				
Male	3 (20%)	11 (65%)	47 (52%)	61 (50%)
Female	12 (80%)	6 (35%)	43 (48%)	61 (50%)
Stage				
I	--	1 (6%)	1 (1%)	2 (2%)
II	--	2 (12%)	6 (7%)	8 (7%)
III	--	13 (76%)	75 (83%)	88 (72%)
IV	--	1 (6%)	8 (9%)	9 (7%)

FIG. 6B shows the initial EV profiling results of samples from pre-surgical CRC patients ($n = 107$) and non-cancer control subjects ($n = 15$; see **FIG. 24** for the complete heatmap). The SCOPE assay identified a subgroup of CRC patients ($n = 17$) whose mutation types matched their tissue *KRAS* status. This finding was further corroborated through tissue staining (immunohistochemistry), which detected mutated *KRAS* protein in tumor tissues (**FIG. 6C** and **FIG. 25**). These observations underlined the rationale for assessing tumor *KRAS* status through EV profiling.

To evaluate the prognostic potential of *KRAS* mutation via SCOPE, we first defined the *KRAS* variant allele fraction (VAF_{KRAS}), a ratio between *KRAS* mutation ($KRAS^{MT}$) and $KRAS^{WT}$ signals. We then set a threshold for VAF_{KRAS} to determine whether a sample is positive for *KRAS* mutation. The VAF_{KRAS} data from EV samples without *KRAS* mutation (*i.e.*, non-cancer subjects and $KRAS^{WT}$ CRC patients) were pooled and fitted to a normal distribution (**FIG. 26**). We then set the threshold for *KRAS* mutation negativity as the 99th percentile of this distribution ($VAF_{KRAS} = 5.5\%$). Applying this threshold, we could effectively categorize samples by their *KRAS* mutation status (**FIG. 6D**). In samples with *KRAS* mutation, only the VAF_{KRAS} value of the mutated gene exceeded the threshold. In contrast, the values of non-mutated genes remained below it.

We further monitored the VAF_{KRAS} of the 17 patients with *KRAS* mutations. Fourteen patients were eventually deemed non-recurrent, with the remaining three considered recurrent; these were confirmed by surgical resection or radiological detection of lesions. Figure 6e summarizes the changes in VAF_{KRAS} values of these patients (see **FIG. 27** for individual patient results) as they received standard clinical care, including curative surgery and chemotherapy. For all 17 patients, VAF_{KRAS} initially decreased after surgery, presumably due to the reduction in CRC-derived EVs in circulation following tumor resection. However, a noticeable divergence emerged over time. Among the non-recurrent patients (**FIG. 6E**, left), VAF_{KRAS} continued to decrease and eventually fell below the non- $KRAS^{MT}$ threshold, aligning

with favorable clinical outcomes (tumor-free status). In contrast, VAF_{KRAS} values rebounded in recurrent patients and ultimately returned to their pre-surgery levels (**FIG. 6E**, right).

Interestingly, VAF_{KRAS} values for mutated mRNA were higher than the threshold (5.5%) in the days soon after surgery. We estimated EV clearing by modeling the VAF_{KRAS} trend in non-recurrent patients (Fig. 6f). The VAF_{KRAS} values decreased over time according to a power law ($VAF_{KRAS} \sim d^{-0.3}$), where d is the number of days after surgery. The model suggested that VAF_{KRAS} values would fall below the mutation threshold about ten days after surgery, which underscored the potential advantage of delaying EV analysis for a more accurate assessment of minimal residual disease and post-surgical prognosis. Furthermore, these analyses present an opportunity to develop an EV-based prognostic metric to guide the selection and duration of adjuvant treatment.

Example 7: Stratifying Glioma Patients

We used SCOPE to detect a panel of genetic alterations in glioma patients, specifically IDH1 mutation, EGFR amplification, and EGFRvIII deletion. Obtaining this information can be crucial to classifying gliomas and guiding effective treatment based on their molecular traits⁴⁸. For instance, patients with IDH1-WT and EGFR amplification (classified as glioblastoma IDH1-wt) are linked to poor prognoses and resistance to radiotherapy⁴⁹. On the other hand, patients with the IDH1 mutation (classified as astrocytoma or oligodendroglioma depending on other markers, including 1p19p co-deletion) exhibit prolonged overall survival and are more likely to respond to temozolomide treatment^{50,51}. The analysis of extracellular vesicles (EVs) in peripheral blood holds particular promise for gliomas, considering the risks associated with complications and morbidity from brain tissue biopsies. Analyzing EVs in peripheral blood is particularly appealing for gliomas, considering the risk of complications and morbidity from brain tissue biopsies.

To investigate EV-based glioma typing, we analyzed EVs in plasma samples taken before surgery. Results were compared to those derived from corresponding tissue samples collected during surgical removal of tumors (**FIG. 7A**). We first designed and validated a new set of SCOPE probes for mRNA targets (**FIG. 7B** and **FIGs. 28A-28D**): wild-type IDH1 ($IDH1^{WT}$) and its point mutation ($IDH1^{R132H}$) most frequently found in gliomas; WT EGFR ($EGFR^{WT}$) and $EGFRvIII$. The designed probes were highly specific (**FIG. 7C**) and enabled SCOPE to genotype different GBM cell lines through EV profiling (**FIG. 7D**).

Next, we analyzed plasma samples from 15 non-cancer controls and 60 glioma patients with different tumor subtypes (see **Table 4** for patient information). We applied

SCOPE to profile EVs for *GAPDH*, *CD63*, *IDH1^{WT}*, *IDH1^{R132H}*, *EGFR^{WT}*, and *EGFRvIII* mRNA. Given that both *EGFR^{WT}* and *EGFRvIII* were designated as target markers, we used the *GAPDH* gene as a reference for normalization purposes^{18,52}. Specifically, we scaled signals from other markers against the *GAPDH* signal rather than estimating VAFs. We also

5 molecularly assessed and classified patients' tumor specimens. The SCOPE results demonstrated the potential of robust and highly sensitive glioma typing through EV analysis (**FIG. 7E** and **FIG. 29**). We observed differential expression patterns of *EGFR^{WT}*, *IDH1^{R132H}*, and *EGFRvIII* across different glioma subtypes. For example, *EGFR^{WT}* expression in EVs was higher in GBM patients with *EGFR* amplification or *EGFRvIII* mutation than in

10 *IDH1^{R132H}* mutant patients. *IDH1^{R132H}* and *EGFRvIII* mRNA were only detected in the EVs from patients with matching mutations in tumor tissue.

Table 4. Clinical information of cohorts involved in glioma detection.

	Non-cancer	Glioma patients	Total
Number	15	60	75
Age			
Median	39	59	59
Range	27–47	27–83	27–73
Sex			
Male	3 (20%)	36 (60%)	39 (52%)
Female	12 (80%)	24 (40%)	36 (48%)
Stage			
I	--	0 (0%)	0 (0%)
II	--	1 (1.7%)	1 (1.7%)
III	--	8 (13.3%)	8 (10.6%)
IV	--	51 (85%)	51 (68%)

DISCUSSION

EVs are a promising source of cell-free RNA for liquid biopsy applications both in preclinical and clinical contexts. Nucleic acids within EVs are shielded from degradation and are more abundant than ctDNA, which renders EV RNA robust analytical targets for molecular diagnostics⁵³. Among the various EV RNA species, microRNAs are highly

20 abundant and thus more commonly studied^{23,24}. In contrast, due to its lower concentration, detecting EV mRNA often requires large sample volumes and advanced instrumentation. The SCOPE technology can overcome these limitations by improving target amplification and signal generation. i) SCOPE exhibits specificity down to a single nucleotide. This precision is achieved through an innovative concept where RNA replication is initiated solely upon

25 recognition of target RNA by Cas13a. ii) SCOPE's concurrent amplification of RNA targets and fluorescent signals leads to analytical sensitivity far surpassing other isothermal

amplification methods (see **Table 5** for comparison)⁵⁴. iii) SCOPE produces these results within a short timeframe (30 min) through a one-pot assay that can be conducted using readily available laboratory tools. These advantages would facilitate SCOPE's integration into routine preclinical and clinical applications across academic and industrial laboratories.

5

Table 5. Comparison of detection sensitivity among different isothermal amplification methods.

Method	Target	Sensitivity (copies/mL)	Enzymes and proteins	Temp (°C)	References
LAMP	DNA, RNA	10^7 – 10^9	Bst DNA polymerase, RTase	60–65	A
NASBA	DNA, RNA	10^6 – 10^9	AVM RTase, T7 RNA polymerase, RNase H	41	B
RCA	DNA, RNA	10^3 – 10^7	DNA polymerases, ligase	65	C
EXPAR	miRNA	10^6 – 10^9	DNA polymerase, NEase	60	D
RPA	DNA	10^3 – 10^6	Recombinase protein, SSB protein, DNA polymerase	37–42	E
SMART	RNA	10^4 – 10^5	RNA polymerase	41	F
SCOPE	RNA	10^2	Cas13a, T7 RNA polymerase	40	Describe herein

LAMP, loop-mediated isothermal amplification; NASBA, nucleic acid sequence-based amplification; RCA, rolling circle amplification; EXPAR, exponential amplification reaction; RPA, recombinase polymerase amplification; SMART, signal-mediated amplification of RNA technology.

A) Notomi, T., Mori, Y., Tomita, N. & Kanda, H. Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *J Microbiol* **53**, 1-5 (2015).

B) Compton, J. Nucleic acid sequence-based amplification. *Nature* **350**, 91-92 (1991).

C) Banér, J., Nilsson, M., Mendel-Hartvig, M. & Landegren, U. Signal amplification of padlock probes by rolling circle replication. *Nucleic Acids Res.* **26**, 5073-5078 (1998).

D) Van Ness, J., Van Ness, L. K. & Galas, D. J. Isothermal reactions for the amplification of oligonucleotides. *Proc. Natl. Acad. Sci. U S A* **100**, 4504-4509 (2003).

E) Piepenburg, O., Williams, C. H., Stemple, D. L. & Armes, N. A. DNA detection using recombination proteins. *PLoS Biol.* **4**, e204 (2006).

F) Hall, M. J., Wharam, S. D., Weston, A., Cardy, D. L. & Wilson, W. H. Use of signal-mediated amplification of RNA technology (SMART) to detect marine cyanophage DNA. *BioTechniques* **32**, 604-6, 608 (2002).

A key innovation of SCOPE lies in its signal template, a hybrid design that combines a cleavable, fluorescent RNA probe with a DNA template. This structure synergistically couples two enzymatic activities (Cas13a collateral cleavage and T7 polymerase reaction) and establishes a self-amplifying cycle to accelerate signal growth. Notably, this reaction is conditional — Cas13a first needs to recognize the RNA target to unlock the subsequent SCOPE reaction. This design was supported by kinetic modeling and experimental data. When Cas13a and T7 reactions were executed individually, their assay products increased linearly over time, consistent with zeroth-order kinetics. Conversely, SCOPE signals displayed exponential growth and rapidly reached saturation, closely resembling first-order rate kinetics. However, the reaction scheme may be susceptible to false positives. A potential contributing factor is the degradation of the fluorescent RNA segment in the signal template.

This segment, designed for specific cleavage by the activated Cas13a, can be degraded by other RNases present in samples. We mitigated such an effect by including a broad-spectrum RNase inhibitor in the SCOPE mixture. We also analyzed technical replicates of each sample to guard against false positives. We may consider chemically modifying the RNA segment, for instance, with phosphorothioate to enhance nuclease resistance⁵⁵. Further investigations would be necessary to evaluate Cas13a *trans*-cleavage efficacy on these modified targets.

We validated SCOPE's performance through systematic pre-clinical and clinical studies. SCOPE's limit of detection extends into the sub-attomolar ranges. Equally impressive was SCOPE's high selectivity, enabling the precise identification of point mutations. In a noteworthy example, SCOPE effectively differentiated *KRAS*^{WT} mRNA from its mutated subtypes (G12C, G12D, G12S, G12V) with minimal crosstalk, surpassing the performance of a commercial *KRAS* mutation assay. Furthermore, SCOPE detected *KRAS* mutant alleles at levels as low as 0.01% VAF, a sensitivity comparable to high-end techniques such as digital PCR and BEAMing PCR⁵⁶. The SCOPE assay achieved this sensitivity without sample partitioning and remained compatible with conventional thermal cyclers, which would enhance its accessibility to a wider research community.

SCOPE demonstrated potential for various clinical applications, including early tumor detection, recurrence monitoring, residual disease assessment, and tumor subtyping. The assays were fast (<30 min for signal generation) and cost-effective (<\$4 per marker). These findings illustrate SCOPE's versatility and affordability as a diagnostic tool to expedite clinical decision-making (same-day turnaround), track cancer recurrence/minimal residual disease, gauge responses to therapies (both conventional and experimental under trial auspices), and achieve timely pre-clinical readouts to support biological and drug development efforts. We note that the current results are pertinent to SEC-processed plasma samples, a commonly used approach across EV studies⁵⁷. We employed SEC throughout the study for consistent sample handling, but this method may co-isolate other extracellular RNA carriers (*e.g.*, lipoproteins)^{58,59} that could have contributed to the overall SCOPE signals. To examine SCOPE's robust performance across diverse use cases, further studies should evaluate SCOPE's applicability with other sample types and pre-processing methods (see **FIGs. 30A-30C**).

By employing the SCOPE technique, researchers could expand their knowledge of EV characteristics. In this work, we detected circulating EVs harboring genetic mutations that mirrored tumor tissues, thereby reaffirming EVs' potential as biomarkers. Longitudinal monitoring yielded further insights into EV dynamics. i) Mutation targets in EVs

progressively increased during tumor initiation and growth. ii) EV mutational loads declined from pre-surgery values following curative surgery in CRC patients. This decline persisted in patients responsive to chemotherapy and receded to the background value. These observations contrasted with non-responsive CRC patients whose EV mutational loads stayed above the background value. These results underscore EVs' potential utility for tumor monitoring, akin to the capabilities demonstrated with ctDNA^{60,61}. We also note unexpected observations that warrant further investigation. Within a week of CRC surgery, EV mutational loads may remain elevated, potentially due to residual microscopic tumors or the release of tumor-derived materials during surgery. In our pilot study, this uncertainty resolved approximately ten days after surgery, a potentially optimal window to begin EV analysis to inform post-surgery prognoses and shape the type and duration of adjuvant interventions. SCOPE can be used to analyze diverse tumor types and multimodal treatment contexts. This could establish cancer-specific timeframes for optimal EV analysis, furthering SCOPE's utility in treatment monitoring and prognostication.

Another potent strategy entails analyzing both EV mRNA and ctDNA in plasma to obtain maximal molecular information. EV mRNA possesses the advantage of higher abundance compared to ctDNA, thereby improving sensitivity for cancer diagnostics⁵⁶. Detecting EV mRNA can also be practical for certain targets. For example, *EGFRvIII* deletion involves variable breakpoints in the *EGFR* gene⁶², which complicates the design of specific ctDNA assays. Alternatively, EV mRNA analysis can target the conserved *EGFRvIII* transcript sequence, facilitating a more straightforward assay. In comparison, ctDNA analyses can provide molecular insights unavailable at the transcript level, such as promoter alterations⁶³ and methylation patterns⁶⁴, which can enhance diagnostic accuracy and indicate the tumor's tissue of origin. Conveniently, both EV nucleic acids and ctDNA can be extracted from plasma using the same isolation process to expedite these synergistic analyses⁵⁶.

We envision further technical improvements to enhance SCOPE's preclinical and clinical impact. One straightforward approach is to encompass other major mutations for wider cancer coverage^{65,66}. These targets could include *PIK3CA* (H1047R; E545K), *TP53* (R175H; R273C; G245S), *NRAS* (Q61R; Q61K), *BRAF* (V600E; V600K; V600R), and *EGFR* (L858R; E746_A750del). Additionally, the panel could be tailored for treatment-related targets, such as *MGMT* for temozolomide resistance^{18,67}, *EGFR* (T790M) for resistance to EGFR tyrosine kinase inhibitors (TKIs)⁶⁸, and *FGFR3* (R248C; S249C; G370C; Y373C) for sensitivity to FGFR TKI treatments⁶⁹. SCOPE's high selectivity will help design these probes. In addition, the assay's "one-target per tube" format will effectively eliminate

potential crosstalk between probes within a reaction vessel; probe testing can thus focus on attaining similar analytical performance among different probes. For example, we prepared *BRAF* mutation probes (**FIGs. 31A-31B**) within two weeks, demonstrating the assay's efficiency for rapid probe development.

We can also incorporate protein detection into the assay workflow, capitalizing on EVs' inherent advantage as a multi-cargo carrier. Detecting proteins will complement mRNA analyses, establishing greater connections between tumor genotypes and phenotypes. Protein markers can offer additional insights into tumors' tissue of origin⁷⁰, crucial information often difficult to obtain solely from driver gene mutations. We propose using aptamers or DNA-
barcoded antibodies as affinity ligands, which will make protein assays compatible with SCOPE. We can further explore employing orthogonal Cas proteins that exhibit distinct preferences for motif sequences during *trans*-cleavage⁷¹. By designing signal templates that are fluorescently distinguishable and compatible with each Cas variant, it would be feasible to detect multiple targets (4 to 6) in a single reaction. In addition, adapting SCOPE to the digital PCR format (*i.e.*, sample partitioning) would be an intriguing direction for absolute target quantification and further enhancements in VAF sensitivity. With these advancements, SCOPE would be positioned as a powerful liquid biopsy tool for precision oncology, enabling comprehensive and reliable molecular characterization of tumors in a single, cohesive, and accessible platform.

REFERENCES

1. Ignatiadis, M., Sledge, G. W. & Jeffrey, S. S. Liquid biopsy enters the clinic - implementation issues and future challenges. *Nat. Rev. Clin. Oncol.* **18**, 297-312 (2021).
2. Heitzer, E., Haque, I. S., Roberts, C. E. S. & Speicher, M. R. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat. Rev. Genet.* **20**, 71-88 (2019).
3. Killingsworth, B., Welsh, J. A. & Jones, J. C. EV Translational Horizons as Viewed Across the Complex Landscape of Liquid Biopsies. *Front Cell Dev. Biol.* **9**, 556837 (2021).
4. Parikh, A. R. et al. Liquid versus tissue biopsy for detecting acquired resistance and tumor heterogeneity in gastrointestinal cancers. *Nat. Med.* **25**, 1415-1421 (2019).
5. Pantel, K. & Alix-Panabières, C. Liquid biopsy and minimal residual disease - latest advances and implications for cure. *Nat. Rev. Clin. Oncol.* **16**, 409-424 (2019).
6. Siravegna, G., Marsoni, S., Siena, S. & Bardelli, A. Integrating liquid biopsies into the management of cancer. *Nat. Rev. Clin. Oncol.* **14**, 531-548 (2017).
7. Verweij, F. J. et al. The power of imaging to understand extracellular vesicle biology in vivo. *Nat. Methods* **18**, 1013-1026 (2021).

8. Shao, H. et al. New Technologies for Analysis of Extracellular Vesicles. *Chem. Rev.* **118**, 1917-1950 (2018).
9. Im, H. et al. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. *Nat. Biotechnol.* **32**, 490-495 (2014).
- 5 10. Jo, A. et al. Inaugurating High-Throughput Profiling of Extracellular Vesicles for Earlier Ovarian Cancer Detection. *Adv. Sci. (Weinh)* **10**, e2301930 (2023).
11. Dixon, A. C., Dawson, T. R., Di Vizio, D. & Weaver, A. M. Context-specific regulation of extracellular vesicle biogenesis and cargo selection. *Nat. Rev. Mol. Cell Biol.* **24**, 454-476 (2023).
- 10 12. Skog, J. et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* **10**, 1470-1476 (2008).
13. Melo, S. A. et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* **523**, 177-182 (2015).
- 15 14. Choi, D. et al. Mapping Subpopulations of Cancer Cell-Derived Extracellular Vesicles and Particles by Nano-Flow Cytometry. *ACS Nano* **13**, 10499-10511 (2019).
15. Hu, J. et al. Overhang molecular beacons encapsulated in tethered cationic lipoplex nanoparticles for detection of single-point mutation in extracellular vesicle-associated RNAs. *Biomaterials* **183**, 20-29 (2018).
- 20 16. Vitale, S. R. et al. Detection of tumor-derived extracellular vesicles in plasma from patients with solid cancer. *BMC Cancer* **21**, 315 (2021).
17. van de Haar, J. et al. Codon-specific KRAS mutations predict survival benefit of trifluridine/tipiracil in metastatic colorectal cancer. *Nat. Med.* **29**, 605-614 (2023).
18. Shao, H. et al. Chip-based analysis of exosomal mRNA mediating drug resistance in glioblastoma. *Nat. Commun.* **6**, 6999 (2015).
- 25 19. Park, J. et al. An integrated magneto-electrochemical device for the rapid profiling of tumour extracellular vesicles from blood plasma. *Nat. Biomed. Eng.* **5**, 678-689 (2021).
20. Noerholm, M. et al. RNA expression patterns in serum microvesicles from patients with glioblastoma multiforme and controls. *BMC Cancer* **12**, 22 (2012).
- 30 21. Chen, Y. et al. Exosome detection via the ultrafast-isolation system: EXODUS. *Nat. Methods* **18**, 212-218 (2021).
22. Wei, Z. et al. Coding and noncoding landscape of extracellular RNA released by human glioma stem cells. *Nat. Commun.* **8**, 1145 (2017).
23. Hong, J. S., Son, T., Castro, C. M. & Im, H. CRISPR/Cas13a-Based MicroRNA Detection in Tumor-Derived Extracellular Vesicles. *Adv. Sci. (Weinh)* **10**, e2301766 (2023).
- 35 24. Yan, H. et al. A one-pot isothermal Cas12-based assay for the sensitive detection of microRNAs. *Nat. Biomed. Eng.* **7**, 1583-1601 (2023).
25. Ramshani, Z. et al. Extracellular vesicle microRNA quantification from plasma using an integrated microfluidic device. *Commun. Biol.* **2**, 189 (2019).
- 40 26. Li, Y. et al. EV-origin: Enumerating the tissue-cellular origin of circulating extracellular vesicles using exLR profile. *Comput. Struct. Biotechnol. J.* **18**, 2851-2859 (2020).
27. Pickar-Oliver, A. & Gersbach, C. A. The next generation of CRISPR-Cas technologies and applications. *Nat. Rev. Mol. Cell Biol.* **20**, 490-507 (2019).
- 45

28. Kaminski, M. M., Abudayyeh, O. O., Gootenberg, J. S., Zhang, F. & Collins, J. J. CRISPR-based diagnostics. *Nat. Biomed. Eng.* **5**, 643-656 (2021).
29. Moon, J. & Liu, C. Asymmetric CRISPR enabling cascade signal amplification for nucleic acid detection by competitive crRNA. *Nat. Commun.* **14**, 7504 (2023).
- 5 30. Liu, T. Y. et al. Accelerated RNA detection using tandem CRISPR nucleases. *Nat. Chem. Biol.* **17**, 982-988 (2021).
31. Bruch, R., Urban, G. A. & Dincer, C. CRISPR/Cas Powered Multiplexed Biosensing. *Trends Biotechnol.* **37**, 791-792 (2019).
32. Abudayyeh, O. O. & Gootenberg, J. S. CRISPR diagnostics. *Science* **372**, 914-915
10 (2021).
33. Potapov, V. & Ong, J. L. Examining Sources of Error in PCR by Single-Molecule Sequencing. *PLoS One* **12**, e0169774 (2017).
34. Kebschull, J. M. & Zador, A. M. Sources of PCR-induced distortions in high-throughput sequencing data sets. *Nucleic Acids Res.* **43**, e143 (2015).
- 15 35. Gootenberg, J. S. et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* **356**, 438-442 (2017).
36. Truong, N. P., Jia, Z., Burges, M., McMillan, N. A. & Monteiro, M. J. Self-catalyzed degradation of linear cationic poly(2-dimethylaminoethyl acrylate) in water. *Biomacromolecules* **12**, 1876-1882 (2011).
- 20 37. Martin, C. T. & Coleman, J. E. Kinetic analysis of T7 RNA polymerase-promoter interactions with small synthetic promoters. *Biochemistry* **26**, 2690-2696 (1987).
38. Maslak, M. & Martin, C. T. Kinetic analysis of T7 RNA polymerase transcription initiation from promoters containing single-stranded regions. *Biochemistry* **32**, 4281-4285 (1993).
- 25 39. Avaro, A. S. & Santiago, J. G. Uncertainty Quantification of Michaelis-Menten Kinetic Rates and Its Application to the Analysis of CRISPR-Based Diagnostics. *Angew. Chem. Int. Ed. Engl.* **61**, e202209527 (2022).
40. Huyke, D. A. et al. Enzyme Kinetics and Detector Sensitivity Determine Limits of Detection of Amplification-Free CRISPR-Cas12 and CRISPR-Cas13 Diagnostics. *Anal. Chem.* **94**, 9826-9834 (2022).
30
41. Newman, A. M. et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat. Med.* **20**, 548-554 (2014).
42. Diehl, F. et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc. Natl. Acad. Sci. USA* **102**, 16368-16373 (2005).
- 35 43. DuPage, M., Dooley, A. L. & Jacks, T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat. Protoc.* **4**, 1064-1072 (2009).
44. Li, R. et al. Therapeutically reprogrammed nutrient signalling enhances nanoparticulate albumin bound drug uptake and efficacy in KRAS-mutant cancer. *Nat. Nanotechnol.* **16**, 830-839 (2021).
- 40 45. Pfirschke, C. et al. Immunogenic Chemotherapy Sensitizes Tumors to Checkpoint Blockade Therapy. *Immunity* **44**, 343-354 (2016).
46. Mendiratta, G. et al. Cancer gene mutation frequencies for the U.S. population. *Nat. Commun.* **12**, 5961 (2021).

47. Welsh, J. A. et al. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *J. Extracell. Vesicles* **13**, e12404 (2024).
- 5 48. Weller, M. et al. EANO guidelines on the diagnosis and treatment of diffuse gliomas of adulthood. *Nat. Rev. Clin. Oncol.* **18**, 170-186 (2021).
49. Guo, G. et al. Ligand-Independent EGFR Signaling. *Cancer Res.* **75**, 3436-3441 (2015).
50. Chen, W. W. et al. BEAMing and Droplet Digital PCR Analysis of Mutant IDH1 mRNA in Glioma Patient Serum and Cerebrospinal Fluid Extracellular Vesicles. *Mol. Ther. Nucleic Acids* **2**, e109 (2013).
- 10 51. SongTao, Q. et al. IDH mutations predict longer survival and response to temozolomide in secondary glioblastoma. *Cancer Sci.* **103**, 269-273 (2012).
52. Batool, S. M. et al. Highly Sensitive EGFRvIII Detection in Circulating Extracellular Vesicle RNA of Glioma Patients. *Clin. Cancer Res.* **28**, 4070-4082 (2022).
- 15 53. Zhou, J. et al. High-throughput single-EV liquid biopsy: Rapid, simultaneous, and multiplexed detection of nucleic acids, proteins, and their combinations. *Sci. Adv.* **6**, eabc1204 (2020).
54. Srivastava, P. & Prasad, D. Isothermal nucleic acid amplification and its uses in modern diagnostic technologies. *3 Biotech.* **13**, 200 (2023).
- 20 55. Ueda, T., Tohda, H., Chikazumi, N., Eckstein, F. & Watanabe, K. Phosphorothioate-containing RNAs show mRNA activity in the prokaryotic translation systems in vitro. *Nucleic Acids Res.* **19**, 547-552 (1991).
56. Krug, A. K. et al. Improved EGFR mutation detection using combined exosomal RNA and circulating tumor DNA in NSCLC patient plasma. *Ann. Oncol.* **29**, 700-706 (2018).
- 25 57. Lane, R. E., Korbie, D., Trau, M. & Hill, M. M. Optimizing Size Exclusion Chromatography for Extracellular Vesicle Enrichment and Proteomic Analysis from Clinically Relevant Samples. *Proteomics* **19**, e1800156 (2019).
58. Woo, H. K. et al. Characterization and modulation of surface charges to enhance extracellular vesicle isolation in plasma. *Theranostics* **12**, 1988-1998 (2022).
- 30 59. Simonsen, J. B. What Are We Looking At? Extracellular Vesicles, Lipoproteins, or Both? *Circ. Res.* **121**, 920-922 (2017).
60. Schraa, S. J. et al. Circulating tumor DNA guided adjuvant chemotherapy in stage II colon cancer (MEDOCC-CrEATE): study protocol for a trial within a cohort study. *BMC Cancer* **20**, 790 (2020).
- 35 61. Tie, J. et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. *Sci. Transl. Med.* **8**, 346ra92 (2016).
62. Koga, T. et al. Mapping of genomic EGFRvIII deletions in glioblastoma: insight into rearrangement mechanisms and biomarker development. *Neuro. Oncol.* **20**, 1310-1320 (2018).
- 40 63. Muralidharan, K. et al. TERT Promoter Mutation Analysis for Blood-Based Diagnosis and Monitoring of Gliomas. *Clin. Cancer Res.* **27**, 169-178 (2021).
64. Shen, S. Y. et al. Sensitive tumour detection and classification using plasma cell-free DNA methylomes. *Nature* **563**, 579-583 (2018).
- 45 65. Chang, M. T. et al. Identifying recurrent mutations in cancer reveals widespread lineage diversity and mutational specificity. *Nat. Biotechnol.* **34**, 155-163 (2016).

66. Miller, M. L. et al. Pan-Cancer Analysis of Mutation Hotspots in Protein Domains. *Cell Syst.* **1**, 197-209 (2015).
67. Wick, W. et al. MGMT testing--the challenges for biomarker-based glioma treatment. *Nat. Rev. Neurol.* **10**, 372-385 (2014).
- 5 68. Sequist, L. V. et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci. Transl. Med.* **3**, 75ra26 (2011).
69. Krook, M. A. et al. Fibroblast growth factor receptors in cancer: genetic alterations, diagnostics, therapeutic targets and mechanisms of resistance. *Br. J. Cancer* **124**, 880-892 (2021).
- 10 70. Cohen, J. D. et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* **359**, 926-930 (2018).
71. Gootenberg, J. S. et al. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science* **360**, 439-444 (2018).

15

OTHER EMBODIMENTS

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

20

What Is Claimed Is:

1. A method of detecting a target nucleic acid sequence, the method comprising:
providing a sample;
contacting the sample with a RNA-guided Cas protein; a CRISPR guide RNA (crRNA), wherein the crRNA comprises a nucleic acid sequence that is sufficiently complementary to a target nucleic acid sequence; a polymerase; and a signal template, wherein the signal template comprises a polymerase promoter sequence, a RNA sequence comprising a Cas protein cleavable sequence, and a DNA sequence that is sufficiently complementary to the target nucleic acid sequence, wherein the polymerase promoter sequence comprises an acceptor moiety and the RNA sequence comprises a donor moiety, or vice versa;
incubating the sample under an isothermal amplification condition and for a time sufficient for nucleic acid amplification; and
detecting a signal from the donor moiety.
2. The method of claim 1, wherein the signal from the donor moiety is indicative of a level of the target nucleic acid sequence in the sample.
3. The method of claim 1 or claim 2, wherein the target nucleic acid sequence comprises a mutation.
4. The method of claim 3, wherein the mutation is an insertion, a deletion, a substitution, or a fusion.
5. The method of claim 3, wherein the mutation is a single nucleotide polymorphism.
6. The method of any one of claims 1-5, wherein the target nucleic acid sequence comprises messenger RNA (mRNA).
7. The method of any one of claims 1-6, wherein the target nucleic acid sequence comprises a nucleic acid sequence from a gene selected from GAPDH, CD63, KRAS, IDH, EGFR, and BRAF.

8. The method of any one of claims 1-7, wherein the target nucleic acid sequence comprises a nucleic acid sequence selected from SEQ ID NOs:2-16.
9. The method of any one of claims 1-8, wherein the Cas protein comprises Cas13a or Cas13b.
10. The method of any one of claims 1-9, wherein the Cas protein comprises an amino acid sequence having at least 80% identity to SEQ ID NO:1.
11. The method of any one of claims 1-10, wherein the Cas protein comprises SEQ ID NO:1.
12. The method of any one of claims 1-11, wherein the crRNA comprises a nucleic acid sequence selected from SEQ ID NOs:17-31.
13. The method of any one of claims 1-12, wherein the polymerase comprises T7 RNA polymerase or SP6 RNA polymerase.
14. The method of any one of claims 1-13, wherein the signal template comprises a nucleic acid sequence selected from SEQ ID NOs:32-46.
15. The method of any one of claims 1-14, wherein the donor moiety and the acceptor moiety comprise a fluorescence resonance energy transfer (FRET) pair or a chemiluminescence resonance energy transfer (CRET) pair.
16. The method of any one of claims 1-15, wherein the donor moiety and the acceptor moiety are positioned to undergo FRET or CRET prior to cleavage of the Cas protein cleavable sequence.
17. The method of any one of claims 1-16, wherein the donor moiety is a fluorophore donor.

18. The method of claim 17, wherein the fluorophore donor is selected from the group consisting of Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 647, Cy2, Cy3, BODIPY, GFP, fluorescein, IEDANS, EDANS, and a lanthanide metal.
19. The method of any one of claims 1-18, wherein the acceptor moiety is a fluorophore acceptor or a fluorescence quencher.
20. The method of claim 19, wherein the fluorescence quencher is selected from the group consisting of black hole quencher (BHQ), deep dark quencher (DDQ), Eclipse quencher, Dabcyl, QSY quencher, Iowa Black FQ, Iowa Black RQ, ZEN Quencher, and TAMRA.
21. The method of any one of claims 1-20, wherein the isothermal amplification condition comprises a temperature of about 35 to 45 °C.
22. The method of any one of claims 1-21, wherein the sample is a biological sample obtained from a subject.
23. The method of claim 22, wherein the subject has a cancer.
24. The method of any one of claims 1-23, wherein the sample comprises blood, plasma, serum, urine, nasal secretions, or a combination thereof.
25. The method of any one of claims 1-24, wherein the sample comprises extracellular vesicles (EVs).
26. The method of any one of claims 1-25, further comprising, prior to the providing: isolating EVs and extracting RNA.
27. The method of any one of claims 1-26, wherein the sample further comprises 3'-amino-2',3'-dideoxyribonucleotide 5'-triphosphates (nNTPs), a divalent ion, a buffer, a salt, or a combination of any of these.

28. The method of any one of claims 1-27, wherein binding of the Cas protein and the crRNA forms a crRNA/Cas complex, and wherein binding of the crRNA/Cas complex to the target nucleic acid sequence activates a trans-cleavage activity of the Cas protein in the crRNA/Cas complex.
29. A method for treating a subject having a cancer, the method comprising:
detecting a level of the target nucleic acid sequence in the sample according to the method of any one of claims 1-28;
comparing the level of the target nucleic acid sequence in the sample to a reference level; and
administering a treatment if the level of the target nucleic acid sequence in the sample is equal to or higher than the reference level.
30. The method of claim 29, wherein the cancer comprises a solid tumor, renal cell carcinoma, anal cancer, bladder cancer, brain cancer, breast cancer, cervical cancer, colorectal cancer, endometrial cancer, esophageal cancer, gastric cancer, glioblastoma, glioma, kidney cancer, liver cancer, lung cancer, lymphoma, melanoma, myeloma, neuroblastoma, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma, skin cancer, thyroid cancer, urothelial cancer, uterine cancer, or a combination thereof.
31. The method of claim 29 or claim 30, wherein the treatment comprises a chemotherapy, an immune checkpoint inhibitor, surgery, radiation, or a combination thereof.
32. A kit comprising:
a CRISPR guide RNA (crRNA), wherein the crRNA comprises a nucleic acid sequence that is sufficiently complementary to a target nucleic acid sequence;
a signal template, wherein the signal template comprises a polymerase promoter sequence, a RNA sequence comprising a Cas protein cleavable sequence, and a DNA sequence that is sufficiently complementary to the target nucleic acid sequence, wherein the polymerase promoter sequence comprises an acceptor moiety and the RNA sequence comprises a donor moiety, or vice versa; and
instructions for performing the method of any one of claims 1-31.

33. The kit of claim 32, further comprising a RNA-guided Cas protein and a polymerase.
34. The kit of claim 32 or claim 33, wherein the crRNA comprises a nucleic acid sequence selected from SEQ ID NOs:17-31.
35. The kit of any one of claims 32-34, wherein the signal template comprises a nucleic acid sequence selected from SEQ ID NOs:32-46.

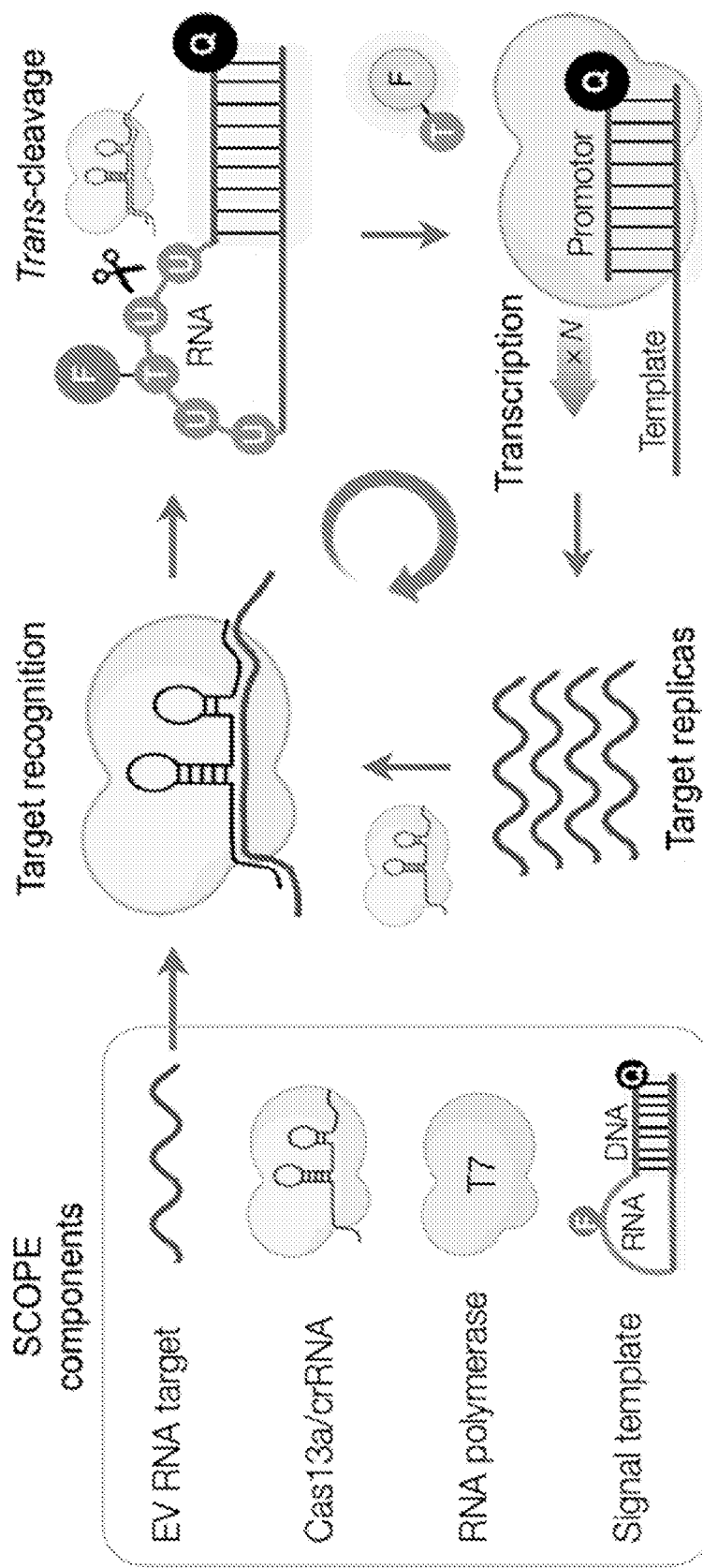


FIG. 1A

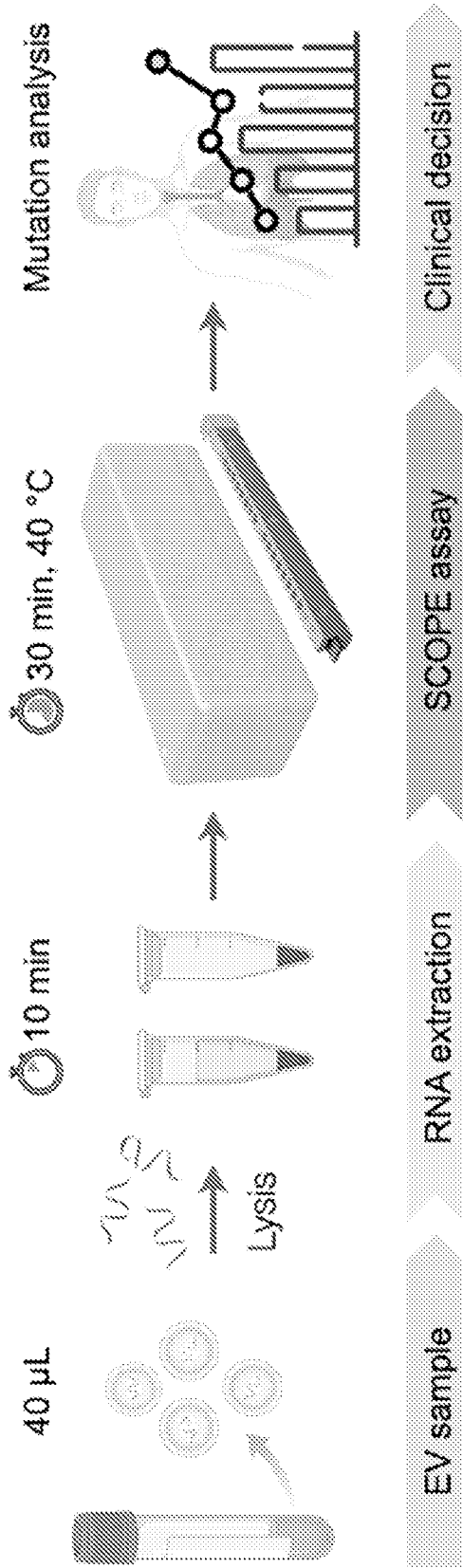


FIG. 1B

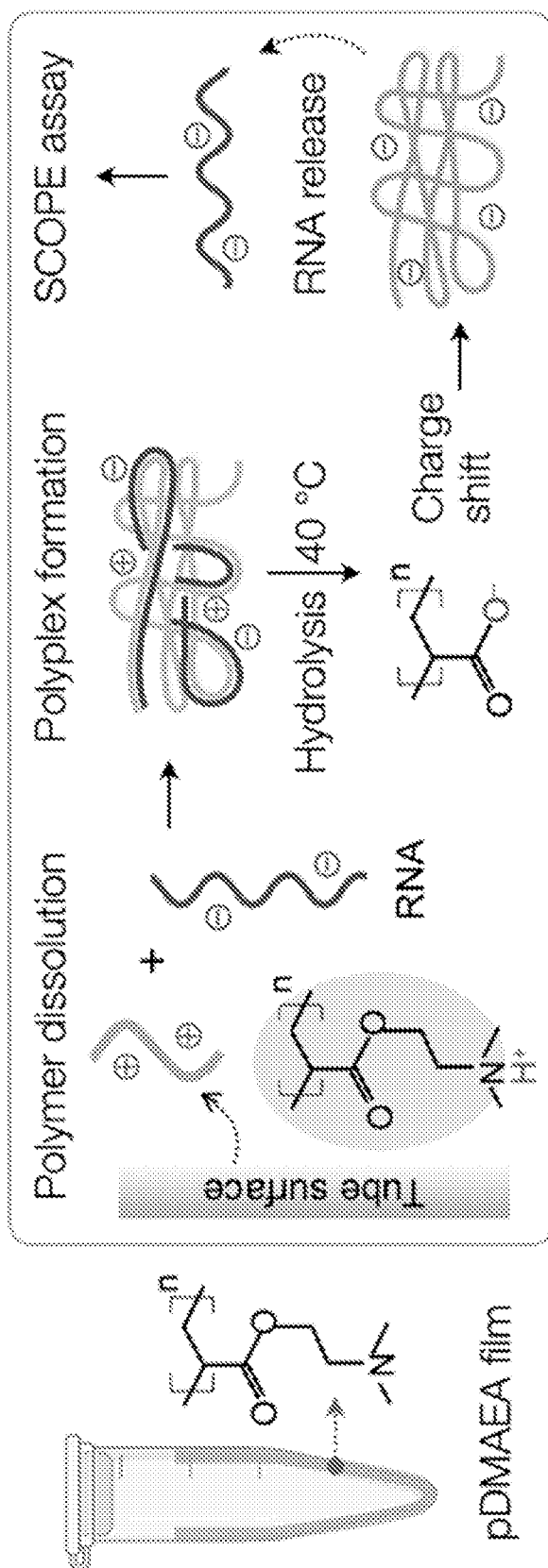


FIG. 2A

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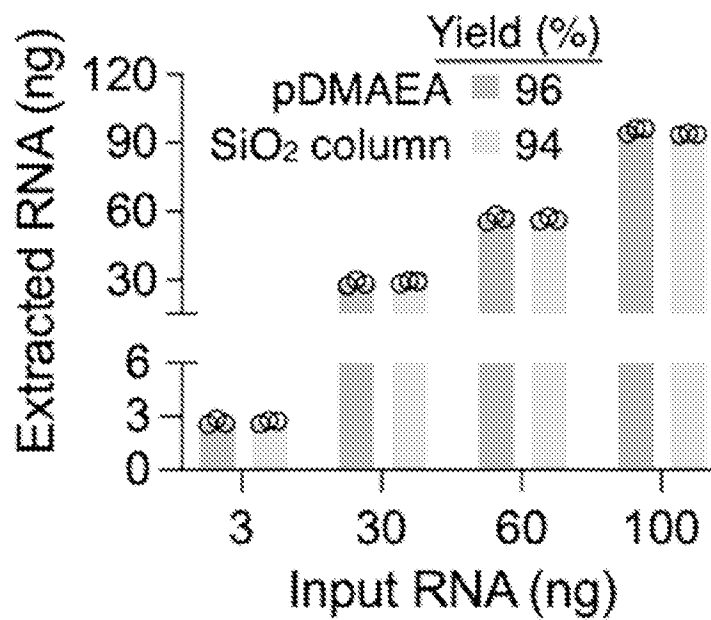


FIG. 2B

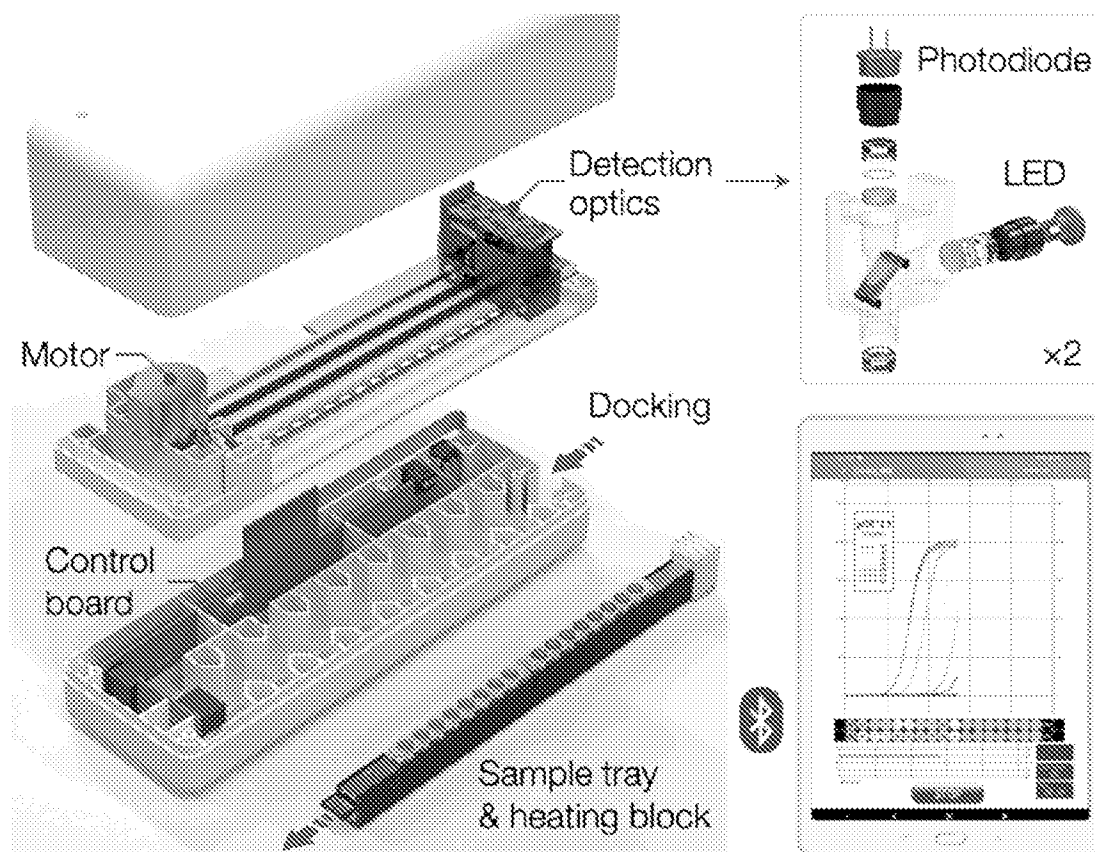


FIG. 2C

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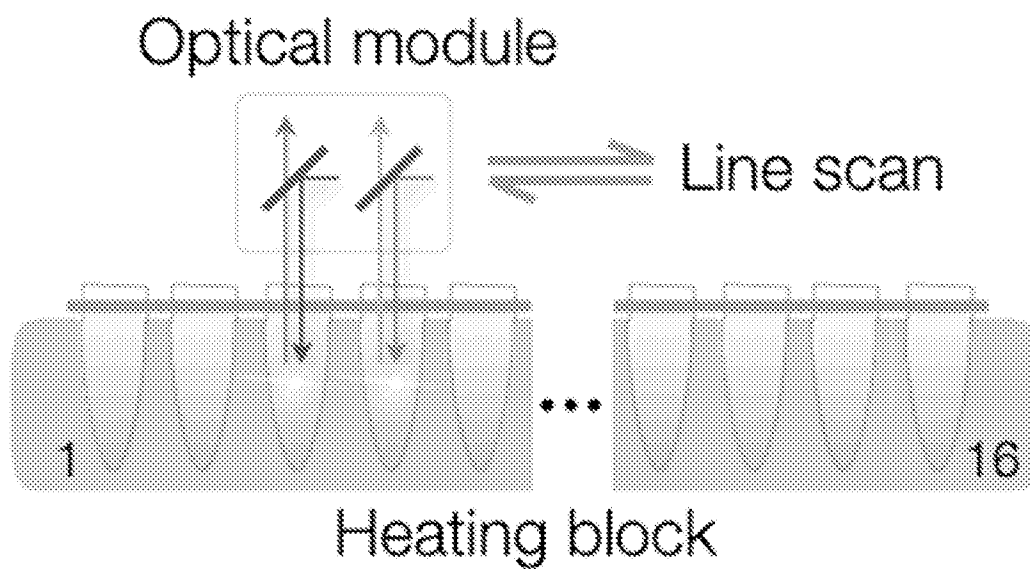


FIG. 2D

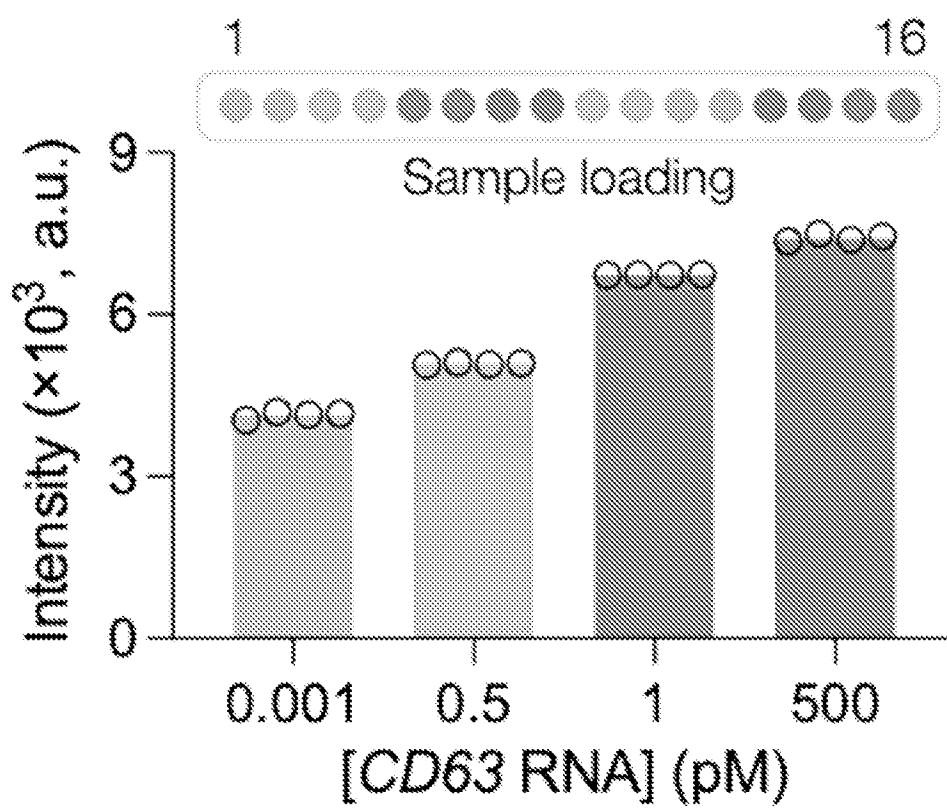


FIG. 2E

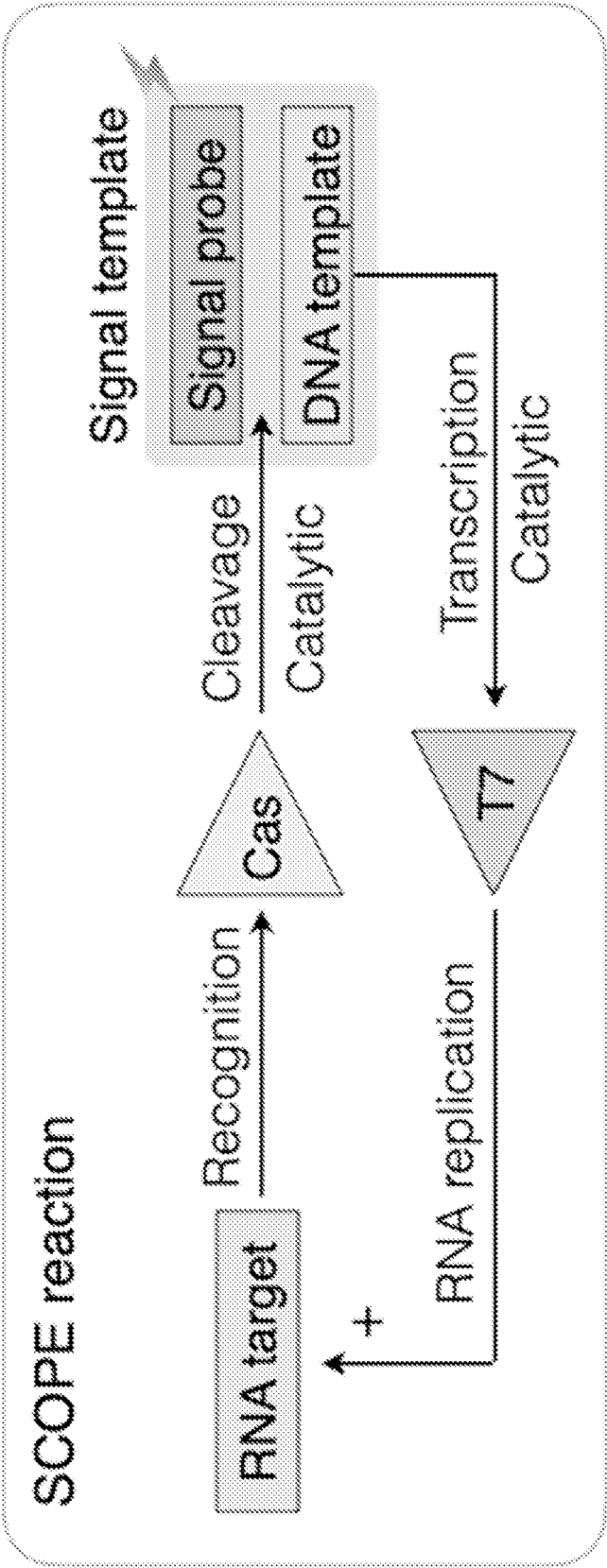


FIG. 3A

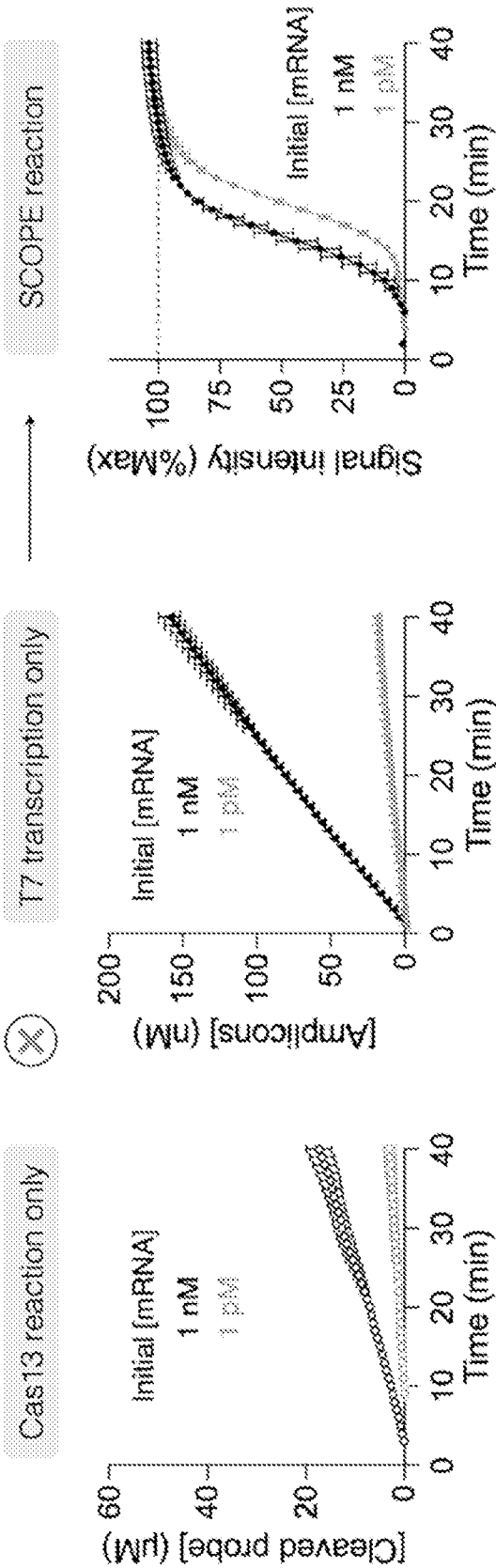


FIG. 3B

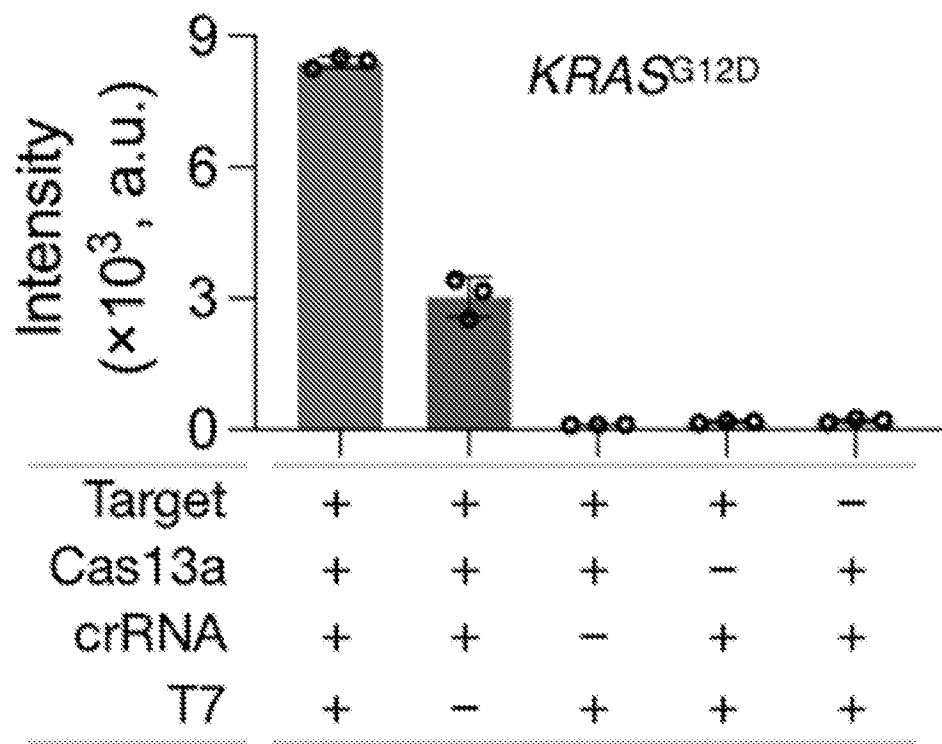


FIG. 3C

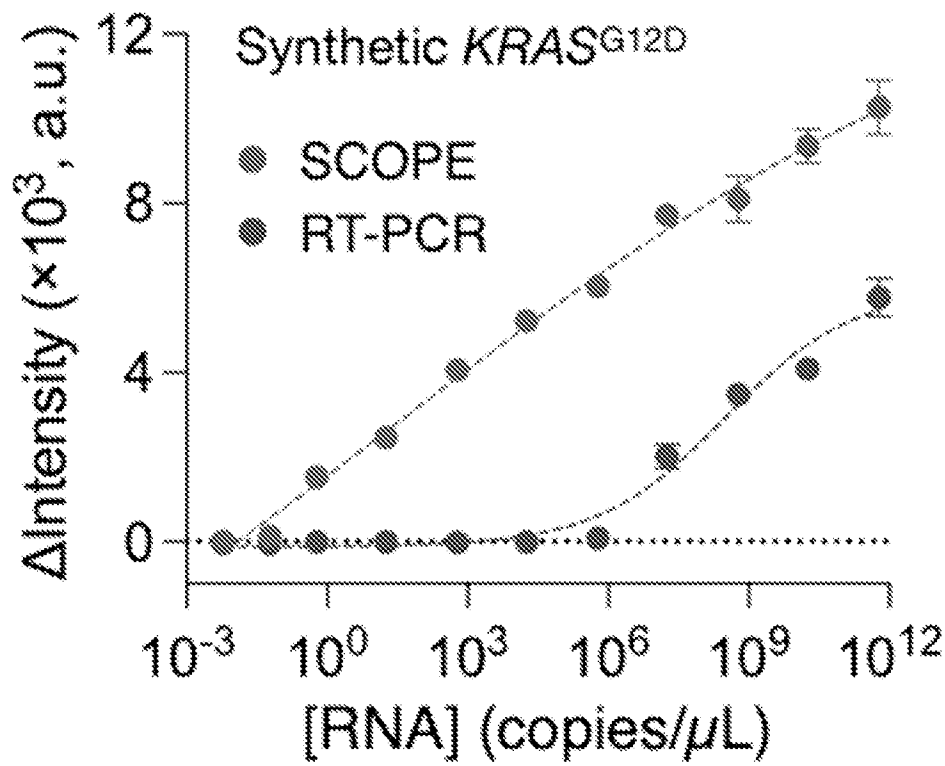


FIG. 4A

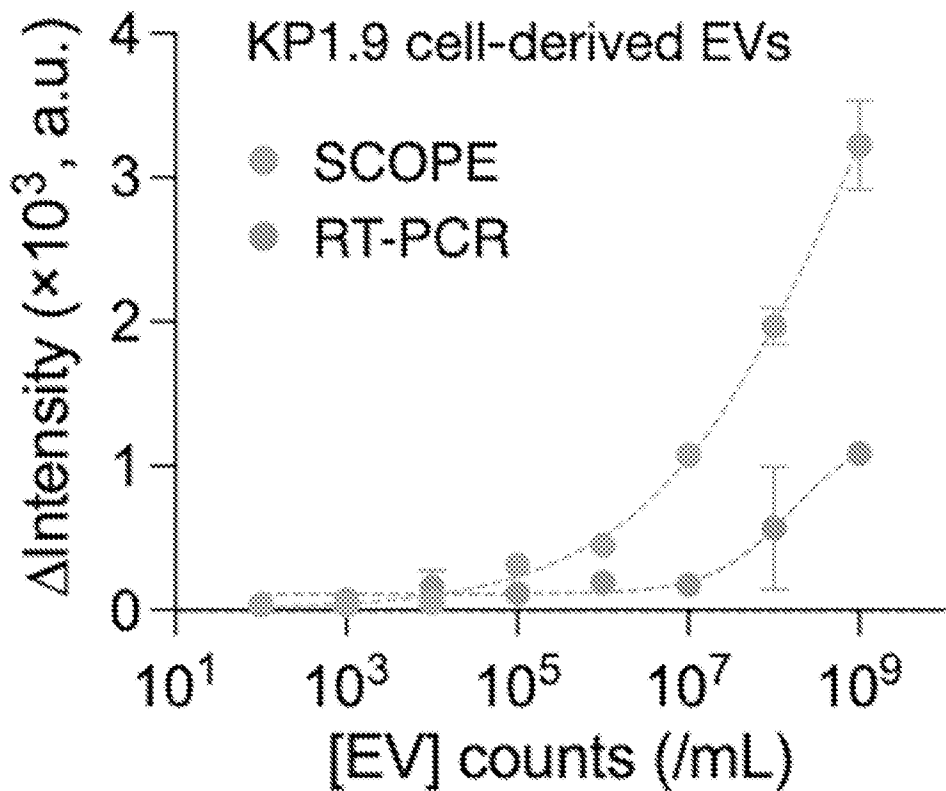


FIG. 4B

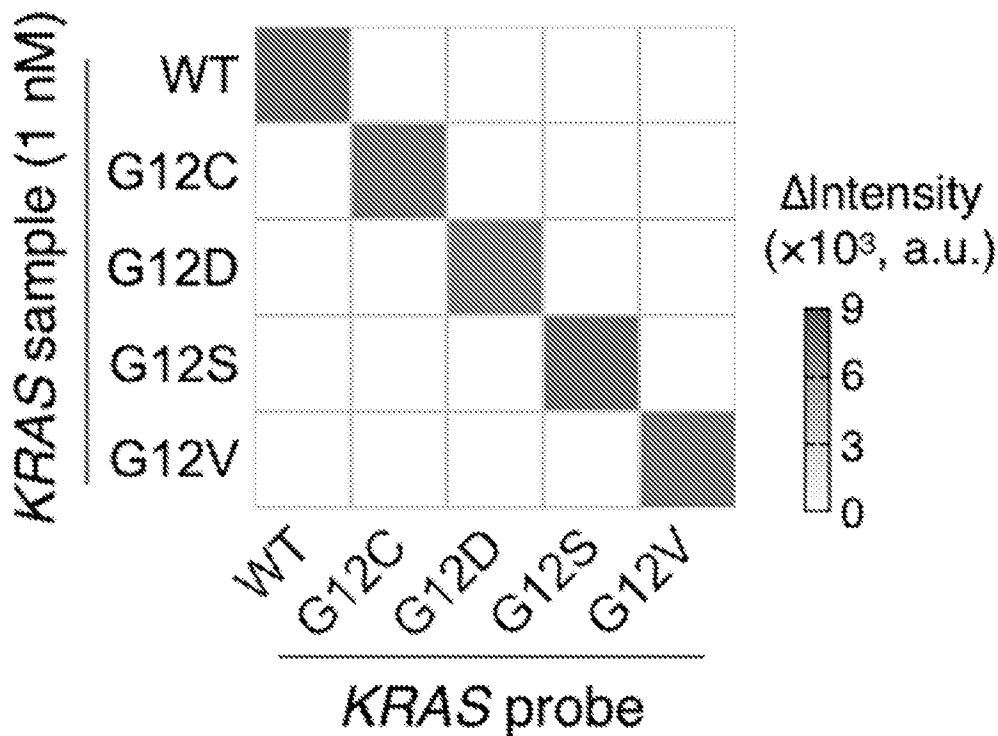


FIG. 4C

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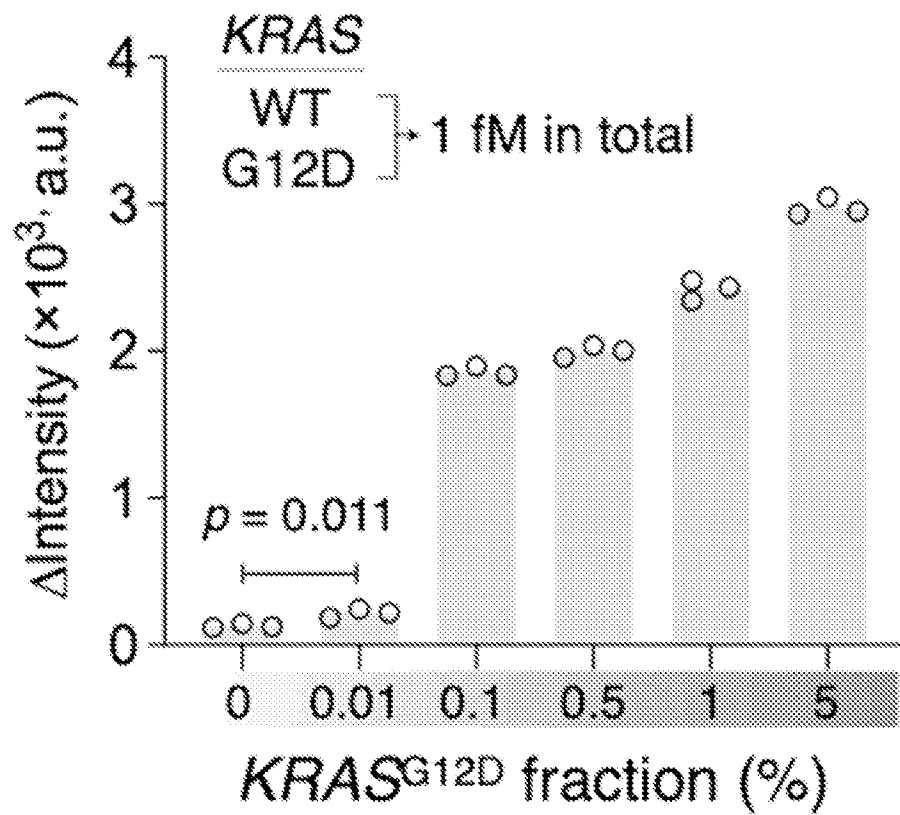


FIG. 4D

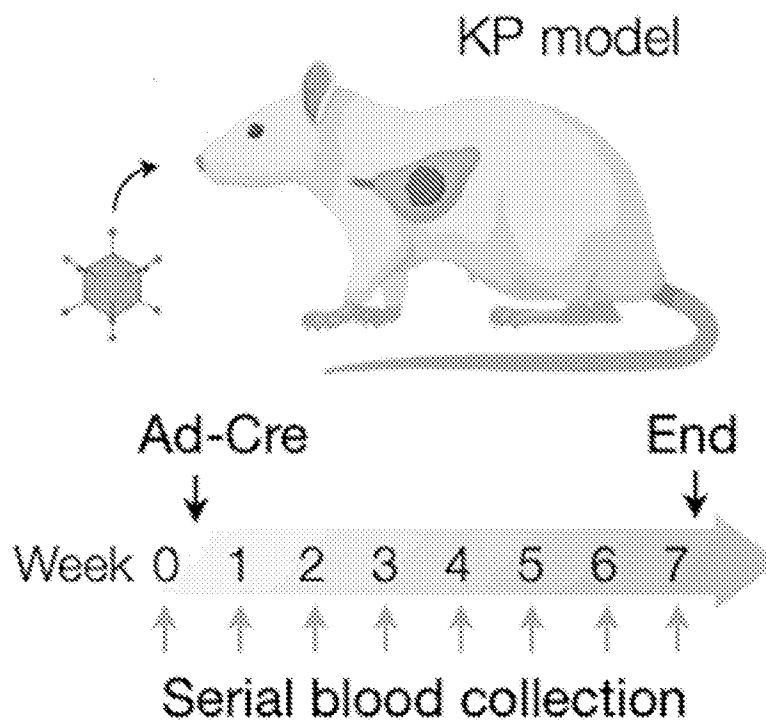


FIG. 5A

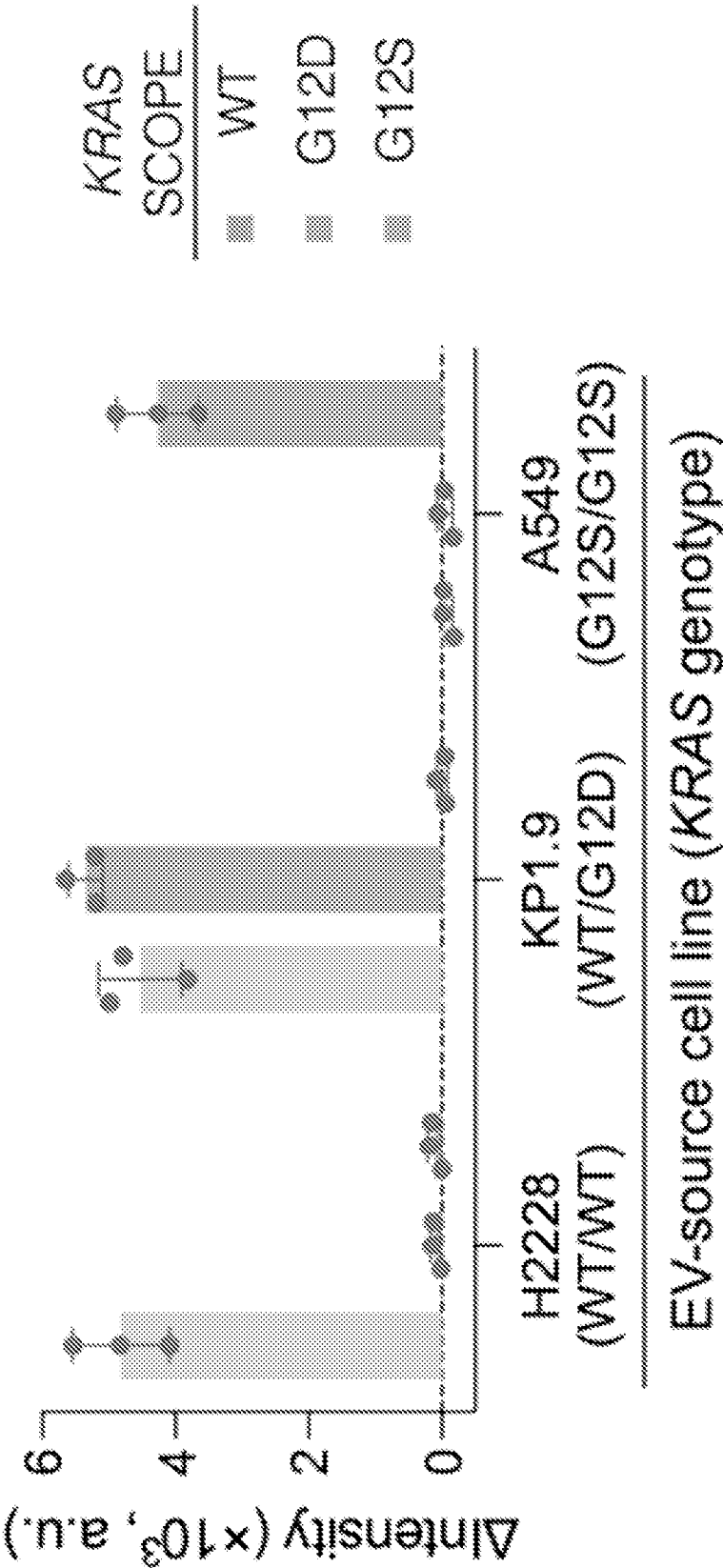


FIG. 5B

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FIG. 5C

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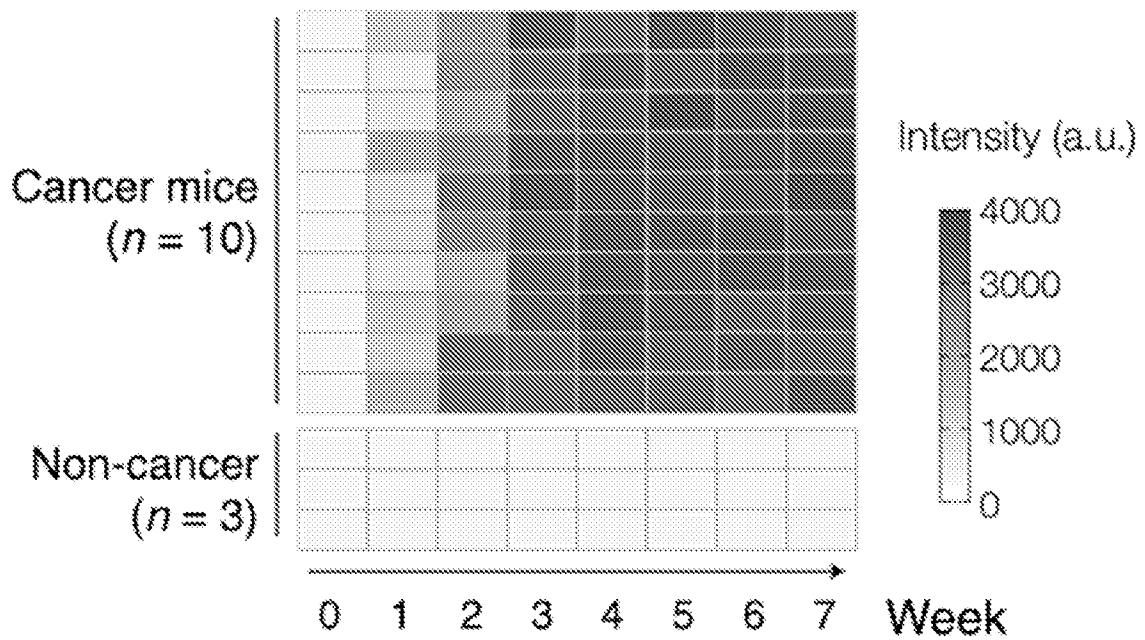


FIG. 5D

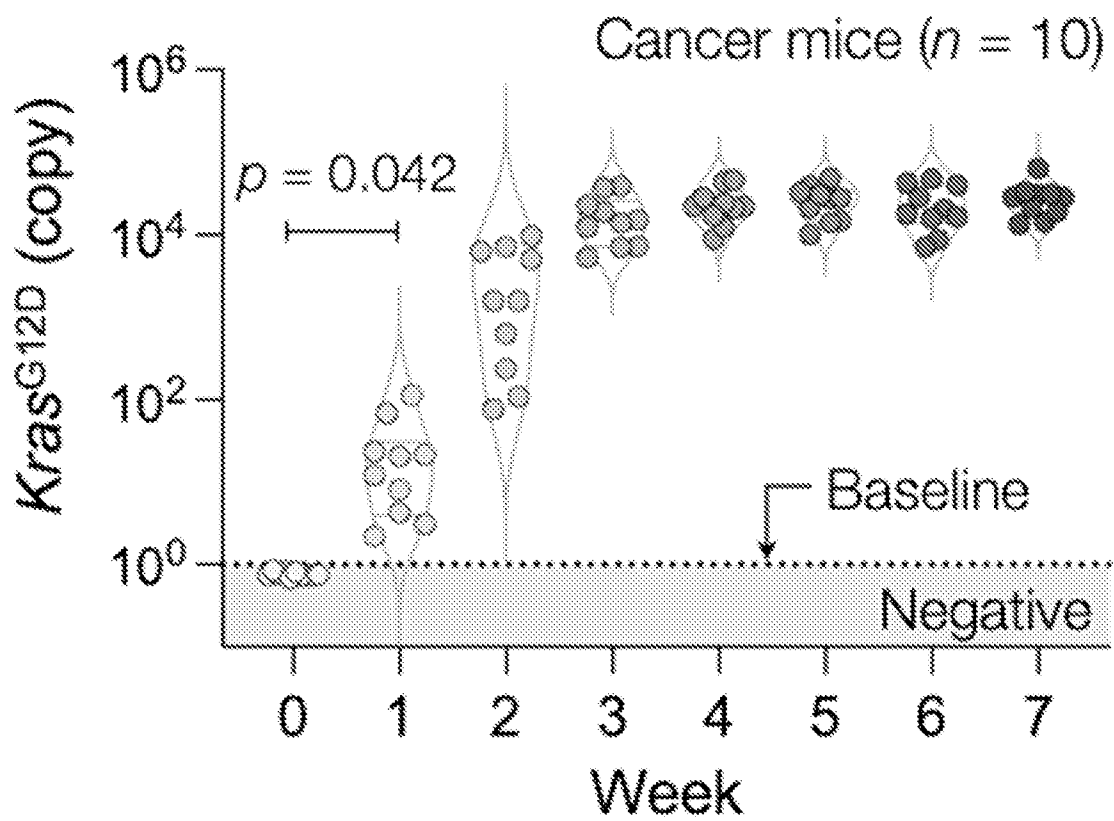


FIG. 5E

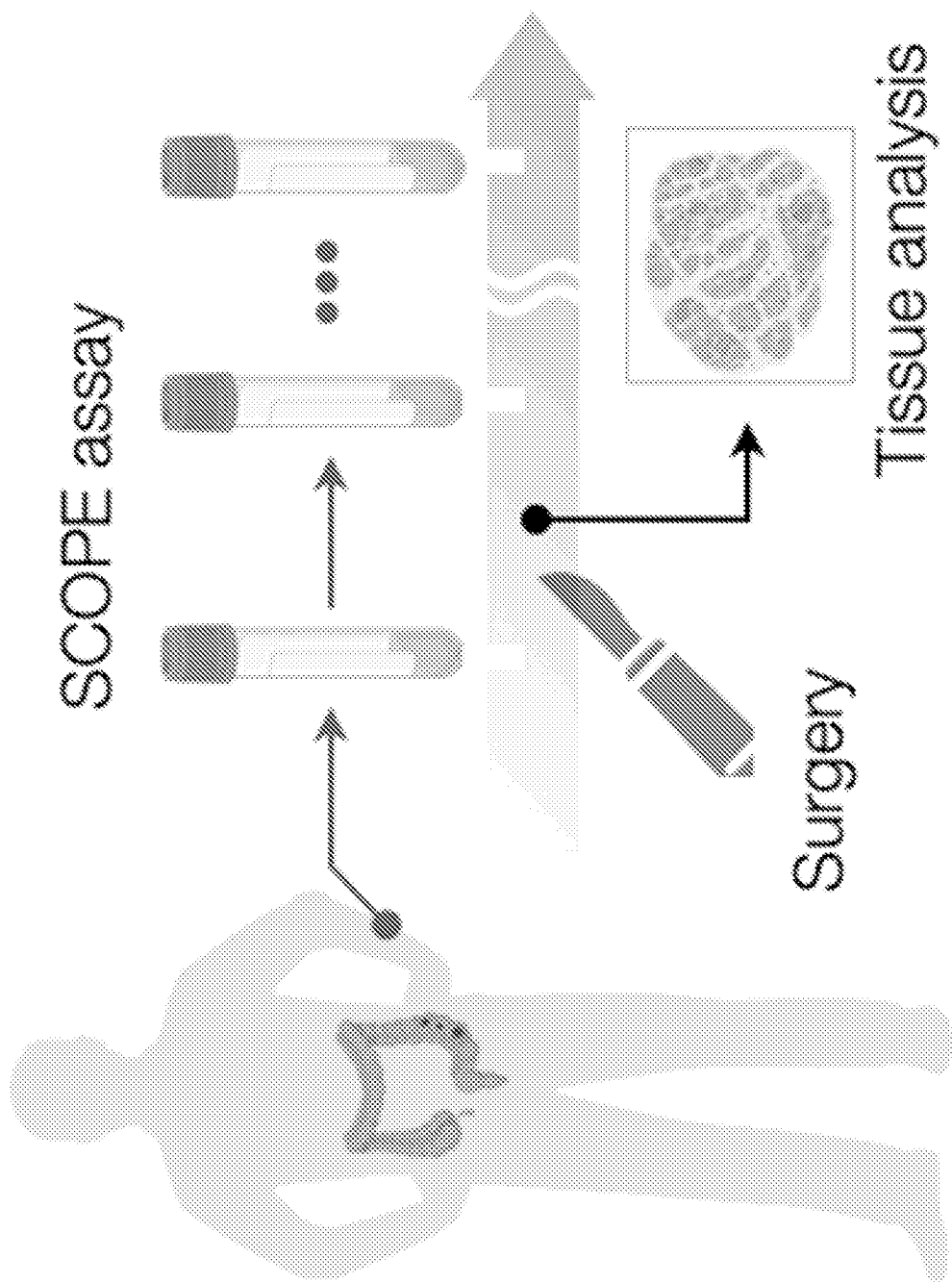


FIG. 6A

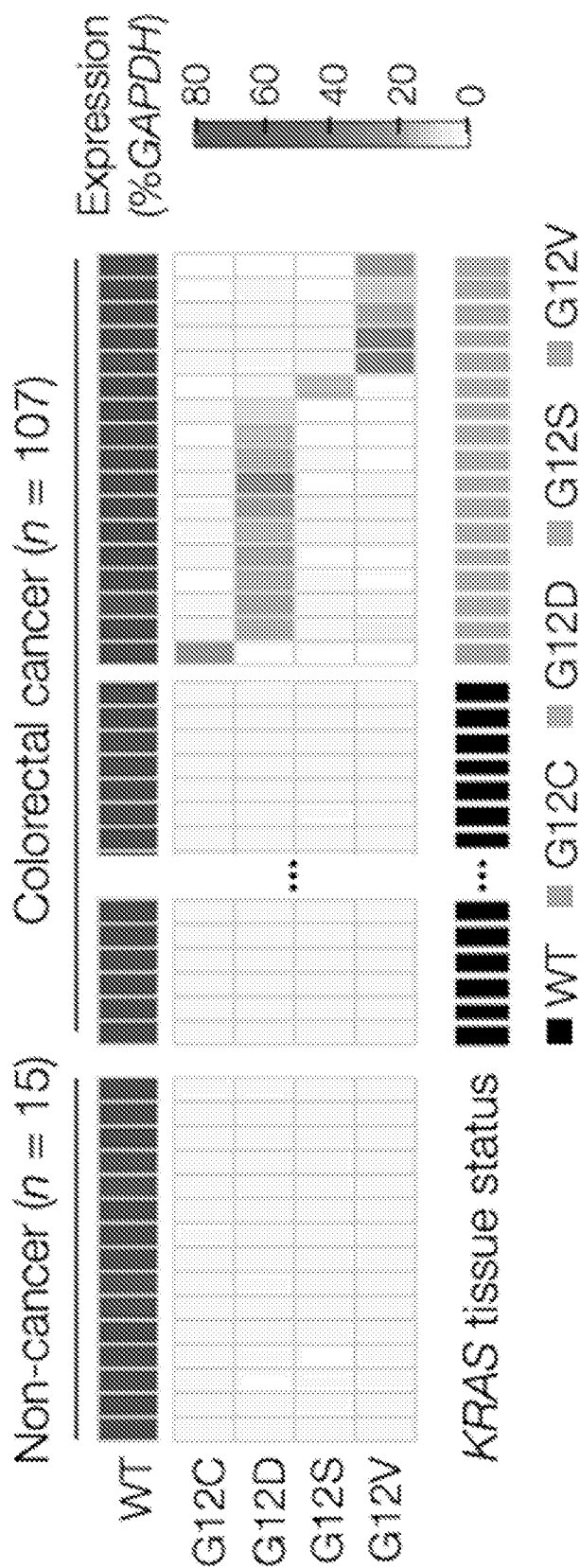


FIG. 6B

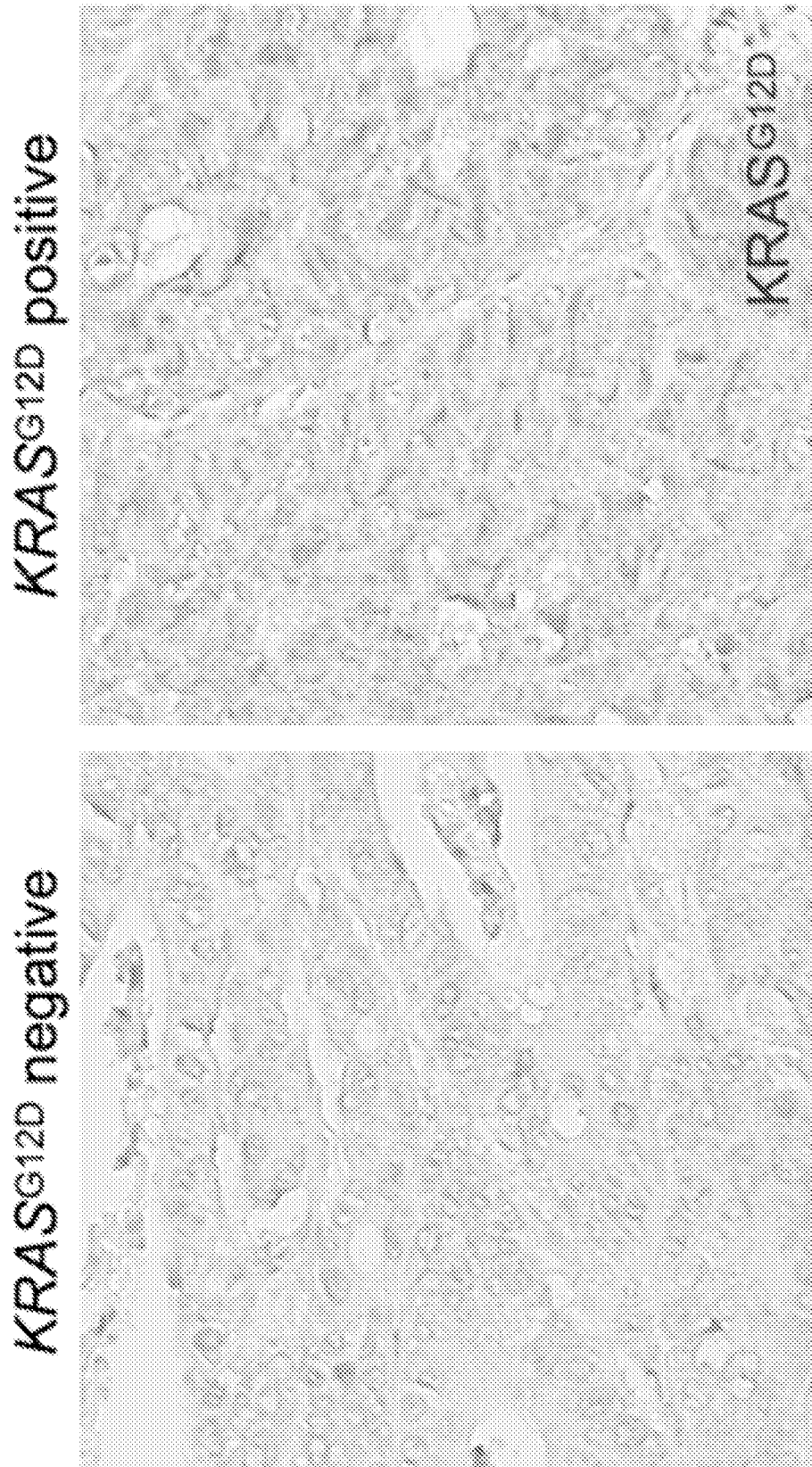


FIG. 6C

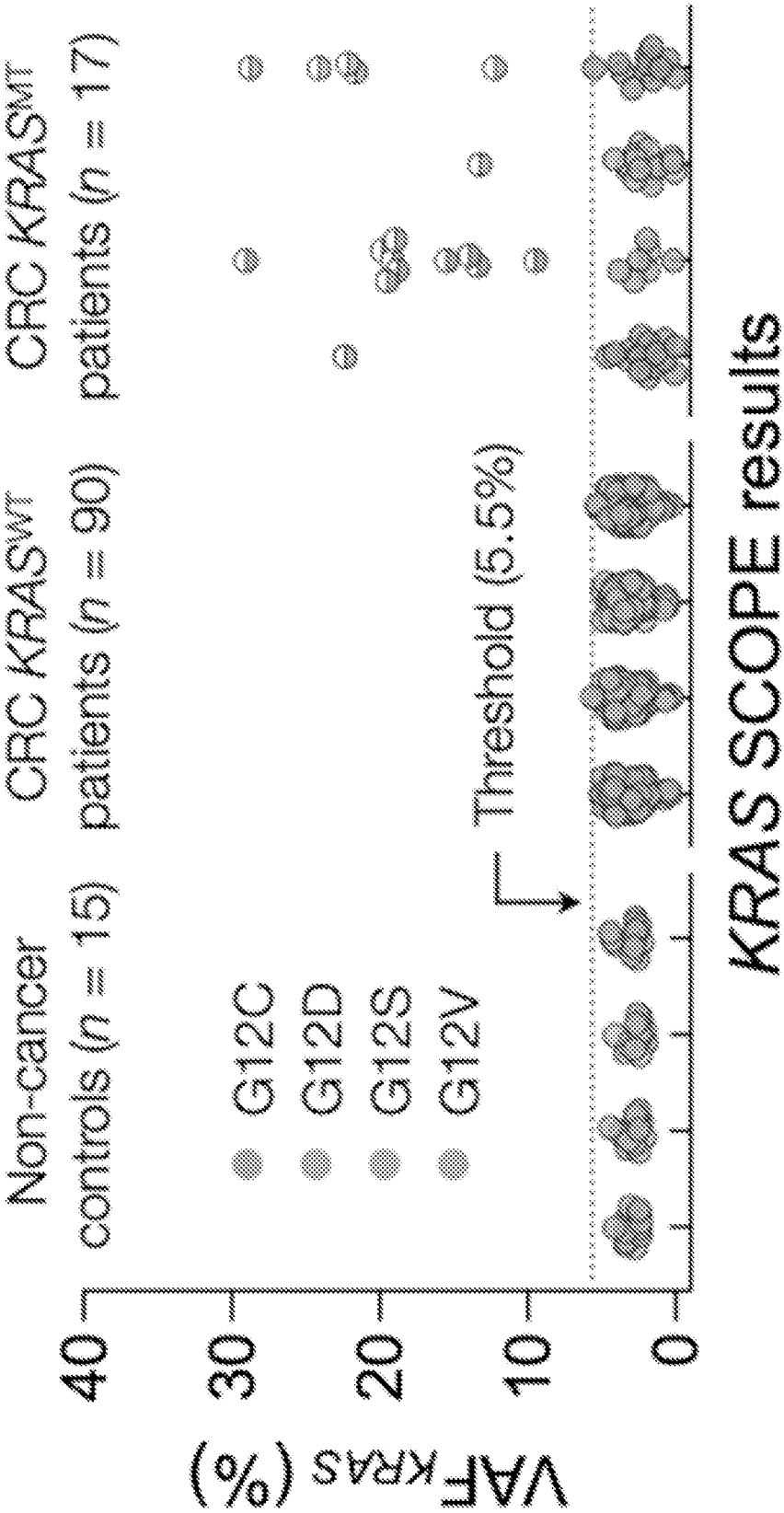


FIG. 6D

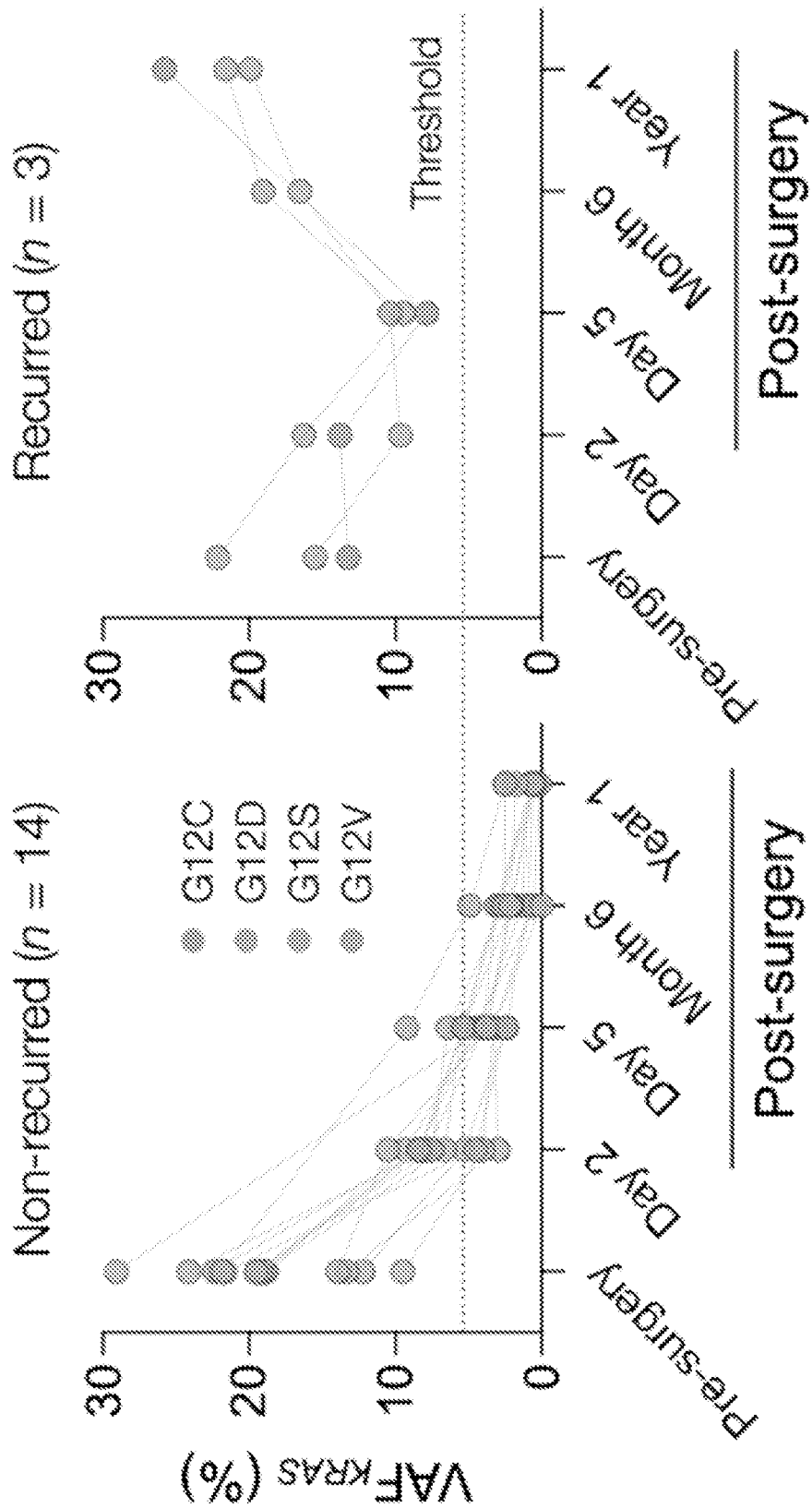


FIG. 6E

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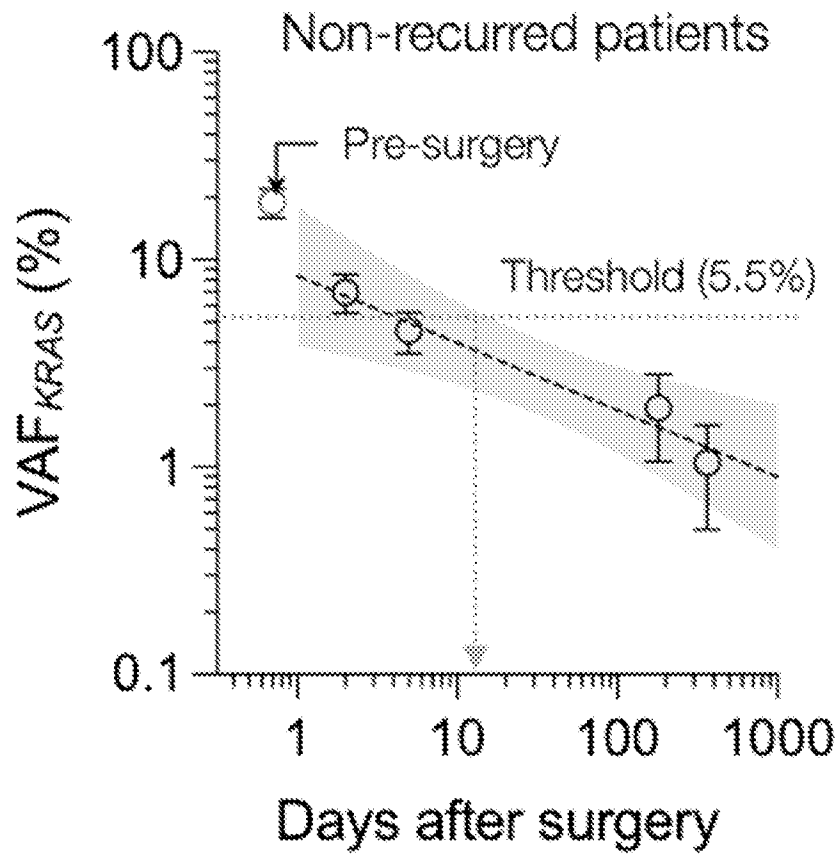


FIG. 6F

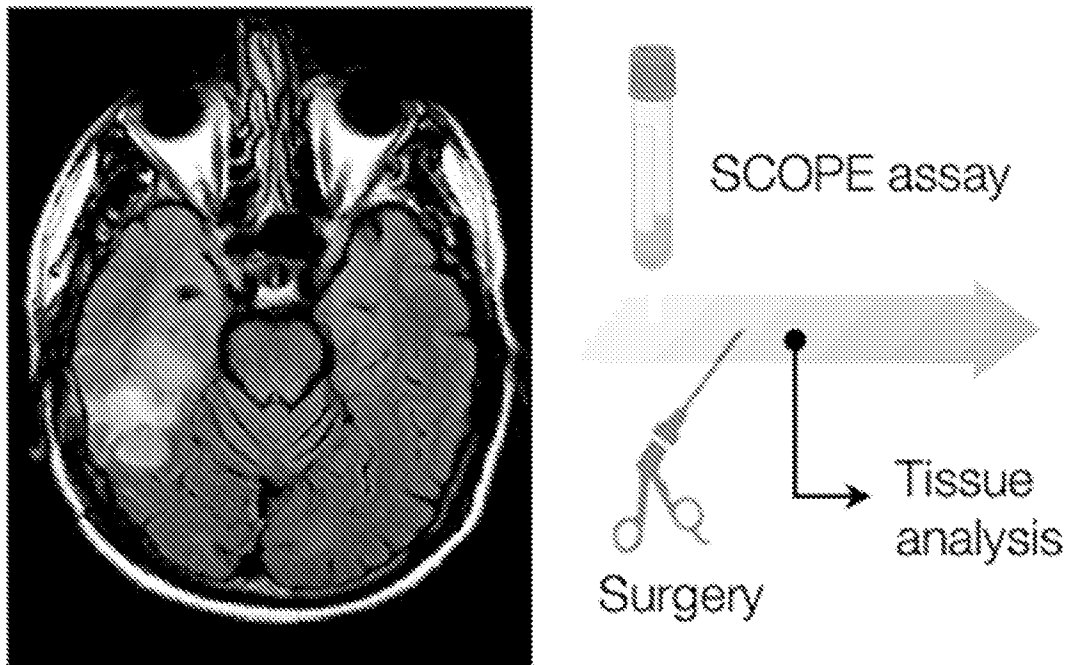


FIG. 7A

<i>IDH1</i> ^{WT}	5'-CCCATCATCATAGGTCGTCATGCTTAT-3'
<i>IDH1</i> ^{R132H}	5'-CCCATCATCATAGGTCATCATGCTTAT-3'

<i>EGFR</i> ^{WT}	5'-GAAAAGAAAGGTAAGGGCGTGTCTCGCC-3'
<i>EGFR</i> ^{vIII}	5'-AAGAAAGGTAATTATGTGGTGACAGATC-3'

FIG. 7B

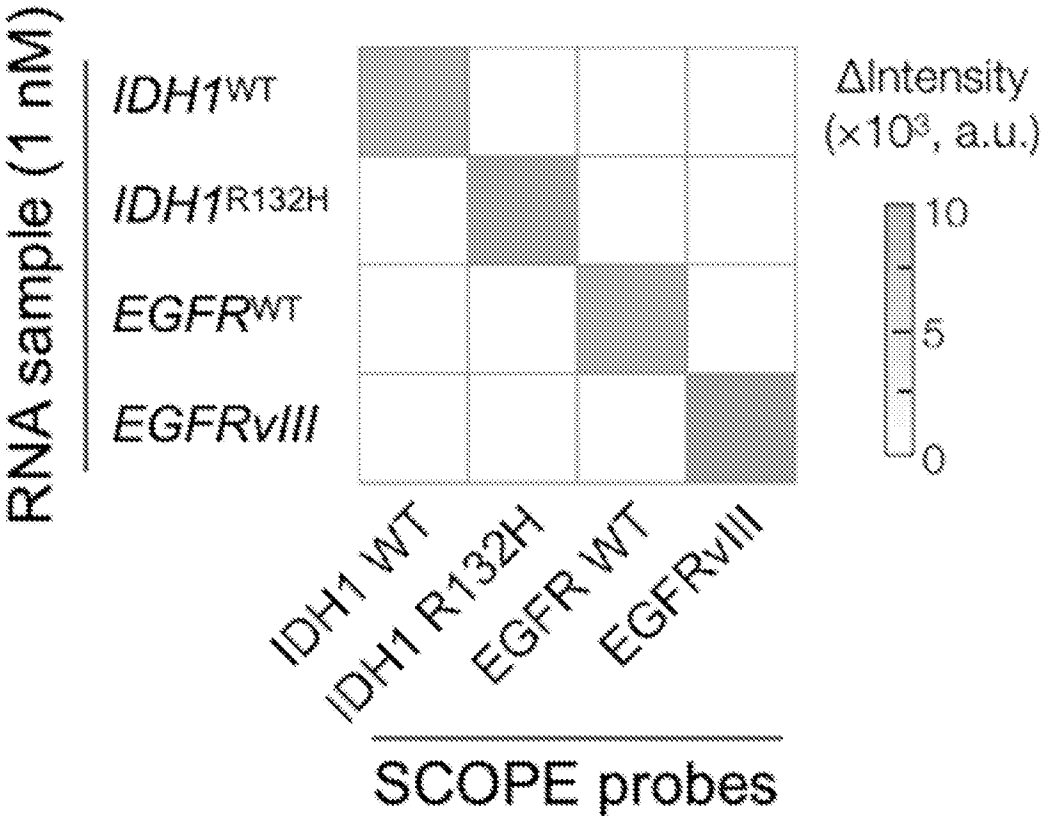


FIG. 7C

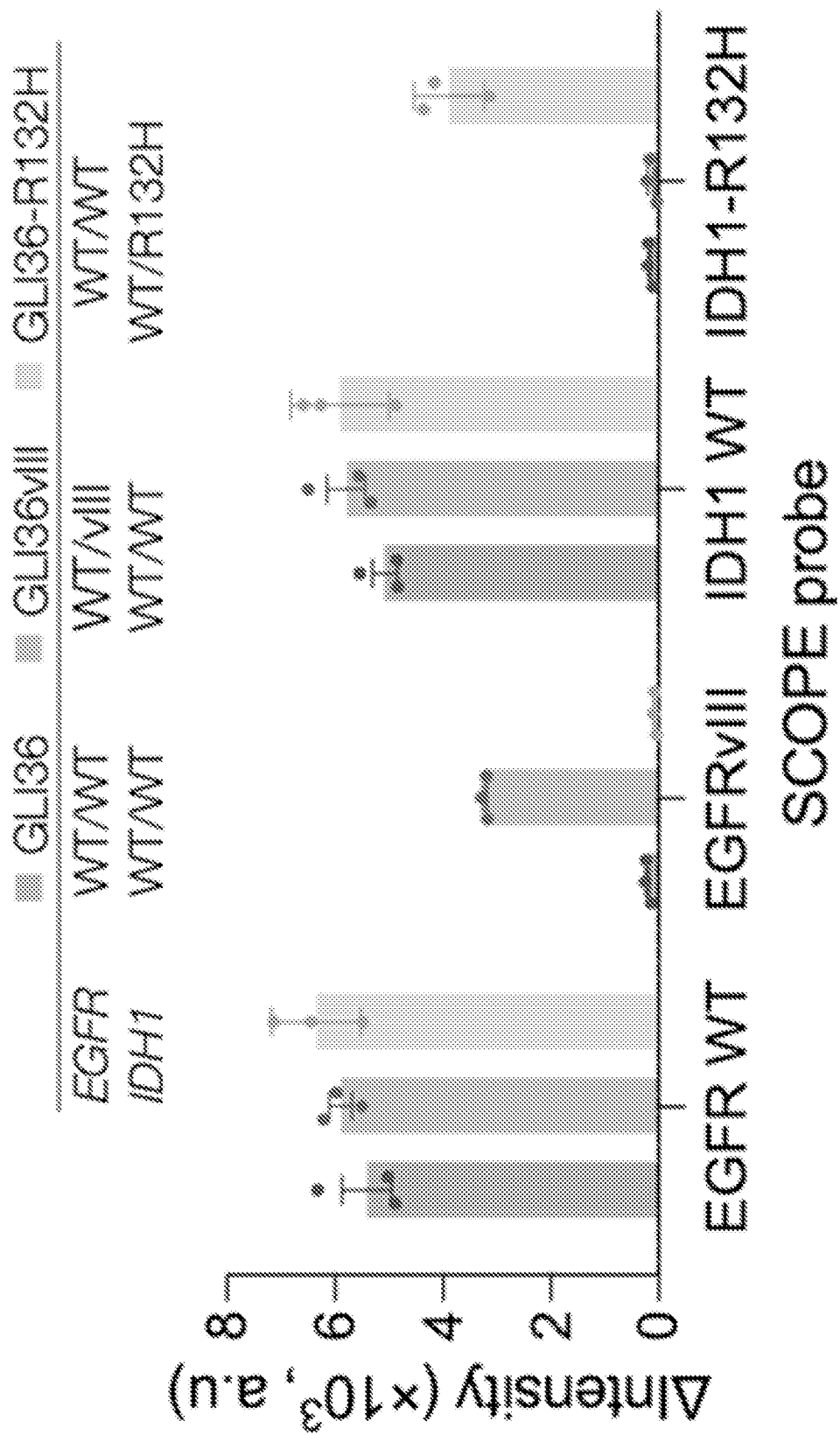


FIG. 7D

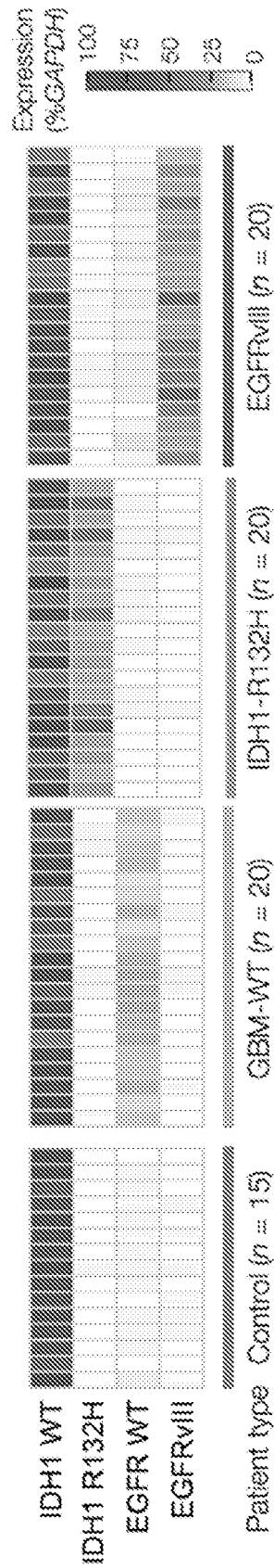


FIG. 7E

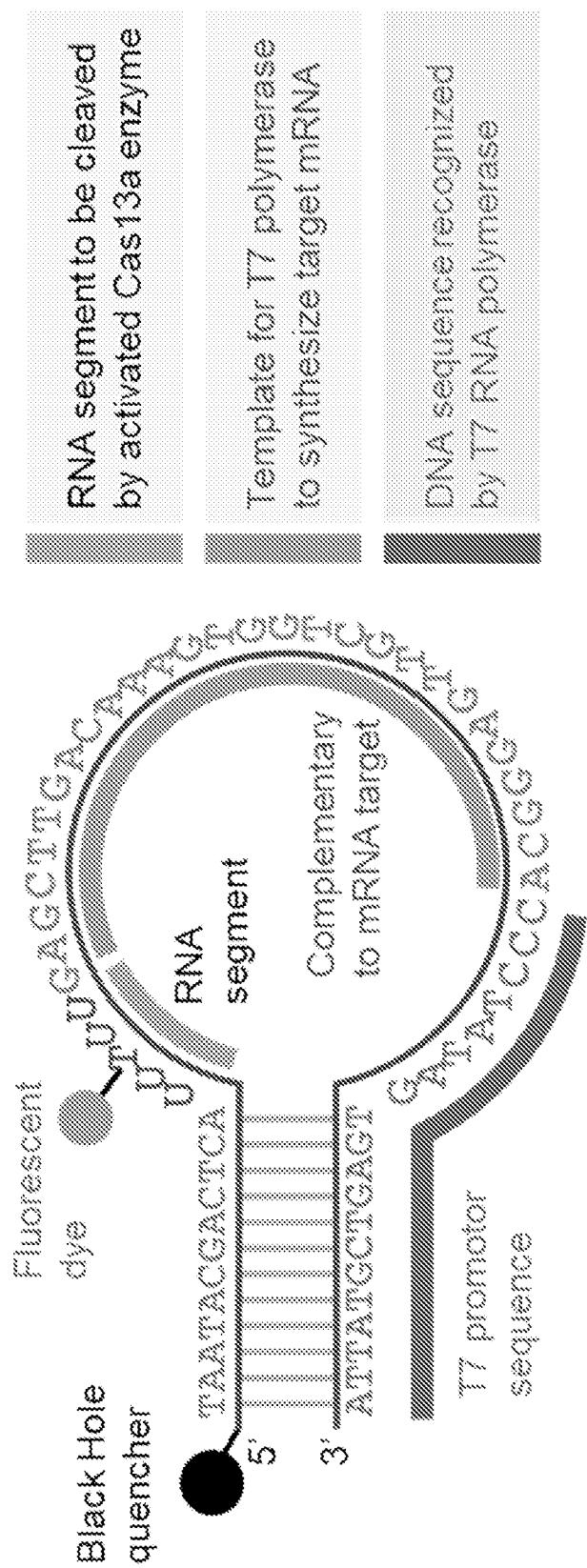


FIG. 8A

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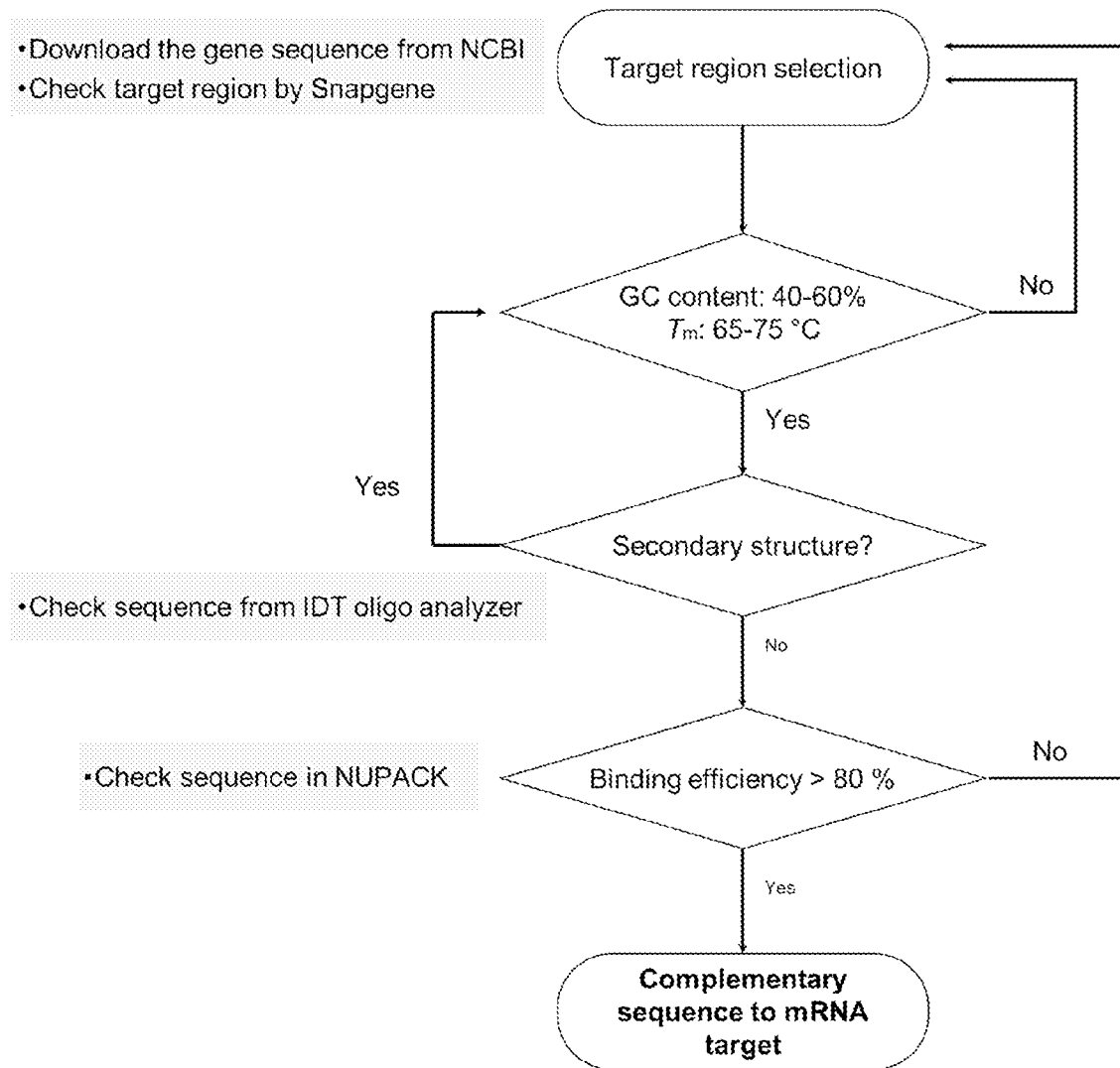


FIG. 8B

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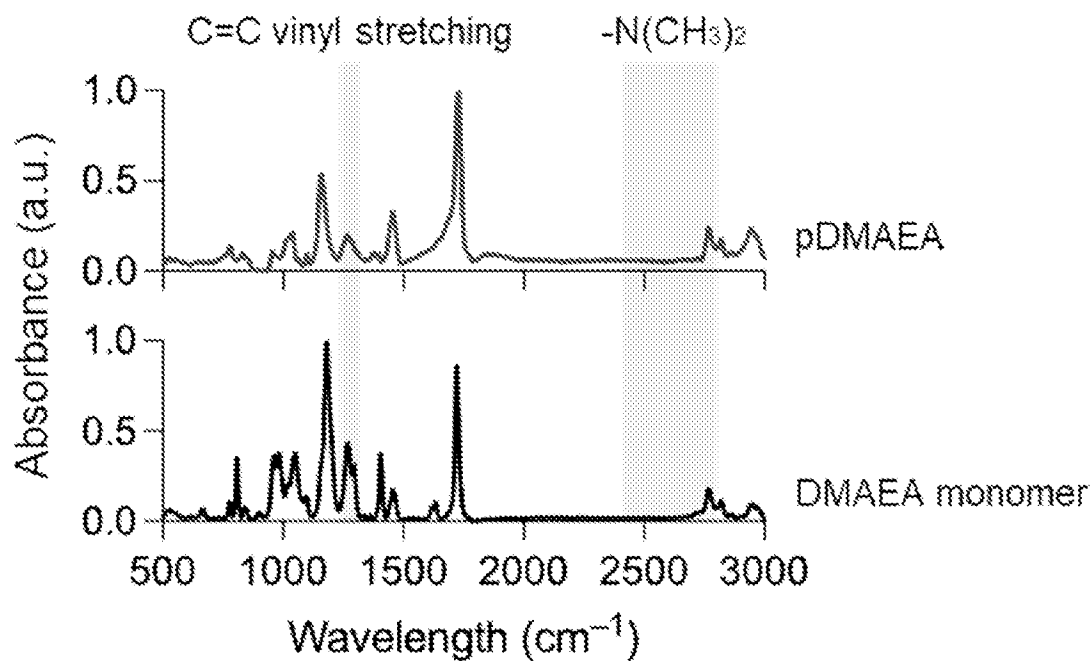


FIG. 10A

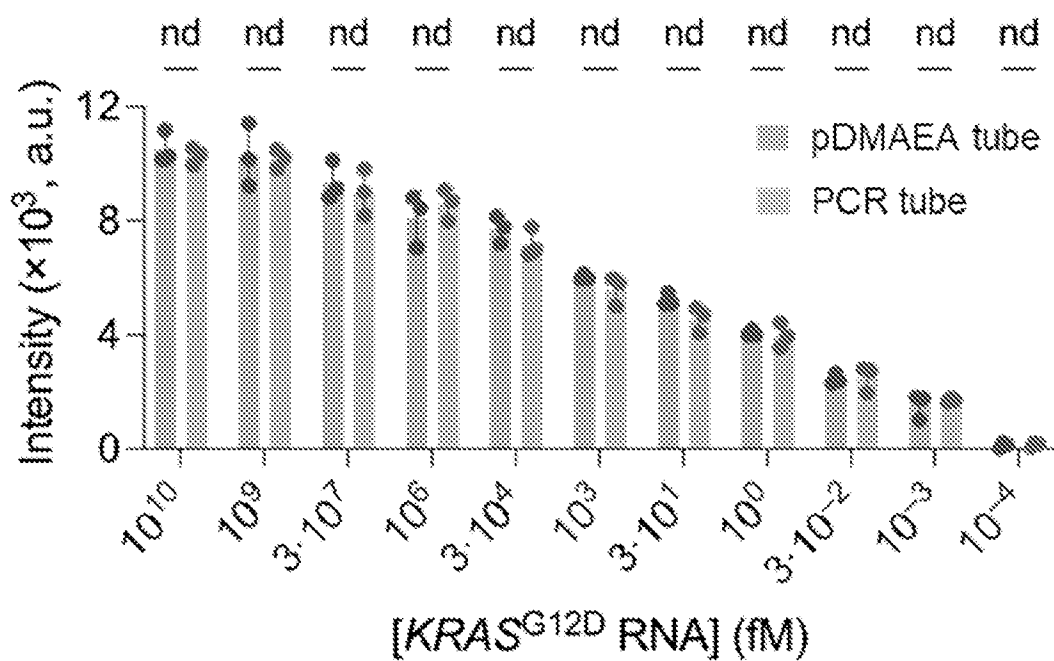


FIG. 10B

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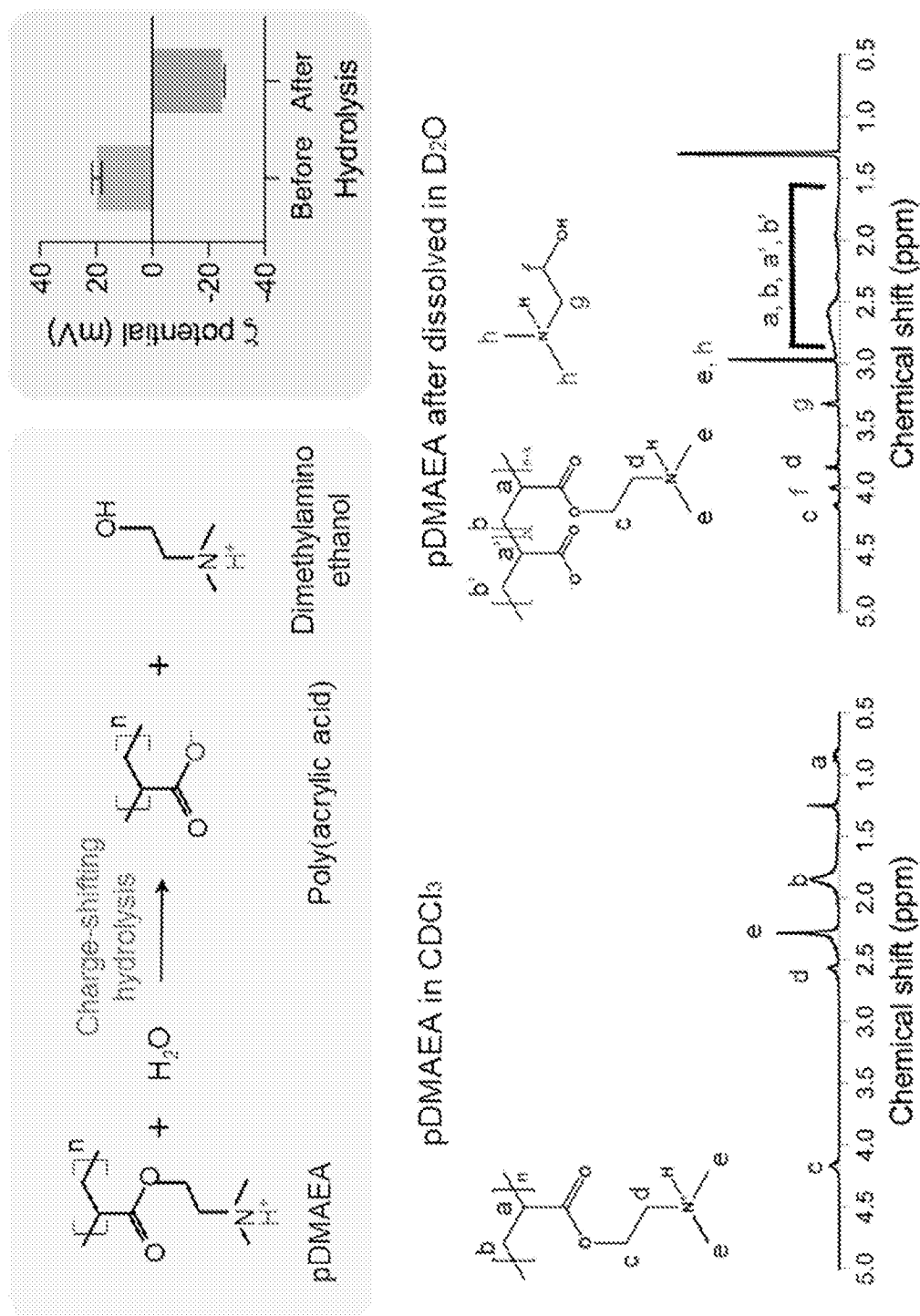


FIG. 11

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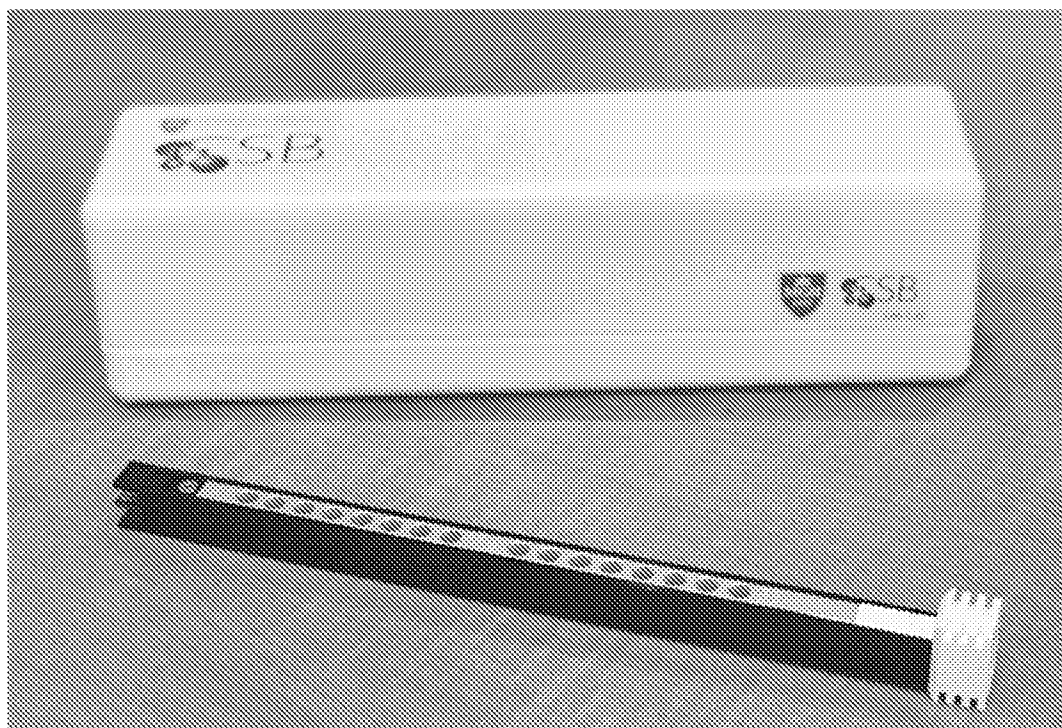


FIG. 12A

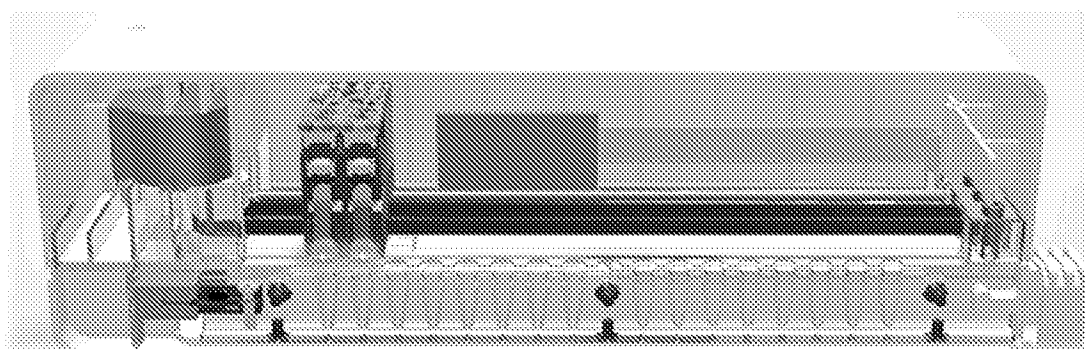


FIG. 12B

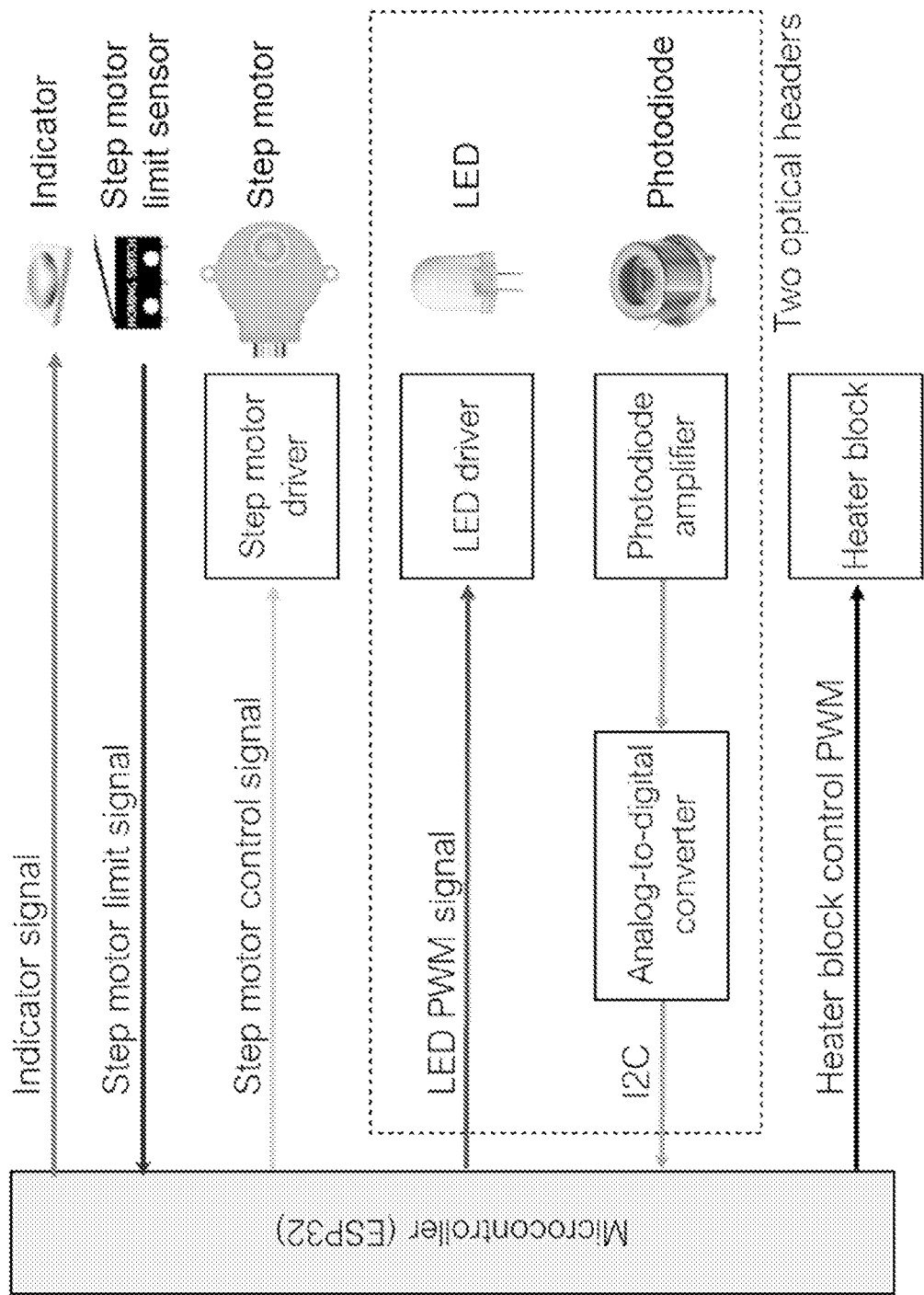
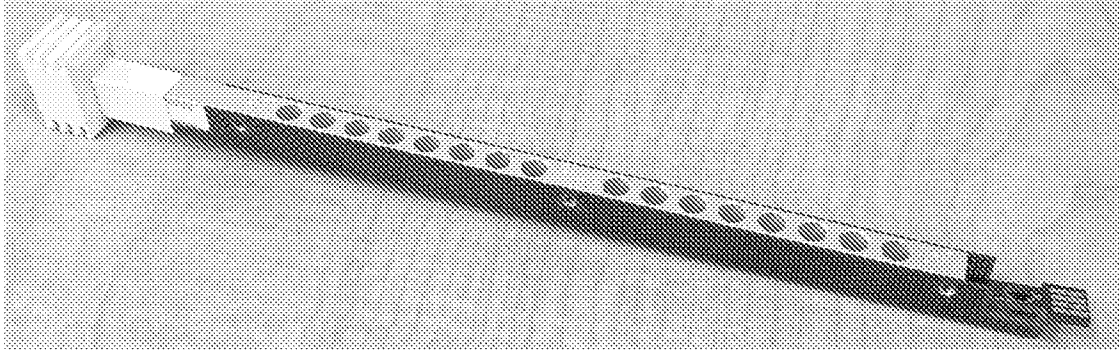
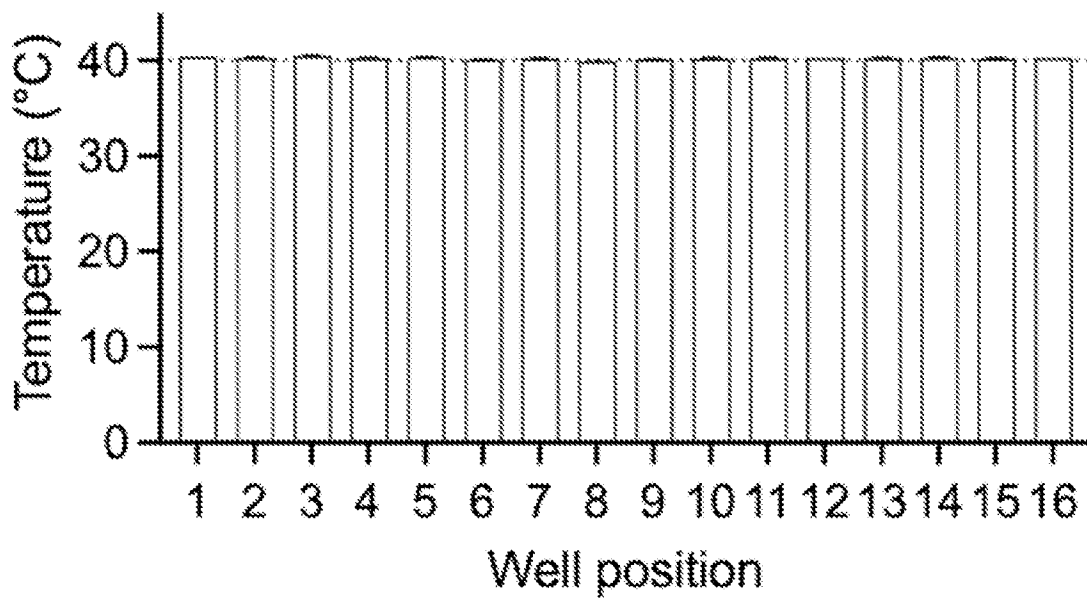


FIG. 12C

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**FIG. 12D****FIG. 12E**

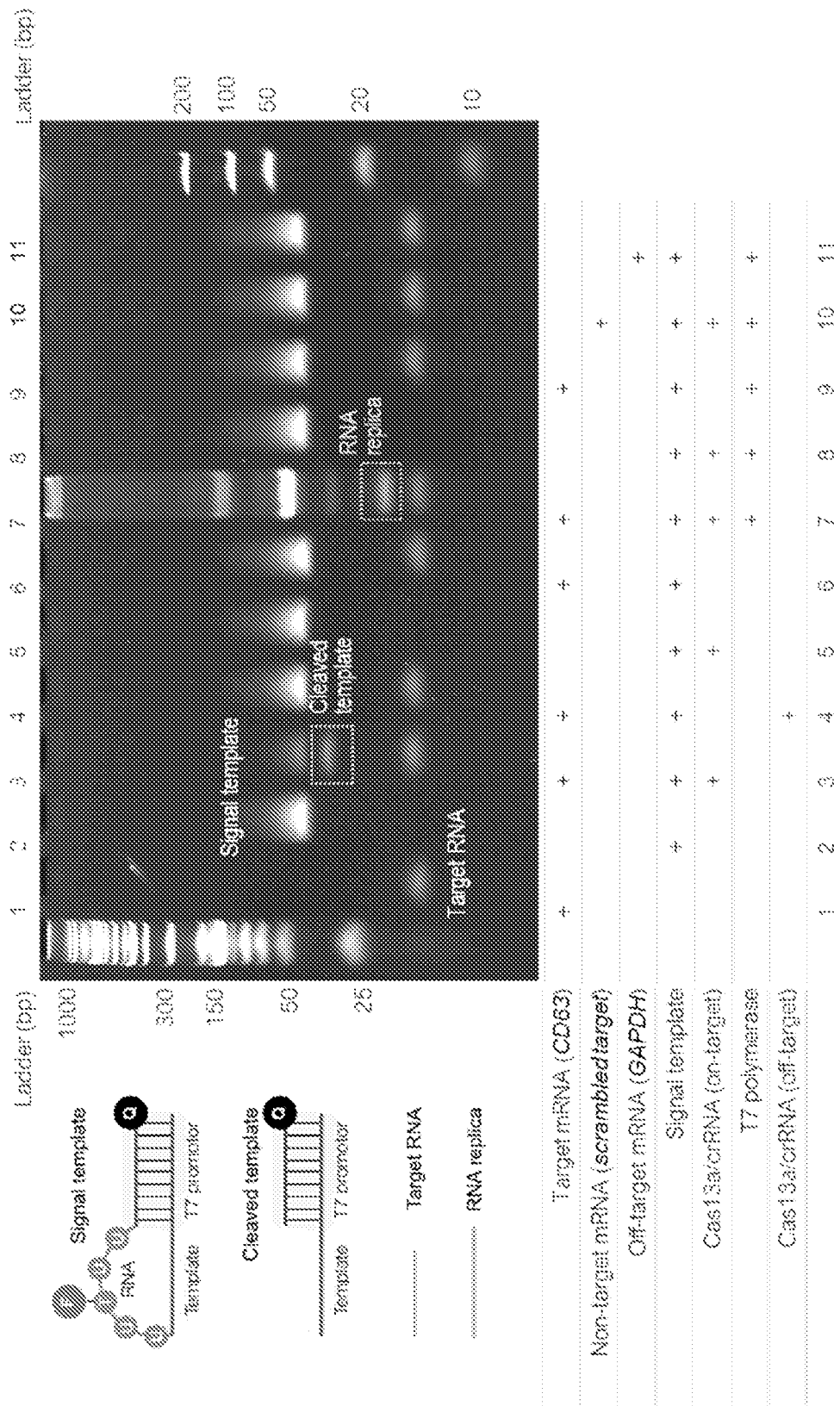
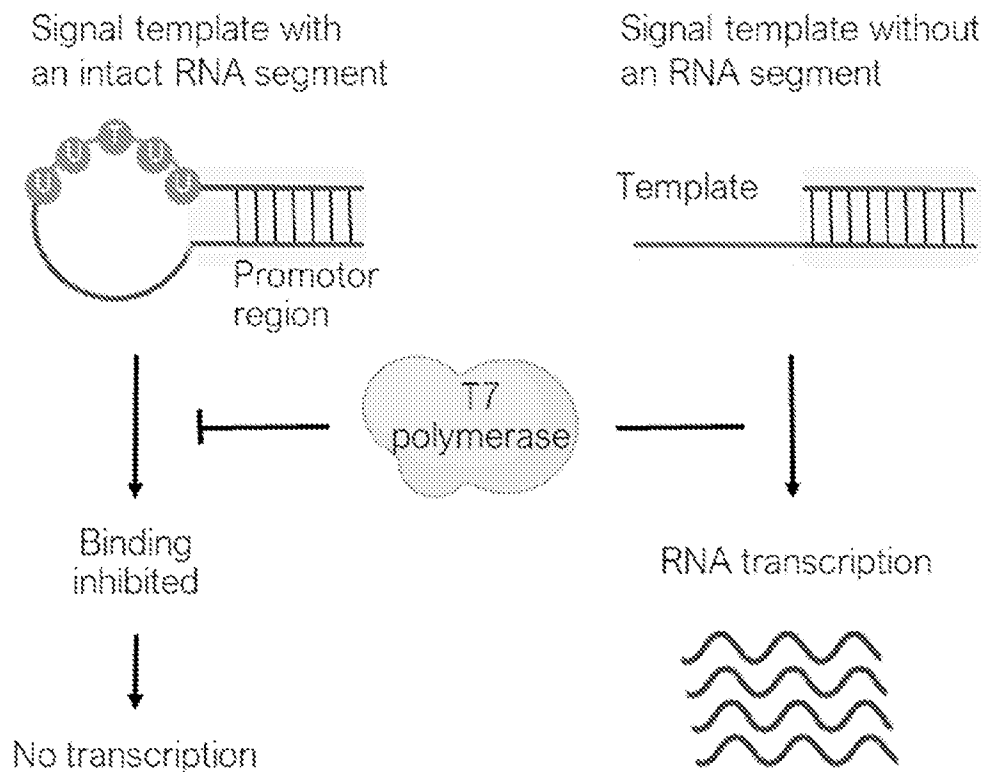
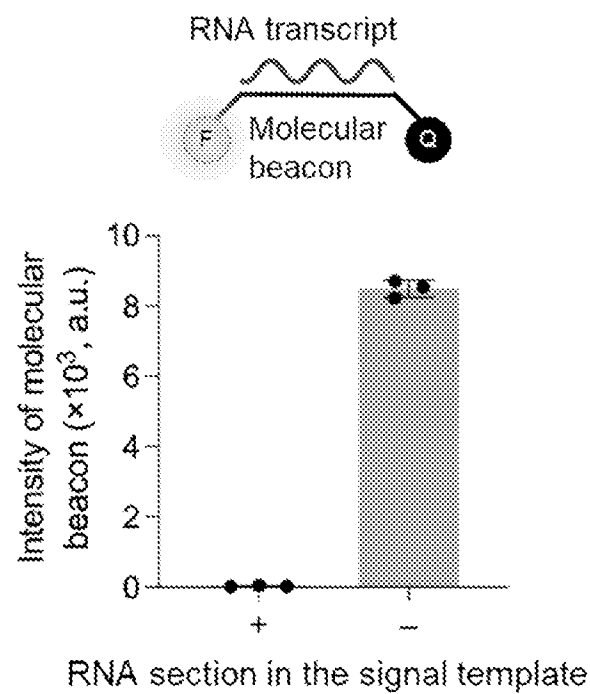


FIG. 13

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**FIG. 14A****FIG. 14B**

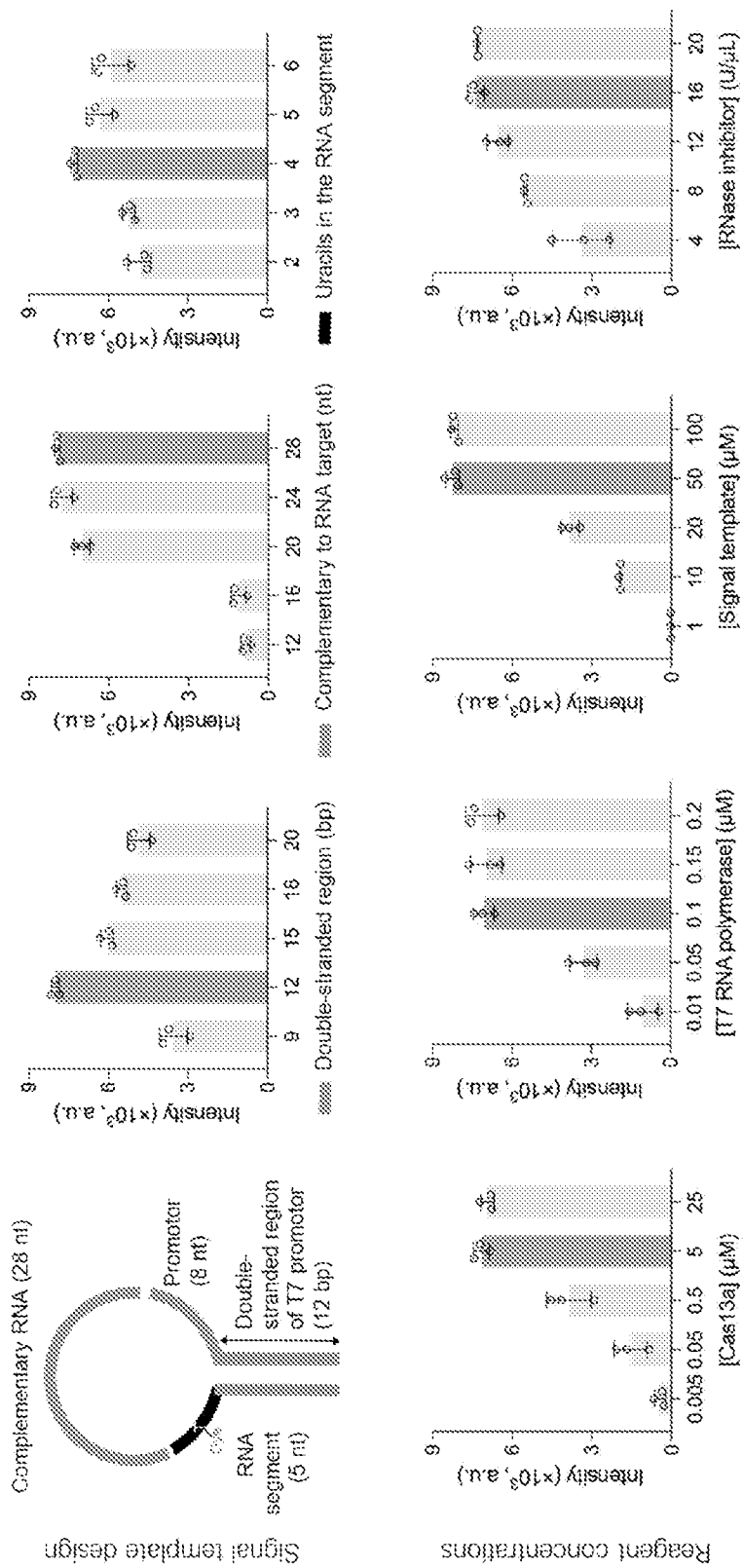


FIG. 15

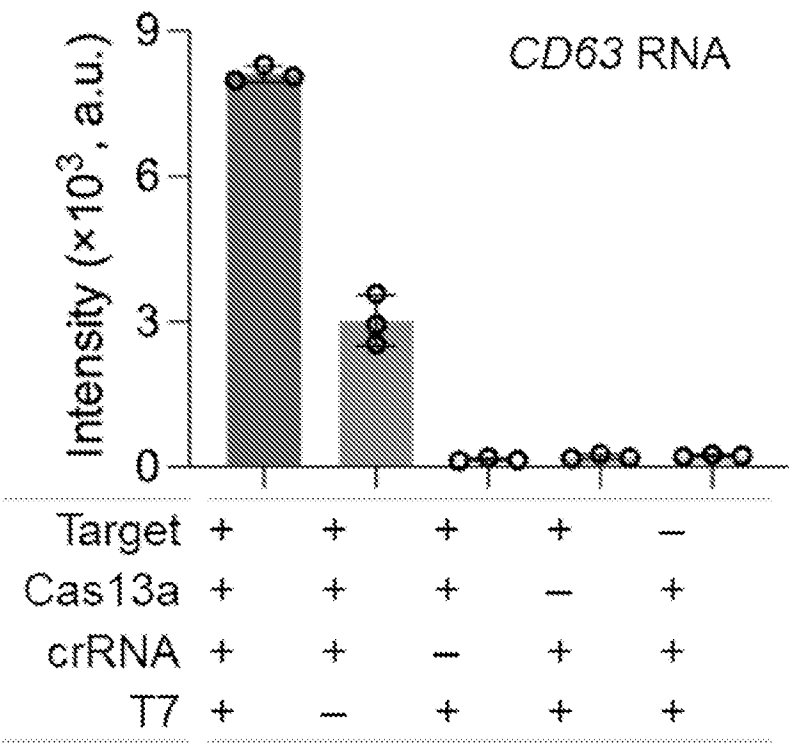


FIG. 16A

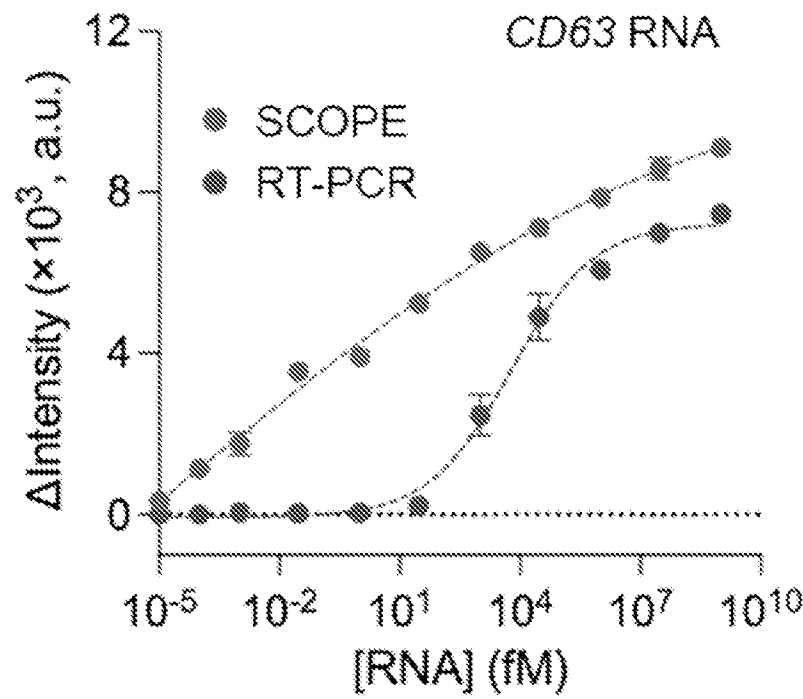


FIG. 16B

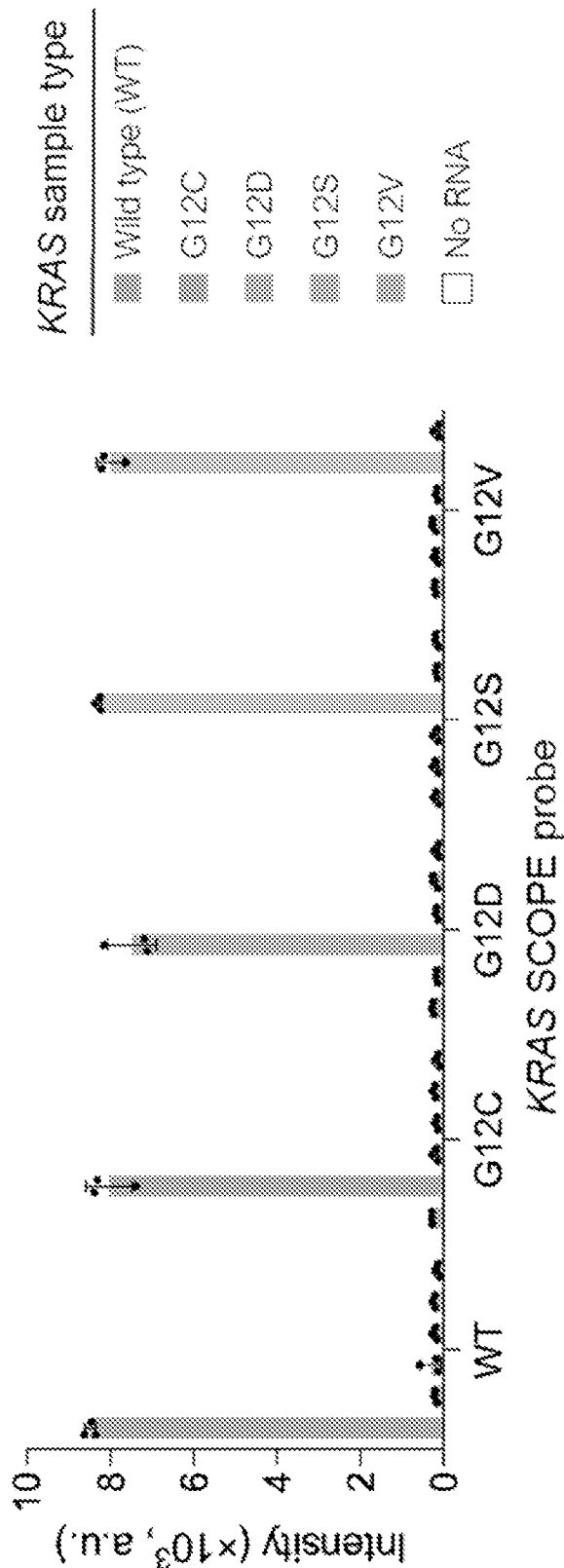


FIG. 17

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Wild type (WT) 5' -GAGCUGGUGGCCGUAGGCAA-3'
G12C 5' -GAGCUUGUGGCCGUAGGCAA-3'
G12D 5' -GAGCUGAUGGCCGUAGGCAA-3'
G12S 5' -GAGCUAGUGGCCGUAGGCAA-3'
G12V 5' -GAGCUGUUGGCCGUAGGCAA-3'

FIG. 18A

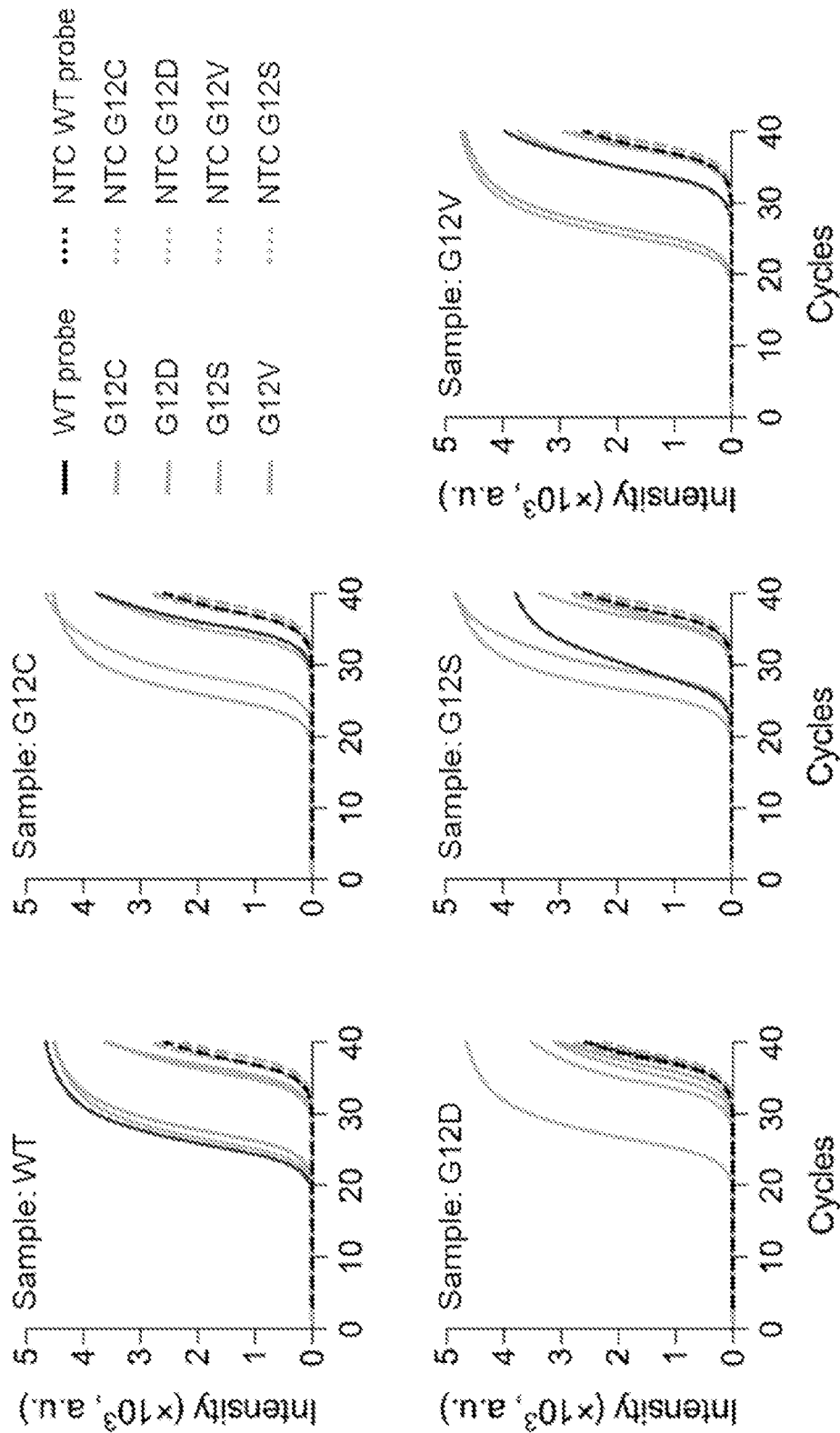


FIG. 18B

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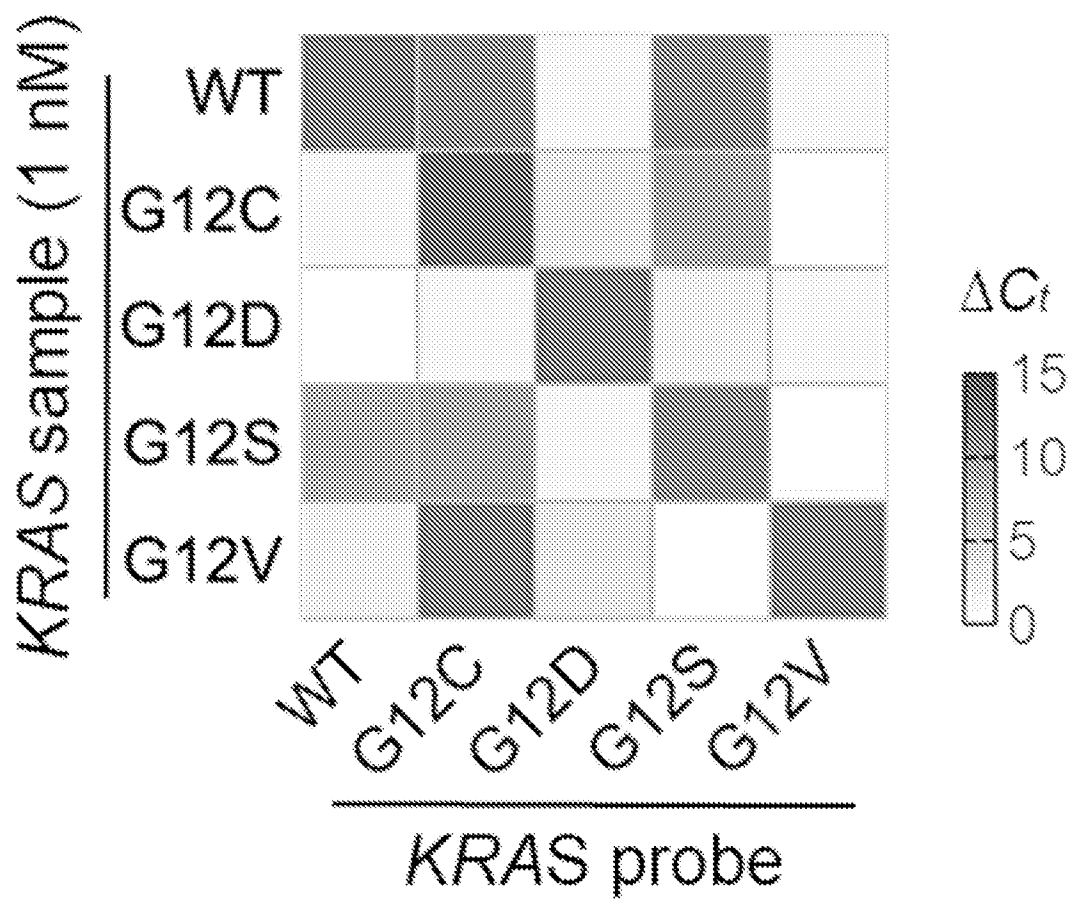


FIG. 18C

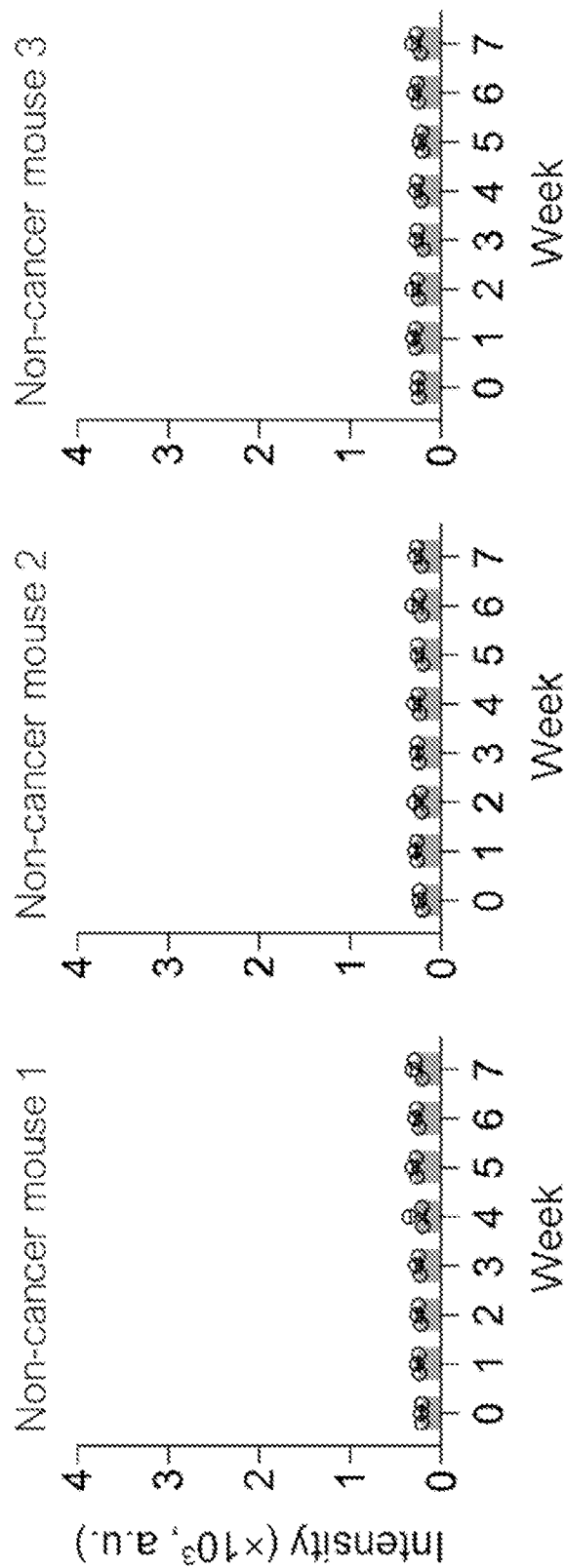


FIG. 19A

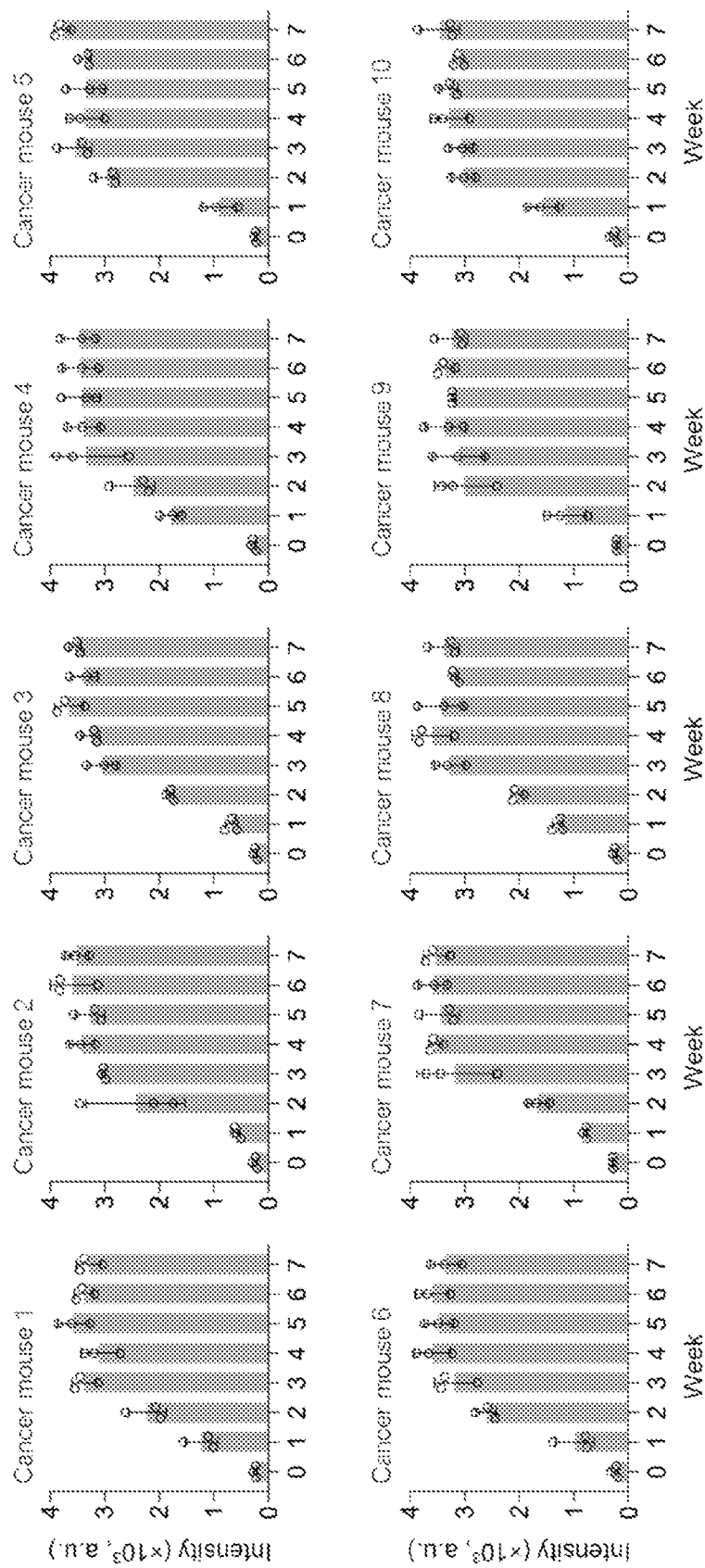


FIG. 19B

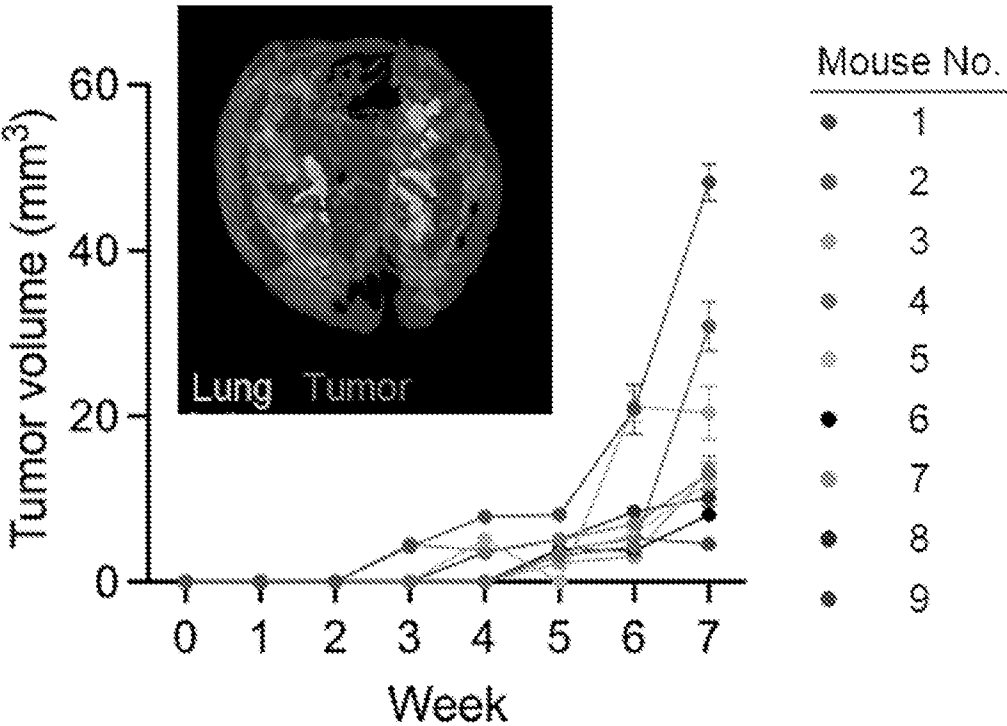


FIG. 20

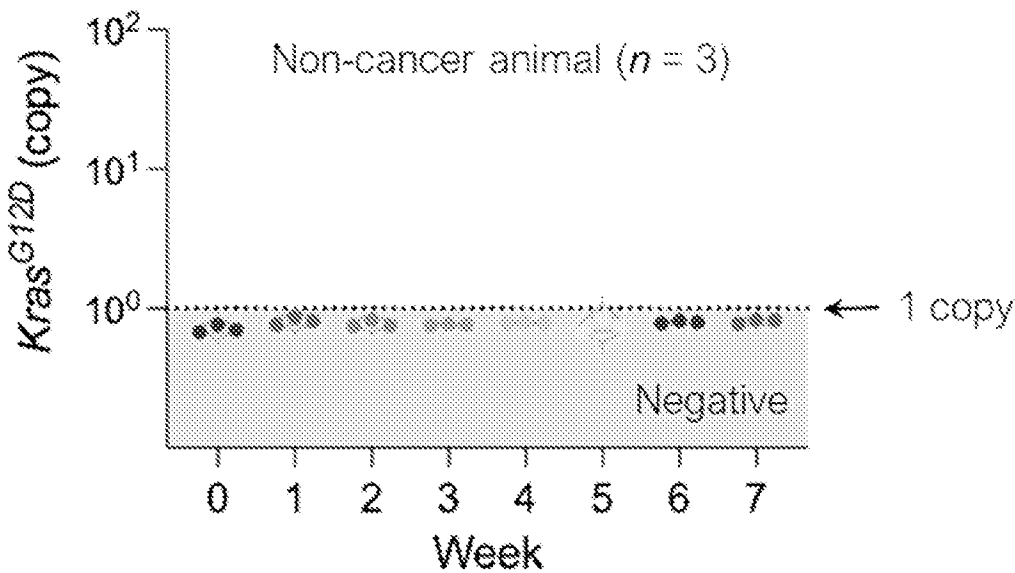


FIG. 21

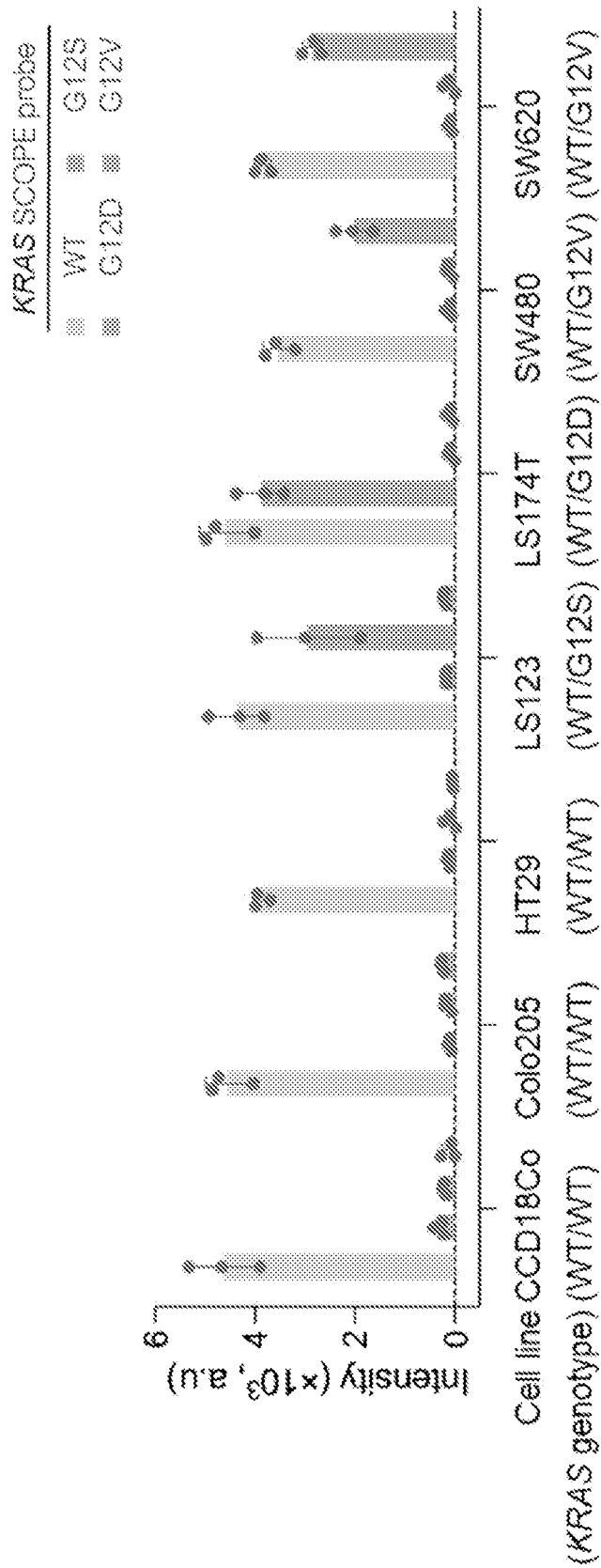
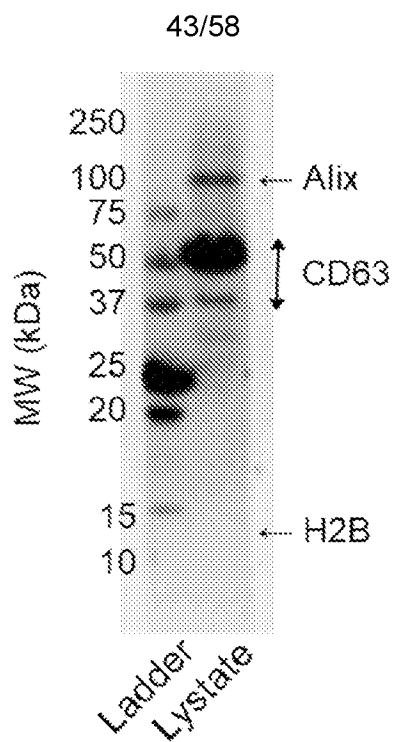
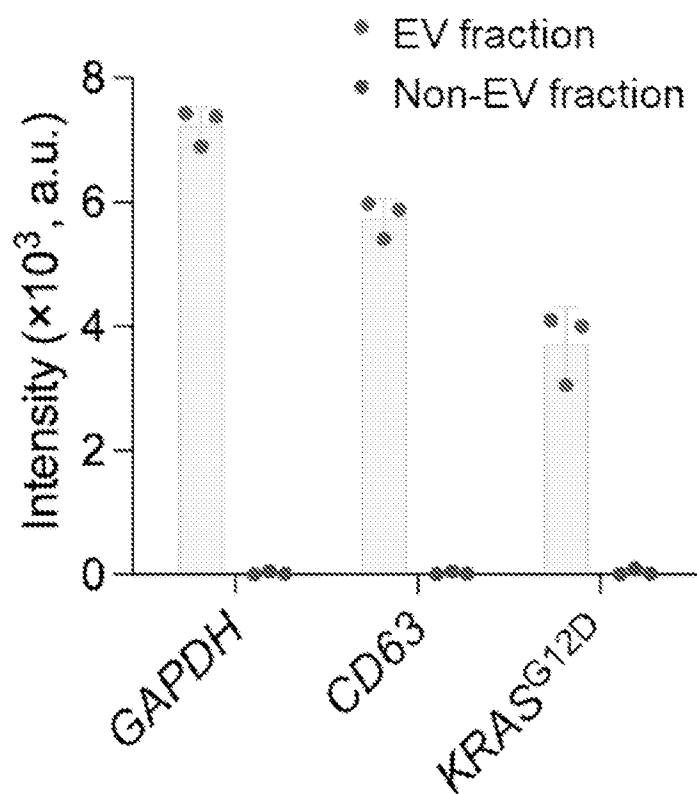


FIG. 22

**FIG. 23A****FIG. 23B**

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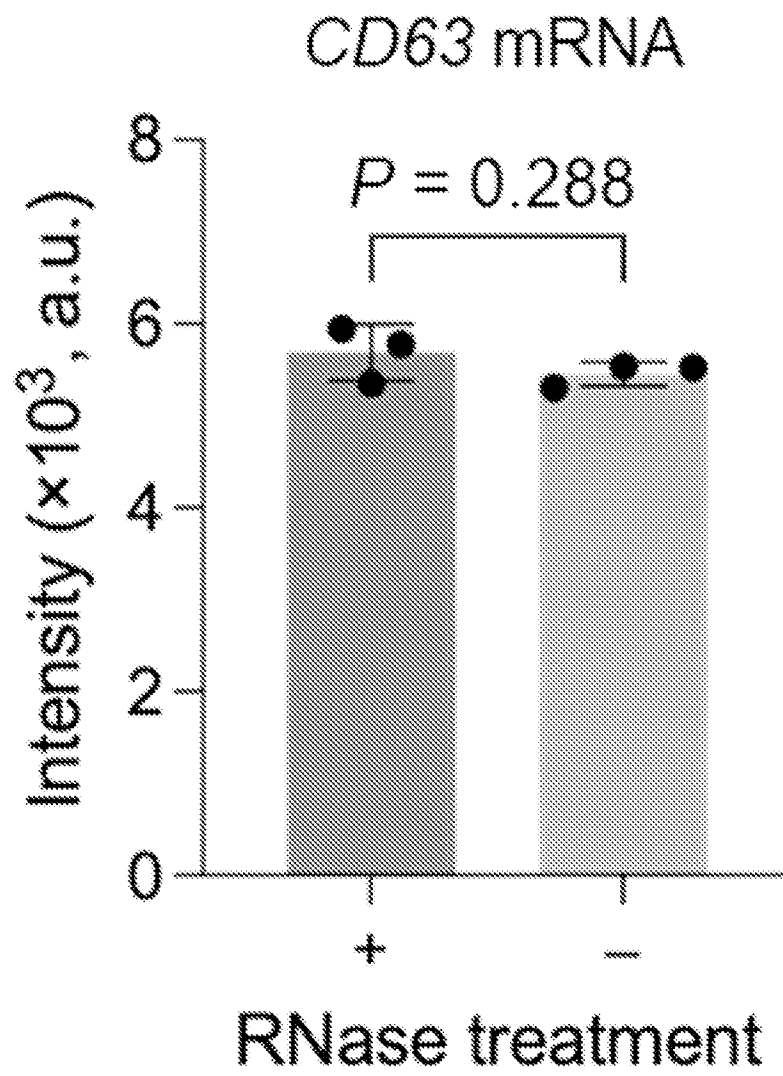


FIG. 23C

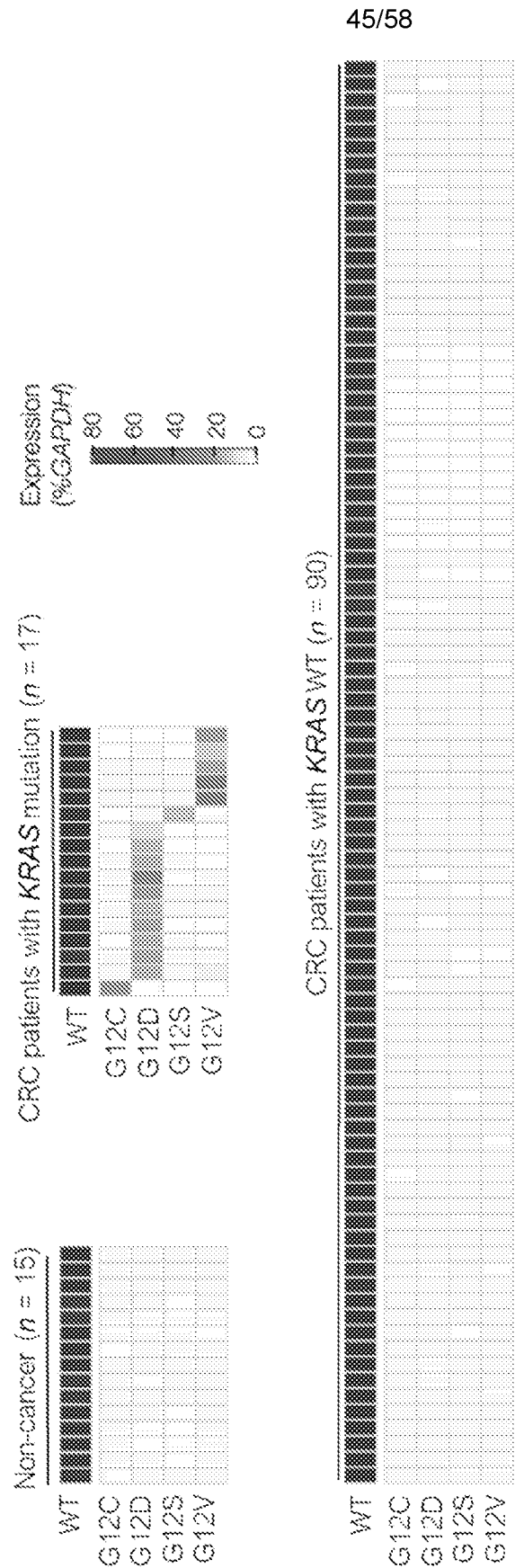


FIG. 24

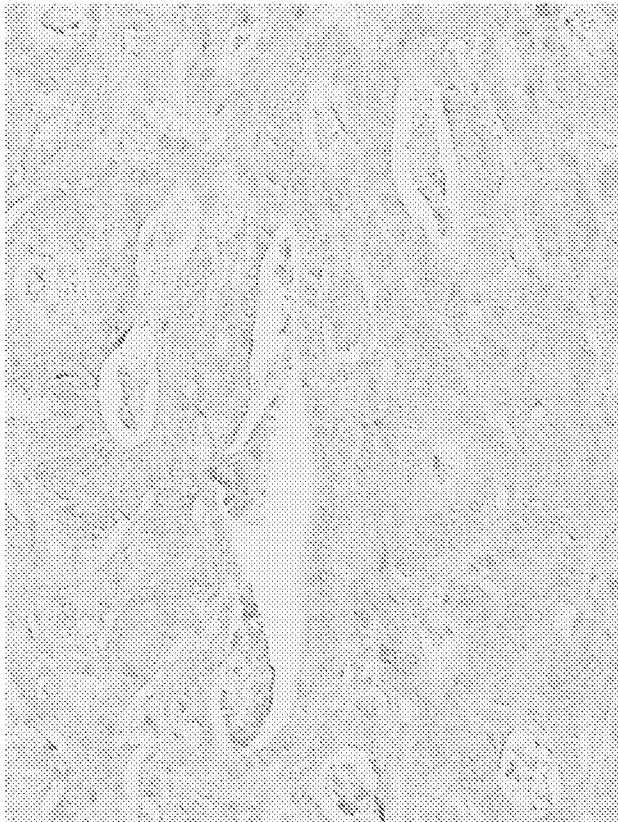


FIG. 25

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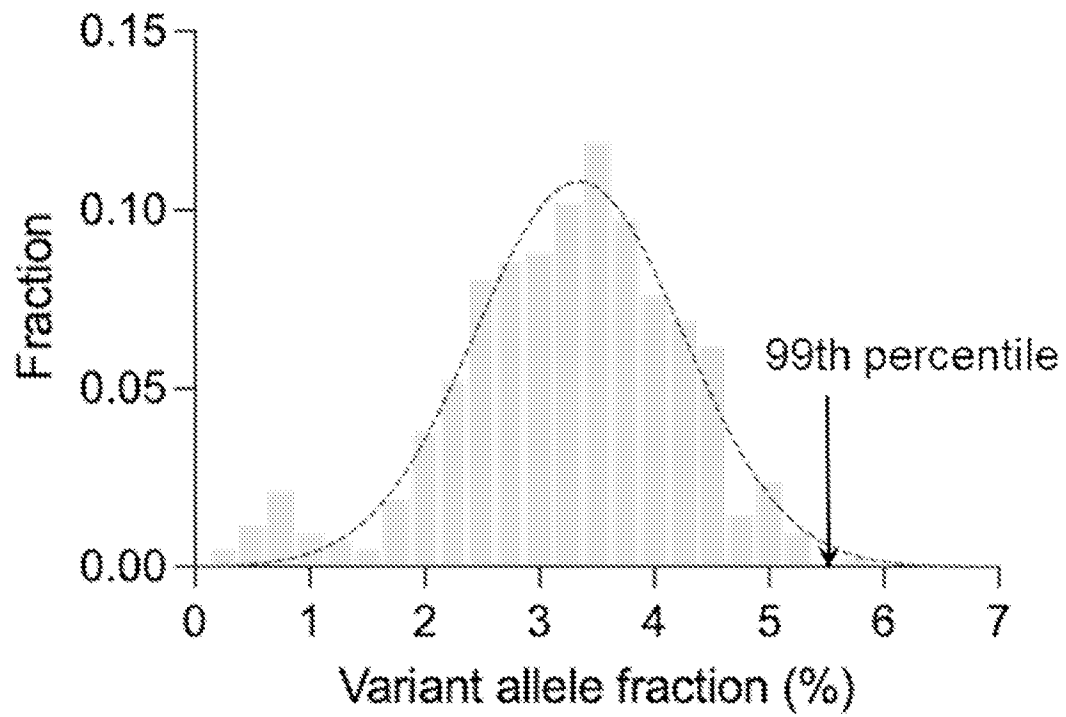
**FIG. 26**



FIG. 27

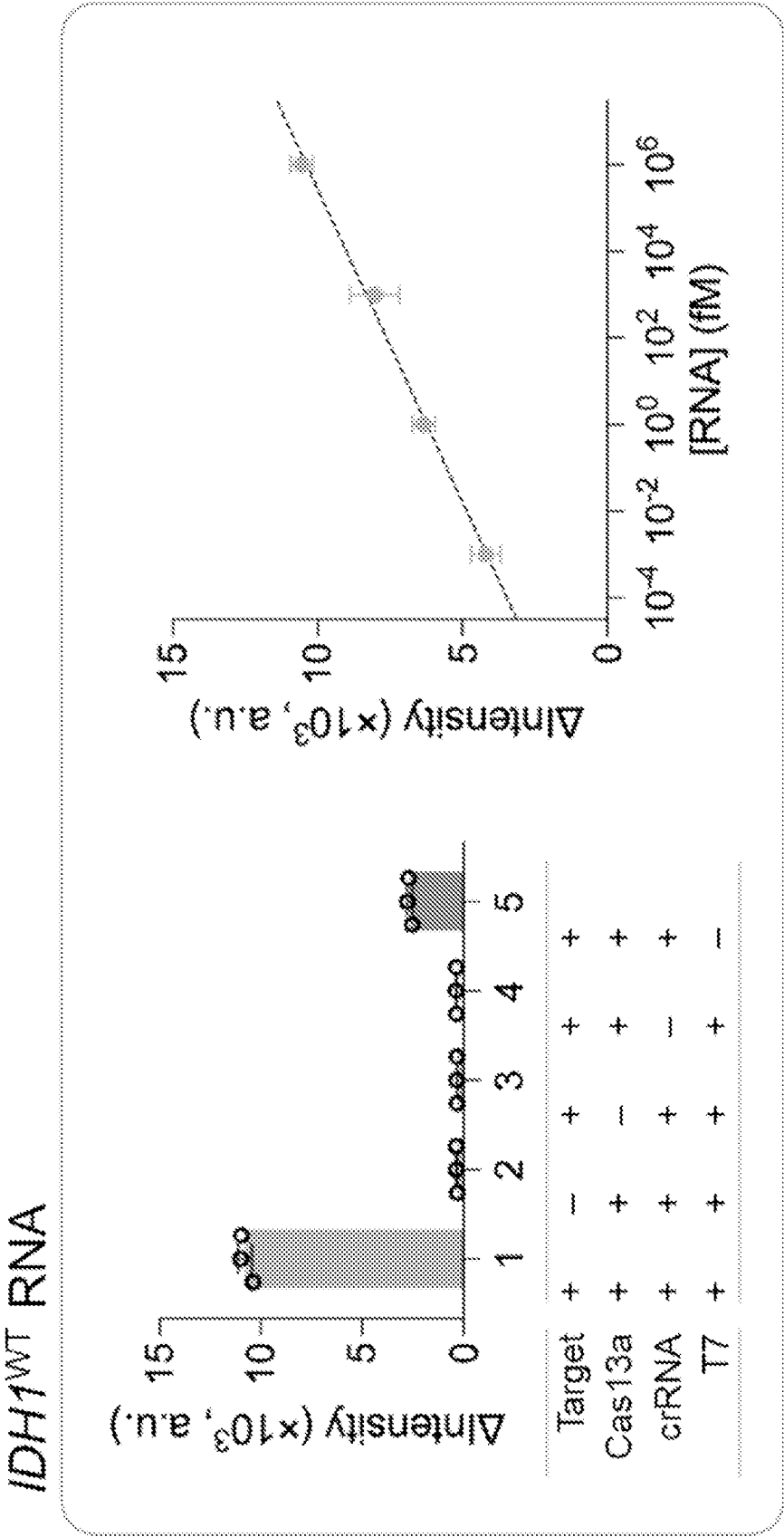


FIG. 28A

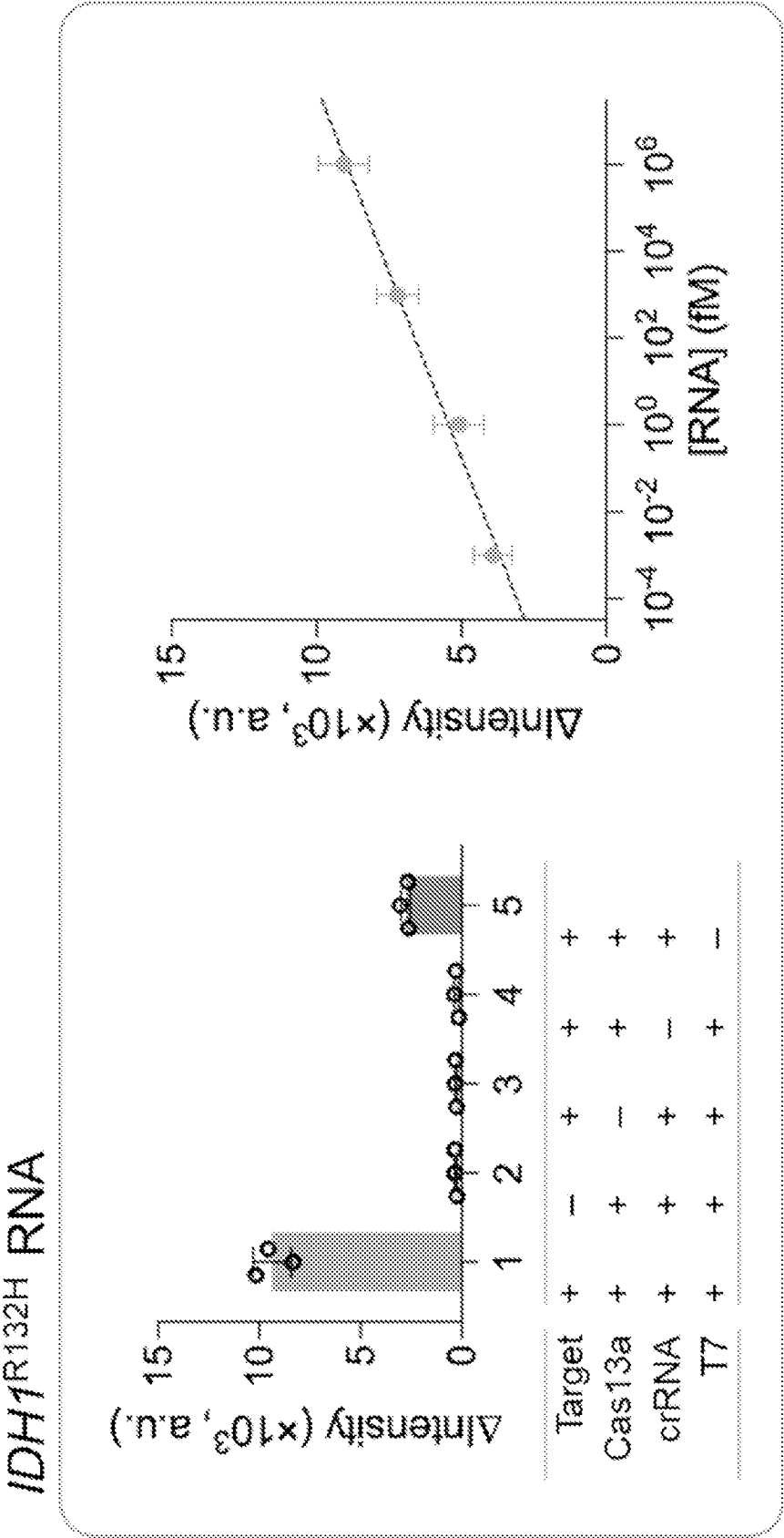


FIG. 28B

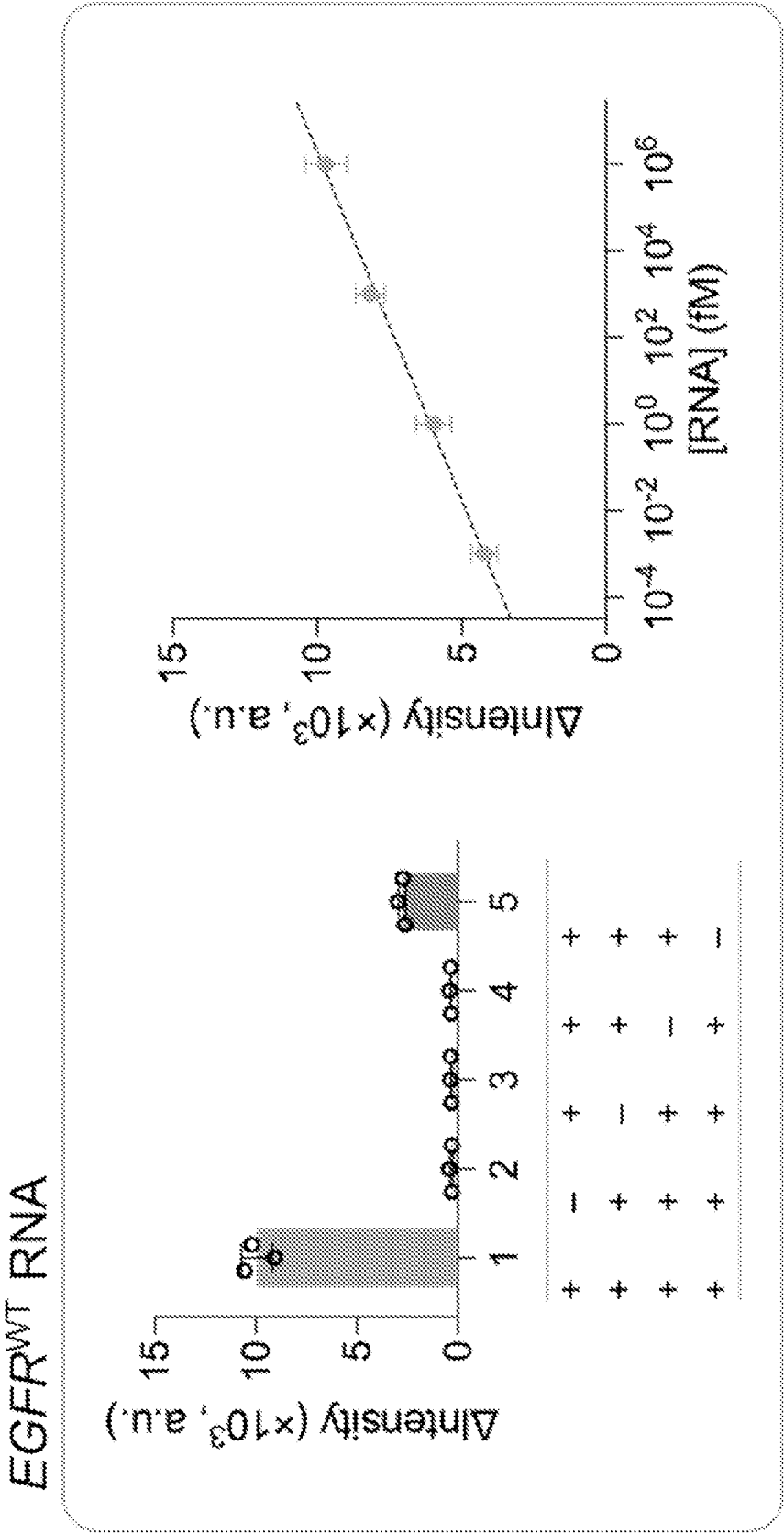


FIG. 28C

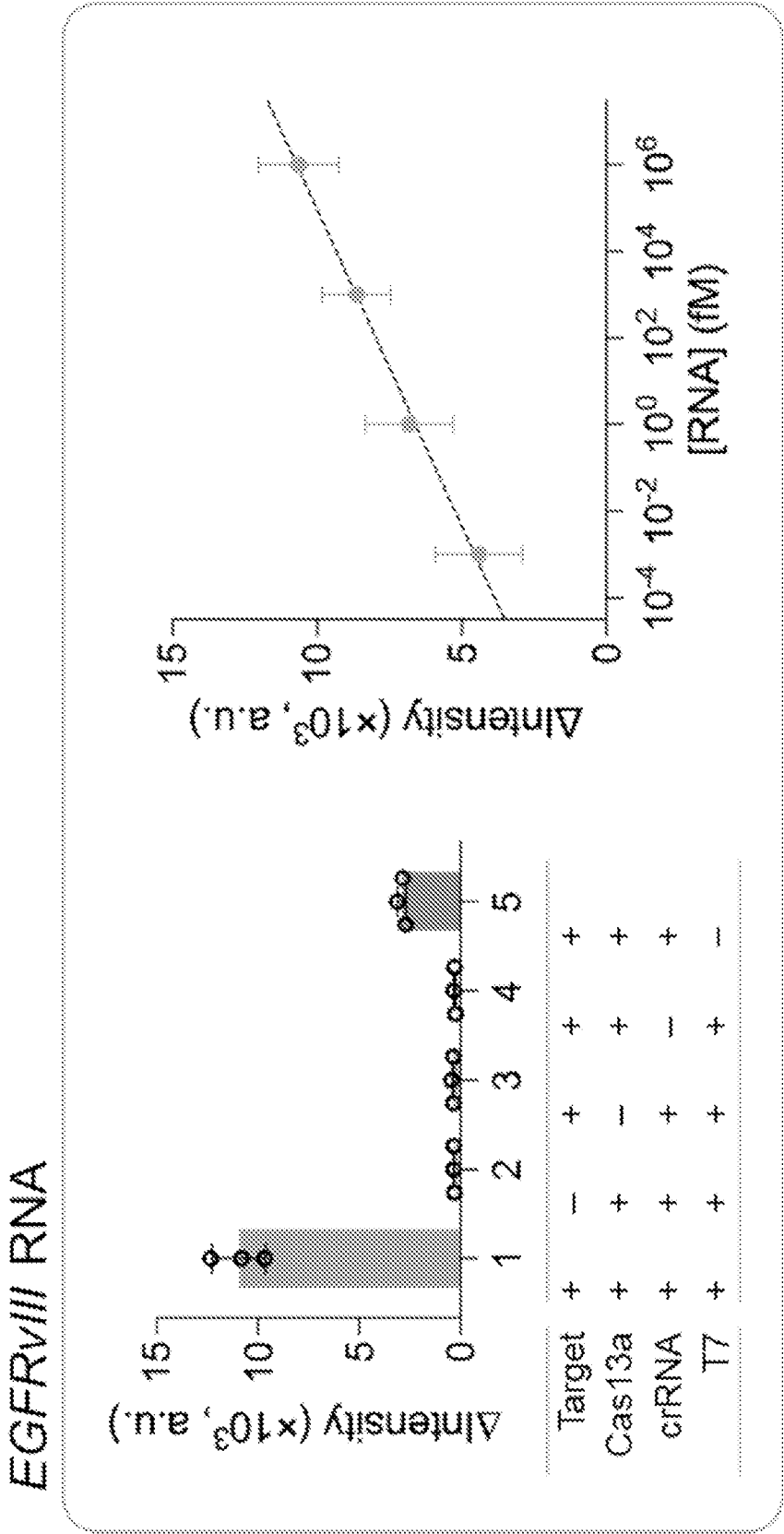


FIG. 28D

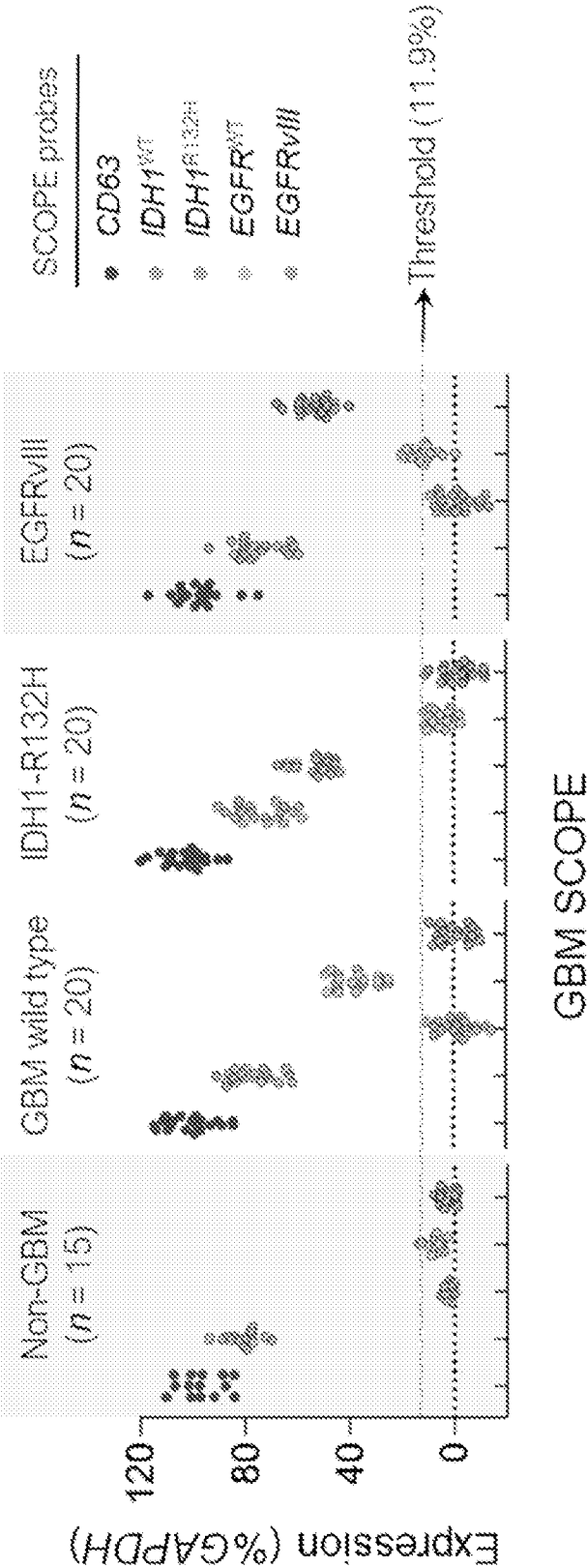


FIG. 29

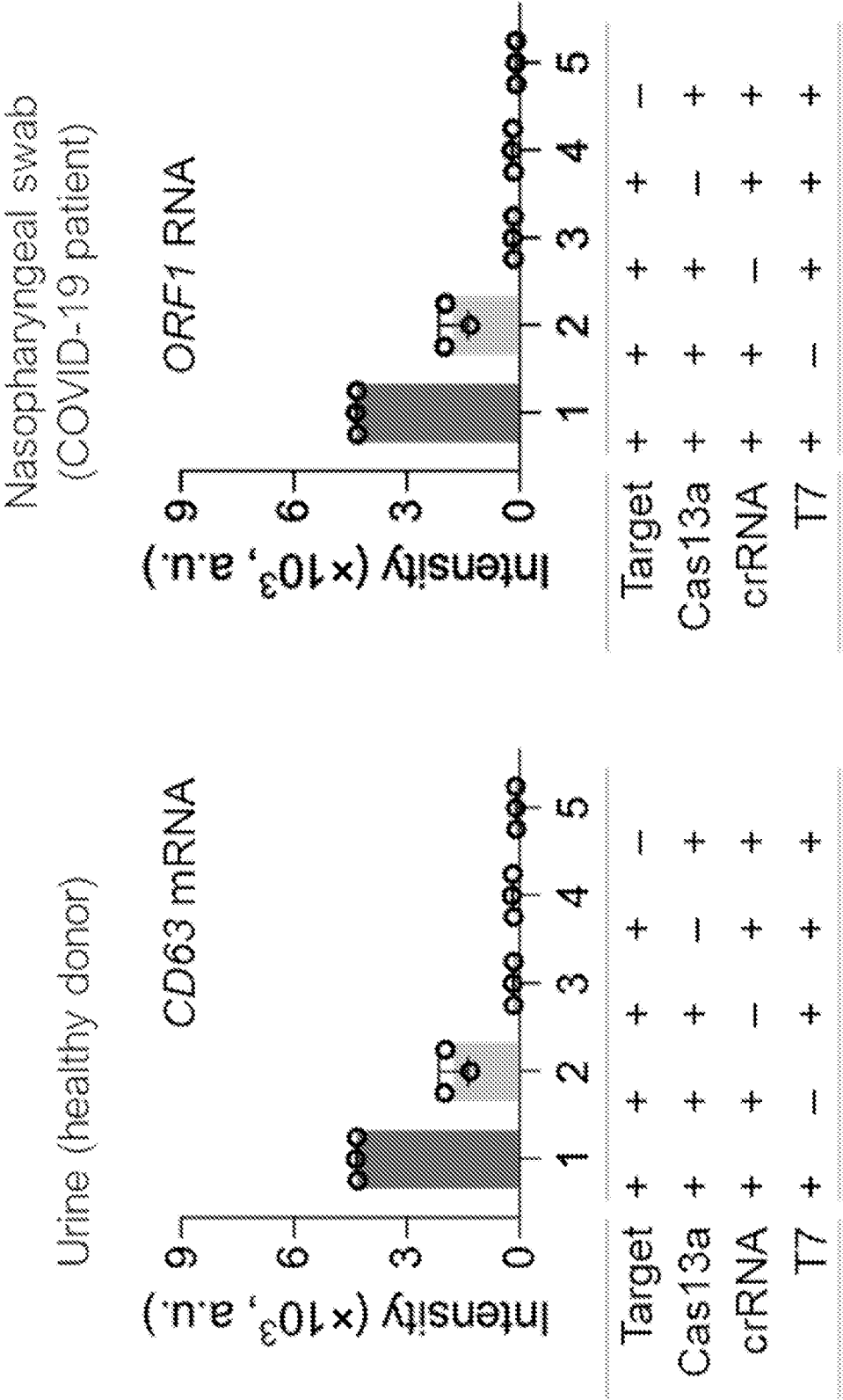


FIG. 30A

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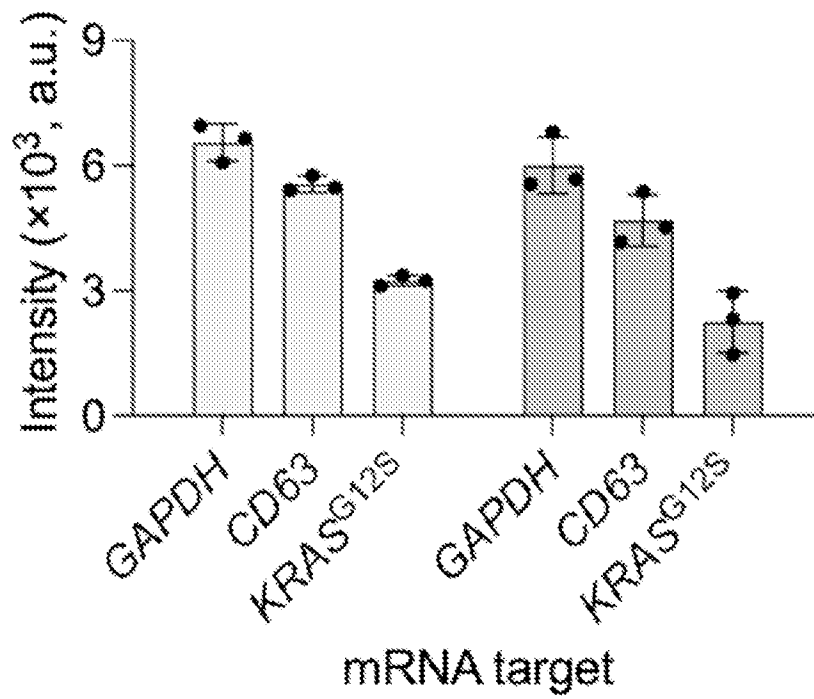


FIG. 30B

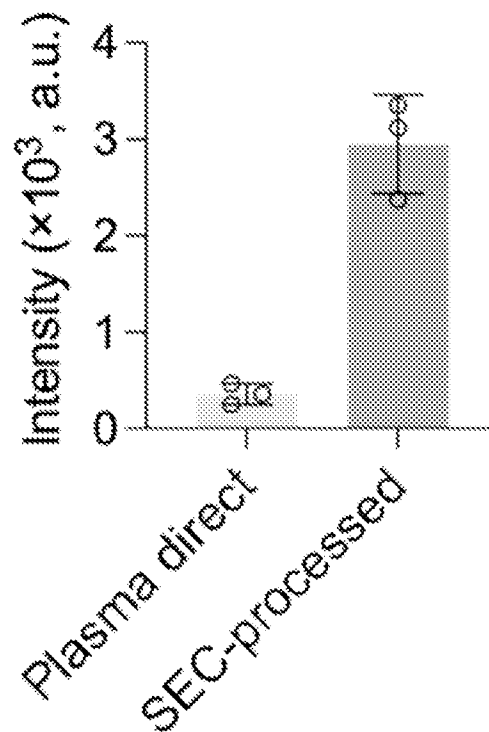


FIG. 30C

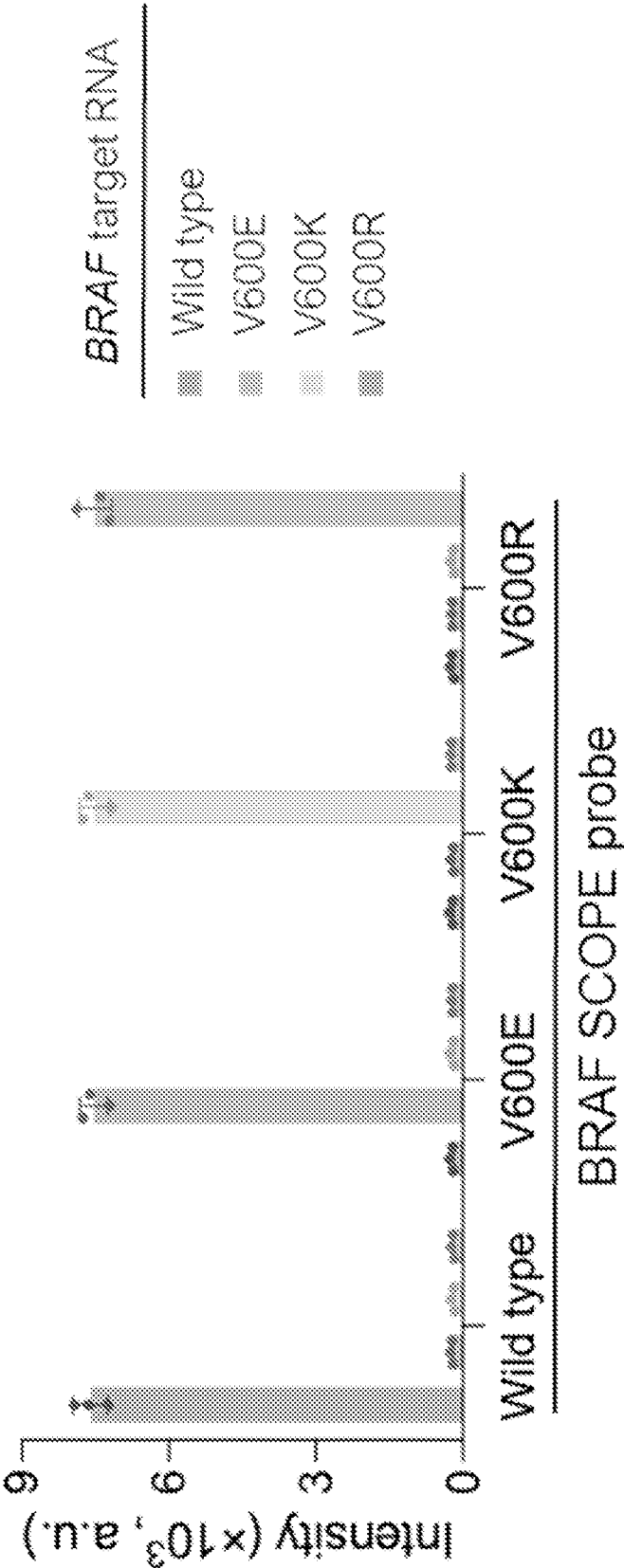


FIG. 31A

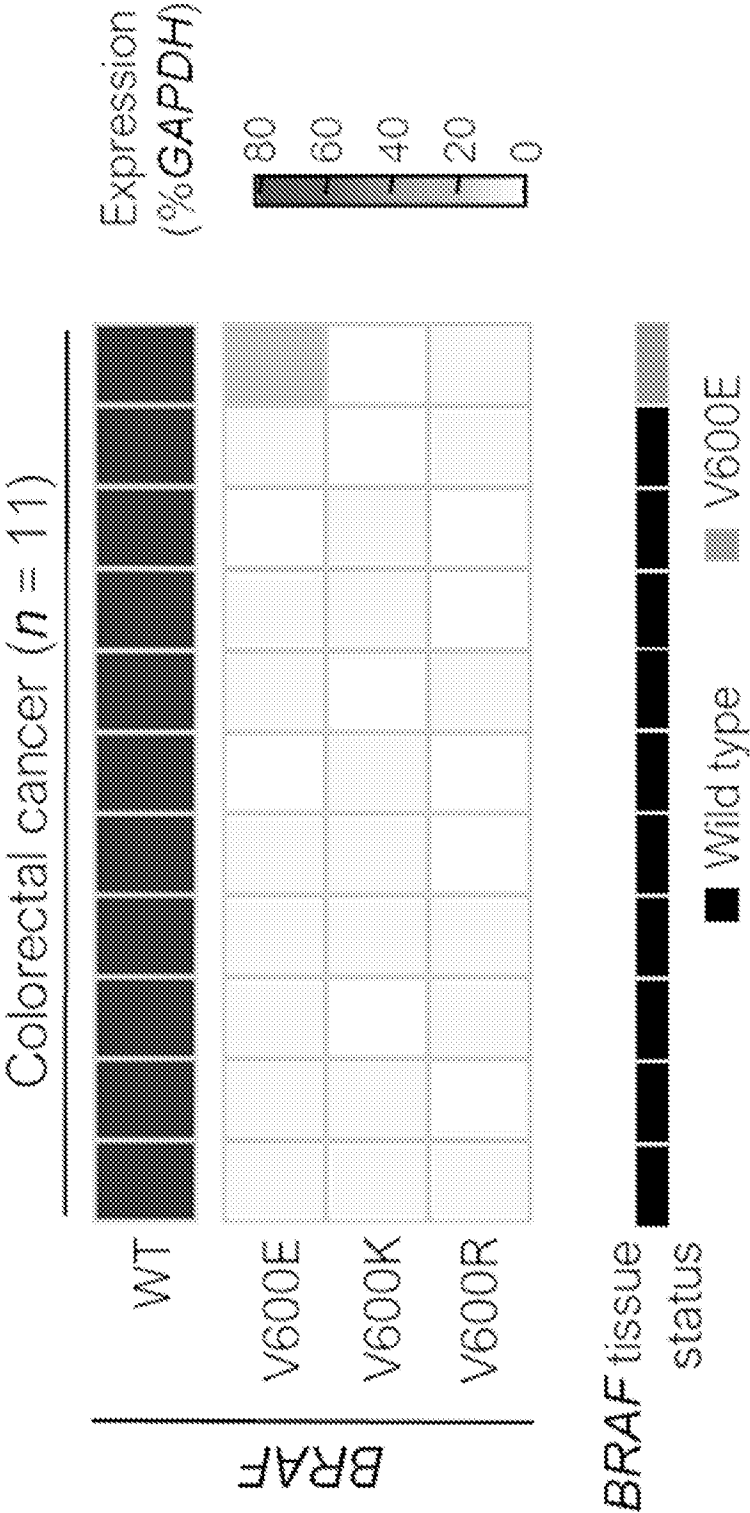


FIG. 31B

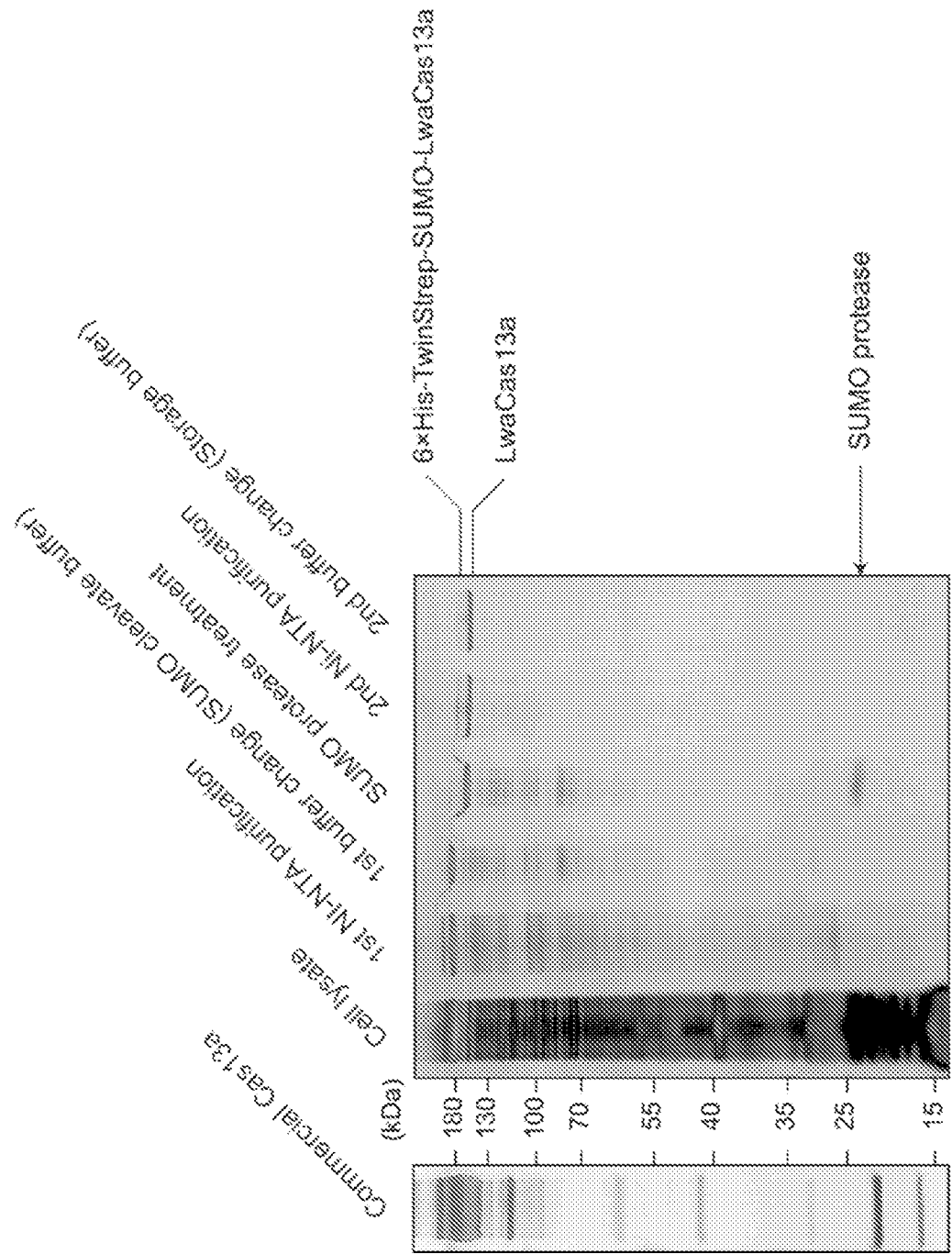


FIG. 32