An Allosteric Inhibitor of KRas Identified Using a Barcoded Rapid Assay Microchip Platform

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Supporting Information

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Materials

Unless otherwise stated all chemicals were used as received.

Barcode Microfabrication and Validation

Chrome masks of the custom barcode design were purchased from University of California, Los Angeles, Nanoelectronics Research Facility, and a Karl Süss MA/BA6 mask aligner (SÜSS MicroTec AG) was used for UV exposure. Silicon wafers (Wafernet Inc.), SU8-2025, and SU8 developer (Microchem Corp) were used for the barcode mold fabrication. Anhydrous dimethylsulfoxide (DMSO), sodium dodecyl sulfate (SDS), and bis(sulfosuccinimidyl)suberate) (BS3) used in barcode fabrication were purchased from American Type Culture Collection (ATCC), Sigma Aldrich, and ThermoFischer Scientific respectively. The Sylgard 184 elastomer, and poly-L-lysine coated glass slides used in DNA barcode microfabrication were purchased from Dow Corning and ThermoFischer Scientific respectively. The poly-L-lysine (PLL) solution (0.1% ($^{w}/_{w}$) used for barcode fabrication was purchased from Sigma Aldrich. All ssDNA used for barcode fabrication and barcode validations were purchased from either Bioneer Inc. or IDT Inc.

Protein Expression, Purification, and Refolding

The Bacto Tryptone (Tryptone) and Bacto yeast (yeast) for the preparation of LB broth media were purchased from Becton, Dickinson, and Company. The ampicillin sodium salt, chloramphenicol, and isopropyl β-D-1-thiogalactopyranoside (1,6-IPTG, dioxane free) used for protein expression from *E. coli* were purchased from Sigma Aldrich. The one-shot B21(D3) *E. coli* cells and PQE80 vector (His_6-tagged human KRas Isoform 4B (residues 1-169)) used for expression of KRas protein were purchased from Life Technologies and Qiagen respectively. Lysozyme (L6876), DNAse I (10104159001), and RNAse A (R6513-10MG) used for lysing cells containing cysteine-modified streptavidin (SAC) were purchased from Sigma-Aldrich.

Cells containing KRas protein were lysed using a constant pressure cell disruptor (Constant Systems Ltd., Scotland, UK). Surfactants Triton X-100 and polysorbate 20 (Tween20) were purchased from Sigma Aldrich. The 20x phosphate buffered saline with 0.05% Tween 20 (PBST) and phosphate buffered saline (PBS) used for protein purification and immunofluorescent assays (IFAs) were purchased from Cell Signaling Technology and Corning respectively. The sodium bicarbonate ($NaHCO_3$), ammonium acetate (NH_4OAc), sodium acetate (NaOAc), sodium chloride (NaCl), imidazole, tris(hydroxymethyl)aminomethane (Tris), tris(hydroxymethyl)aminomethane hydrochloride salt (Tris•HCl), guanidinium chloride (Guan•HCl), magnesium chloride pentahydrate ($MgCl_2•5H_2O$), and beta-mercaptoethanol (βME) used in protein purification and IFA assays were purchased from Sigma Aldrich. The 2-imminobiotin agarose resin, Superdex 75 (10/300) increase column, and Ni-NTA superflow cartridge used for fast protein liquid chromatography (FPLC) purification were purchased from Sigma Aldrich, GE Healthcare Life Sciences, and Qiagen respectively. The Amicon Ultra-15 and Ultra-4 centrifugal filters used to concentrate protein samples were purchased from EMD Millipore.

SAC-DNA Conjugation and Validation of Biotin Binding Affinity

The tris-(2-carboxyethyl)phosphine hydrochloride (TCEP), anhydrous N,N-dimethylformamide (DMF), N-succimidly-4-formyl benzaldehyde (S-4FB) and maleimide 6-hydrazino-nicotinamide (MHPH) used for the conjugation of ssDNA to cysteine-modified streptavidin (SAC) were purchased from Sigma Aldrich and Solulink. The biotin-A_{20}-Cy3 (Biotin*) probe used to test the biotin binding ability of the DESL set and used as a biotinylated blank for IFA assays was purchased from IDT Inc. The complementary ssDNA' used for conjugation to SAC were purchased from Bioneer Inc.

In Situ Library Screen and Hit Bead Sequencing

The mouse anti-biotin-alkaline phosphatase conjugated antibody (ab) (#A6561), goat anti-rabbit-alkaline phosphatase conjugated ab (#A8025), rabbit anti-Ras ab (CST #3965), used for the combined anti screen/pre-clear and the subsequent product/target screens were purchased from Sigma Aldrich and Cell Signaling Technology respectively. The combined 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/ nitro blue tetrazolium (NBT) (#S3771) used to develop hits during the library screens was purchased from Promega. The concentrated hydrochloric acid used to quench the BCIP/NBT development was purchased from Sigma Aldrich. Sequencing of bead hits occurred via Edman degradation sequencing on a Procise Protein Sequencer (Applied Biosystems, California).

Peptide Synthesis and Purification

Fmoc-protected amino acids were purchased from Anaspec, AAPTec, Bachem, ChemPep, and Sigma-Aldrich. Biotin NovaTag™ resin was obtained from EMD Chemicals, Inc. and used for the synthesis of biotinylated peptides and epitopes used for the screens using standard Fmoc/^{t}Bu coupling and cleavage protocols. The peptide one-bead-one compound (OBOC) library was prepared on Tentagel Resin purchased from RAPP Polymere. The Fmoc-protected propionic acid polyethylene glycol (PEG_n) linkers were purchased from ChemPep Inc. The L-ascorbic acid and copper (I) iodide (CuI) used for click reactions were purchased from Sigma Aldrich. The N-methyl pyrrolidine (NMP), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), N, N'-dimethyl formamide (DMF), and *N, N'*-diisopropylethylamine (DIPEA) used in peptide synthesis were bought from EMD Chemicals, Inc., ChemPep, and Sigma-Aldrich respectively. Piperidine, trifluoroacetic acid (TFA), and triethylsilane (TESH) were purchased from Sigma-Aldrich. The Cy3 carboxylic acid used to

prepare dye-labeled switch epitopes (lot #20007) was purchased from CyanDye LLC. The diethyl ether used to precipitate crude peptide was purchased from JT Baker. The Omnisolv grade acetonitrile (MeCN) used for peptide purification was purchased from EMD Millipore. Unless otherwise stated, peptide preparation was performed using a Titan 357 Automatic Peptide Synthesizer (AAPPTec, Louisville, KY) or a Liberty 1 Automated Peptide Synthesizer (CEM, North Carolina). Mass analysis was performed using a Voyager De-Pro matrix assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) (Applied Biosystems, California). The crude peptides were dissolved in either DMSO (Sigma Aldrich) or (1:1) MeCN/doubly distilled water (MQ H_2O) w/ 0.1% TFA before purification by a gradient of 0% to 50% acetonitrile in MQ H_2O with 0.01% ($^{v}/_{v}$) TFA using a RP-HPLC (Beckman Coulter System Gold 126 Solvent Module and 168 Detector) using a C18 reversed phase semi-preparative column (Phenomenex Luna 10 µm, 250 × 10 mm). The concentration of peptides and epitopes was determined using a Nanodrop 2000 Spectrophotomer (ThermoFischer Scientific Inc., Massachusetts).

B-RAP Immunofluorescent Assays and Multi-Well Enzyme-Linked Immunosorbent Assays

The Bovine Serum Albumin (BSA, Biotin free A1933-25G) used in the IFAs and multi-well enzyme-linked Immunosorbent assays (ELISAs) was purchased from Sigma-Aldrich. The non-fat dry milk powder used in the enzyme-linked Immunosorbent assays (ELISAs) and the epitope binding study was purchased from Best Value. The rabbit anti-Ras (CST #3965), Goat anti-rabbit IgG HRP-linked (CST #7074), goat anti-rabbit HRP-linked (CST #7074), rabbit anti-MEK1/2 (CST #9122S), and goat anti-rabbit-Alexafluor 647 conjugated (ab150079) were purchased from Cell Signaling Technologies and Abcam respectively. The recombinant human HRas protein (ab93949) and recombinant human MEK1 protein (#MI14420) for the protein

selectivity study were purchased from Abcam and Fischer Scientific respectively. The ELISAs were run on either 96-well clear Pierce Neutravidin Plates (#15129) or Pierce Neutravidin Coated Plates (#15127) purchased from ThermoFischer Scientific. The epitope binding study was run on 96-well Pierce Neutravidin coated black plates (#15217) from ThermoFischer Scientific. The TMB Microwell Peroxidase Substrate System (#50-76-00) that was used to develop ELISAs was purchased from KPL. The sulfuric acid ($H_2SO_{4(aq)}$) used to quench the enzymatic amplification reaction in the ELISAs was purchased from JT Baker. The 96-well ELISA plates were read using a Flexstation 3 plate reader (Molecular Devices LLC, Sunnyvale, CA). All barcode slides were scanned using an Axon GenePix 4400A (Molecular Devices LLC, Sunnyvale, CA).

Measuring the Functional Effect of the Allosteric Ligands on KRas Protein GTPase Activity

The intrinsic GTPase activity of WT KRas protein was measured using the GTPase-Glo Assay Kit (#V7681) from Promega Corporation (Madison, WI) on opaque white 96-well plates (#6005290) from Perkin Elmer Life Sciences (Waltham, MA). Luminescence was recorded on the Flexstation 3 plate reader used for multi-well ELISAs.

Protocols

DNA Barcode Chip Patterning

The DNA barcode chips were prepared by micro channel-guided flow patterning as described in **References 27** and **28** of the main text. A PDMS slab having the micro-channels was made by soft lithography on a silicon wafer. Its mold was designed as Figure S1 and prepared with SU8 2025 negative photoresist. The fabricated mold contained microfluidic circuits of 20 parallel channels with 50 μm width and ~40 μm height. Sylgard® 184 PDMS pre-polymer and curing agents were mixed in a 10:1 ratio, degassed, ~60 g of the mixture poured onto the mold, and

baked for two hours at 80 °C for curation. The cured PDMS slab was peeled off from the mold, cut into individual microfluidic molds, and the inlet and outlet holes of the microfluidic circuits were punched with the sizes of two mm and 0.5 mm respectively. The number of the inlets and outlets punched out were determined by the number of single stranded DNAs (ssDNAs) used in the assay, and fifteen orthogonal ssDNAs (**B-Q**, Table S2) were used in this study. The slab was then aligned with a PLL glass slide, and bonding occurred with baking at 80 °C for two hours. After cooling briefly, the inlet wells were loaded with 3 μL of a PLL solution (0.1% ($^{m}/_{m}$) in H_2O) , and the PLL solution was flowed and dried by 13.8 kPa nitrogen gas blowing through the solution-loading device overnight. The next day, C6 amine-modified DNA solutions (300 μM in (3:2 ($^{v}/_{v}$)) PBS/DMSO) were individually mixed (1:1) with a 2 mM BS3 cross-linker solution in PBS. Each freshly prepared mixture was flown through a channel under 13.8-20.6 kPa of nitrogen gas using the solution-loading device for 1 hour, and then only the assembled PDMS slab and the bonded PLL slide was incubated at room temperature for 2 hours in a humidified chamber. After incubation, the PDMS slab was removed, and the DNA patterned PLL slides were washed with a 0.02% aqueous SDS solution, doubly distilled water (MQ H_2O) (3x), and spun dry.

Barcode Validation Procedure

To validate the DNA barcode chips, a 5'-modified Cy3-labeled complementary ssDNA cocktail was prepared in 1% BSA in PBS (50nM each ssDNA). The validation occurred over two rounds (B, D, F, H, K, N, P, M then C, E, G, I, L, O, Q) in order to check for channel leaks and crossover. A 120 μL aliquot of the validation solution was applied to a small region at the bottom edge of the DNA barcode before incubating at 37 °C for one hour. After incubation, this region

was washed with 1% BSA in PBS followed by PBS (2x), and the slide was spun dry before being scanned by Axon GenePix 4400A (532 nm, PMT 450, Power 15% (23W)) (Figure S2).

Expression of Cysteine-Modified Streptavidin (SAC) Protein

The SAC protein was expressed using a modification of the procedure reported by Sano and Cantor.[1] A 100 mL starter culture of autoclaved LB media (10.0 g Tryptone, 5.00 g yeast, 10.0 g NaCl per L H_2O) was prepared by inoculating with 50 µL of 100 $^{mg}/_{mL}$ of ampicillin (final concentration 50 $^{\mu g}/_{mL}$) and 100µL of 34 $^{mg}/_{mL}$ chloramphenicol (final concentration 34 $^{\mu g}/_{mL}$) followed by a sterile pipet scraping of a 50% ($^{v}/_{v}$) glycerol stock containing transformed *E. coli* BL21(D3) cells. The starter culture incubated overnight at 37 °C and 250 RPM before adding 10.0 mL of starter culture aliquots to six 2800mL Fernbach-Style Culture Flasks containing 1.00 L autoclaved LB media with 500 µL of 100 $^{mg}/_{mL}$ of ampicillin (final concentration 50 $^{\mu g}/_{mL}$), 1000 µL of 34 $^{mg}/_{mL}$ chloramphenicol (final concentration 34 $^{\mu g}/_{mL}$), and 1000µL of 40% ($^{w}/_{w}$) autoclaved glucose (final concentration 0.4% ($^{w}/_{w}$). The flasks were left to culture at 37.0 °C, 250 RPM until A_{600} = 0.500, and induction was triggered with 1000 µL of a 400 mM 1,6-IPTG solution (final concentration 400 µM). Expression continued at 37.0 °C, 250 RPM for four hours before spinning down the cells at 6000 RPM, 5 minutes at 4 °C. The cells were resuspended in 50 mL of a 10 mM Tris, 1 mM EDTA, 130 mM NaCl buffer at pH=8.0 and spun down (2x). The cells were then flash frozen in $N_{2(l)}$ and stored at -80.0 °C until needed.

Isolation of SAC Inclusion Bodies from e. Coli Cells

The cell pellet was thawed in ice before resuspending in two 50-mL falcon tubes with 40 mL of TEX buffer (30mM Tris, 2mM EDTA, 0.1% TritonX). Each tube was charged with 40 mg fresh lysozyme powder (Final concentration 1.0 $^{mg}/_{mL}$), vortexed until mixed, and allowed to lyse for 30min while tumbling at RT. The solution was very viscous after lysis. The DNA and RNA

were degraded by adding 400 µL of 1.0 $^{mg}/_{mL}$ DNAse and 1.0 $^{mg}/_{mL}$ RNAse in TE Buffer (10 mM Tris, 130 mM NaCl, 1 mM EDTA) (final concentration 10 $^{µg}/_{mL}$), 960 µL of 500 mM $MgCl_2$ (final concentration 12 mM), and 40 µL of 1 M $MnCl_2$ (final concentration 1 mM) to each tube of cell lysate, and the solution was allowed to digest for 30 minutes while tumbling at RT. After digestion, the solution was spun down at 7800 RPM, RT for 10 minutes. The resulting inclusion body (IB) pellets were both washed in 40 mL TEX buffer and spun down at 7800 RPM, 5 minutes at RT. Pellets were washed with 40 mL buffer minus Triton X again before spinning down at 7800 RPM, 5 minutes at RT once more. Each pellet was taken up in 10 mM Tris and spun down at 7800RPM, 10 minutes at RT, aliquotted, and stored at -80.0 °C until needed. If the final pellet is light brown then some DNA is still present. This will be removed at the beginning of the refolding procedure.

Refolding and Purification of SAC Protein

The procedure described here is a modification of the procedure developed by Sano and Cantor.[1]

*****After the initial denaturing keep all solutions at 4 °C*****

An IB aliquot was dissolved in 1000 µL denaturing buffer (6 M Guanidine • HCl at pH=1.5 with 10 mM βME), vortexed, spun down at 13,000 RPM, 2min at RT, and filtered using a 0.45 µM low-protein binding filter. **The resulting solution should be clear and nearly colorless.** The A_{280} was measured on a Nanodrop2000 spectrophotometer, and the concentration of denatured SAC monomer was calculated.[2] The denatured SAC solution was diluted to 1000 µL in denaturing buffer and added dropwise to a rapidly stirring solution of refolding buffer (50 mM NH_4OAc, 150 mM NaCl, and 10 mM βME at pH=6.0) (Final [denatured SAC] ~4 µM). The stir rate was then decreased to about half of its original value, and the solution was covered by

aluminum foil to refold overnight. After sterile filtration with a 0.45 μm low-protein binding filter the resulting solution was concentrated to 10-15 mL using Amicon Millipore filters (10,000-30,000 MWCO) before dialyzing the refolded SAC protein in buffer A (50 mM $NaHCO_3$, 500 mM NaCl, 10 mM βME at pH=11.0) until the solution had a pH of ~11 (about 2 hours). The crude protein was then diluted (1:1) with buffer A, mixed with 2 mL of 2-iminobiotin agarose resin, and allowed to incubate with tumbling in the cold room for one hour. After incubation the supernatant was eluted (3x) before eluting with buffer A until the A_{280} went to baseline. Pure SAC was eluted with buffer B (50 mM NaOAc, 50 mM NaCl at pH=4.0) until the A_{280} went to baseline again. Fractions with pure SAC were pooled and dialyzed against a PBS solution (PBS, 10 mM βME, pH=7.5) overnight. The SAC was concentrated to ~1 $^{mg}/_{mL}$ final concentration, divided into 100 μL aliquots, and stored at -80 °C.

Preparation of SAC-DNA Conjugates

For each planned SAC-DNA conjugation, two Zeba columns were prepared (3 x 300 μL of 5 mM TCEP in PBS, 3.9k RPM, 1 min at RT). Each 100 μL aliquot of SAC was desalted in two separate Zeba columns to remove the βME (3.9k RPM, 2 min at RT). After transferring to eppendorf tubes, 6 μL anhydrous DMF was added followed by 6 μL MHPH (100 mM in anhydrous DMF). Separate eppendorf tubes were charged with 80 μL of 500 μM of conjugation ssDNA in PBS followed by 15 μL anhydrous DMF and 20 μL S-4FB (100 mM in anhydrous DMF). The SAC and DNA solutions were vortexed gently, briefly spun down, and left to react at RT in the dark for four hours. For each conjugation in progress, four Zeba columns were buffered exchanged with citrate buffer (150 mM NaCl, 50 mM sodium citrate, pH=6.0) (3 x 300 μL citrate buffer, 3.9k RPM, 1 min at RT). The SAC and DNA solutions were desalted separately (2 x 3.9k RPM, 2 min, at RT) before combining each SAC protein aliquot with a

unique ssDNA solution. The solutions were vortexed gently, briefly spun down, and left to react in the dark at RT overnight. The reactions were quenched by placing at 4 °C. Each SAC-DNA conjugate was purified by FPLC using a Superdex75 Increase column (isocratic in PBS, 0.5 $^{mL}/_{min}$, 0.5 mL fractions, 75 minutes). Fractions containing pure SAC-DNA were pooled and concentrated using Amicon Ultra-4 Centrifugal filters (30k MWCO): 3900 RPM, 30 minutes at 4 °C. The concentrated SAC-DNA proteins were quantified[3] using a Nanodrop2000 spectrophotometer in the ssDNA nucleic acid mode (using two for the average number of ssDNA strands conjugated as previously established) and stored at 4 °C until needed.

Biotin Binding Test of SAC-DNA Conjugates

Buffers used:

Wash buffer: PBS + 0.05% Tween20 (PBST)

Blocking Buffer: PBS + 1% BSA

***Wash steps used 50 $^{\mu L}/_{well}$**

***Incubation steps used 30 $^{\mu L}/_{well}$**

****After loading the Biotin* probe change pipette tips every time that you aspirate or add solution to a well to prevent cross contamination****

A prefabricated PDMS template was aligned onto the DNA barcode and the microchip slide was taped into a 10 cm petri dish. The wells were washed with PBST before loading blocking buffer and placing the platform into a 37 °C incubator for one hour. A cocktail containing 50 nM of each SAC-DNA in PBS was prepared and added to the pre-blocked wells. The SAC-DNA conjugates were allowed to hybridize to the DNA barcode at 37 °C for one hour before washing the wells with PBST (3x). Each well was loaded with 50 nM, 100 nM, 150 nM, 200 nM, 300 nM, or 400 nM Biotin* in PBS (Figure S5), and the platform was left to shake covered at RT for one

hour. The wells were washed with PBST (3x) before peeling off the PDMS slab and dipping the barcode into PBS, (1:1) PBS/MQ H_2O, MQ H_2O (2x). The barcode was then spun dry and read on the Genepix (532 nm, PMT 450, Power 15% (23 W)).

WT KRas Protein Expression and Purification

The KRas protein was expressed and purified using a modification of the procedure reported by Kuriyan.[4] A starter culture of 100 mL of autoclaved LB media was inoculated with 100 μL of 100 $^{mg}/_{mL}$ of ampicillin (final concentration 100 $^{\mu g}/_{mL}$) followed by a scraping of a 25% ($^{v}/_{v}$) glycerol stock containing transformed *E. coli* (BL21(DE3)) cells. The starter culture was left in an incubator at 37.0 °C, 250 RPM overnight before adding 10.0 mL starter culture aliquots to six 2800 mL Fernbach-Style Culture Flasks containing 1.00 L autoclaved LB media with 1000 μL of 100 $^{mg}/_{mL}$ of ampicillin (final concentration 100 $^{\mu g}/_{mL}$). The flasks were left to culture at 37.0 °C, 250 RPM until A_{600} = 0.500-0.600 and induction was triggered with 1000 μL of a 250 mM 1,6-IPTG solution (final concentration 250 μM). The cells were then left to express overnight at 18.0 °C, 250 RPM before being spun down, resuspended in buffer A (20 mM Tris, 500 mM NaCl, 20 mM imidazole, 5 mM $MgCl_2$, pH=8.0), flash frozen in $N_{2(l)}$, and stored at -80.0 °C until needed. After thawing and douncing, the cells were lysed using a cell disruptor, the cell wall lysate spun down at 8000 RPM, 4 °C for 20 minutes, sterile filtered with a 0.45 μm low-protein binding filter, and purified using FPLC with a Ni-NTA superflow cartridge and a gradient of buffer A to buffer B (20 mM Tris, 300 mM NaCl, 250 mM imidazole, 5 mM $MgCl_2$, pH=8.0). Fractions containing pure KRas were pooled and dialyzed against Tris buffered saline (TBS) (25 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, pH=7.5) overnight. The resulting solution was concentrated using Amicon Ultra-15 centrifugal filters (10k MWCO), quantified,[2] separated into aliquots, flash frozen in $N_{2(l)}$, and stored at -80.0 °C until needed.

In Situ Library Click Screen Combined Preclear/Anti-Screen

The *in-situ* click dual SynEp library screen followed a procedure similar to the one outlined in **Reference 1** from the main text using 450 mg of Pra-capped one-bead-one-compound (OBOC) library. Blocking was performed overnight at 4 °C with blocking buffer (1% BSA and 0.1% Tween20 in TBS). After washing with blocking buffer (3 x 3 minutes) incubation with 25 μM of each scrambled SynEp in binding buffer (0.1% BSA and 0.1% Tween20 in TBS) occurred overnight at 4 °C. The library was washed with TBS (3 x 1 minute) then stripped with 7.5 M Gua•HCl (pH=2.0) at RT for one hour to remove any non-covalently bound scrambled SynEps. Ten rinses with TBS preceded another incubation with blocking buffer at RT for one hour. After five quick rinses of the library with blocking buffer the library was incubated with a cocktail of a (10,000:1) dilution of the mouse anti-biotin-alkaline phosphatase conjugated ab (#A6561), (1,000:1) dilution of the rabbit anti-Ras ab (CST #3965), and a (10,000:1) dilution of the goat anti-rabbit-alkaline phosphatase ab (#A8025) in binding buffer to perform the preclear and anti-screen in one assay. Washes (5 x 3 minutes) with a high salt buffer (25 mM Tris•HCl, 10 mM $MgCl_2$, 700 mM NaCl, pH=7.4), and a low salt buffer (5 x 3 minutes) (25 mM Tris•HCl, pH=7.4). The developing buffer was prepared with 66 μL of BCIP (50 $^{mg}/_{mL}$ in 70% DMF) in 10mL of developing buffer (100 mM Tris•HCl, 150 mM NaCl, 1 mM $MgCl_2$) and incubated with the library beads in a 20cm petri dish for ten minutes before adding 66 μL of NBT (50 $^{mg}/_{mL}$ in 70% DMF) and incubating for an additional fourteen minutes. The beads were then washed 5x with TBS, and stored in 0.1 M $HCl_{(aq)}$ in a 20 cm petri dish. Any beads that turned purple during the combined preclear/anti-clear were promiscuous binders and consequently were picked out using a 10-μL micropipette and discarded. After removing all of the sticky beads the remaining beads were washed with 7.5 M Guan•HCl (pH=2.0) for 30 minutes, rinsed with MQ H_2O (10x),

and incubated in NMP overnight to remove any trace purple coloring. Final rinses with MQ H_2O (3x), TBS (7x) preceded an overnight incubation at 4 °C with blocking buffer.

In-Situ Library Click Screen Product/Target Screens

The pre-blocked library was washed with blocking buffer (3 x 5 minutes) before loading 25 μM of each SynEp in binding buffer and incubating at RT overnight. After rinsing with TBS (3x) the library was incubated with 7.5 M Guan•HCl (pH=2.0) for one hour at RT before rinsing with TBS (10x). The library then underwent an additional one hour incubation with blocking buffer at RT before rinsing with blocking buffer (5x), and incubating with a (10,000:1) dilution of the mouse anti-biotin-alkaline phosphatase conjugated ab in binding buffer for one hour at RT. Development of the library followed the same procedure as the preclear/anti-screen, and the darkest beads were set aside for Edman degradation sequencing. The remaining ~50 light purple beads from the product screen were prepped following the same procedure after the preclear/anti-screen and screened again, using appropriately scaled amounts of reagents, against 25 μM of the full-length KRas protein. After developing additional beads were picked for a total of seven dark beads from the product/target screens of which five beads yielded readable sequences by Edman degradation sequencing.

Peptide Synthesis Protocols

All cyclic peptides and epitopes were prepared following the procedures outlined in **Reference 1**.

The peptides and epitopes were isolated using the following procedure. The resin was rinsed with DCM (5x) and dried under vacuum. A 20 mL scintillation vial was charged with a stir-bar, resin, and 3-5 mL cleavage solution (95% TFA, 2.5% TESH, 2.5% H_2O) and allowed to stir at room temperature for 2-2.5 hours. The solution was then filtered into 40 mL of cold diethyl ether, vortexed for 10 seconds, and stored at 4 °C overnight. The precipitated protein was

centrifuged into a pellet at 4500 RPM for 10-15 minutes prior to decantation of the supernatant. The crude peptides were dissolved in either DMSO or (1:1) MeCN/H_2O w/ 0.1% TFA before HPLC purification, and lyophilization of desired fractions. The resulting lyophilized powder was dissolved in DMSO, quantified at λ= 280 nm,[2] and stored at 4 °C when not in use.

The Cy3- dye was coupled onto the N-terminus of the PEG_2-conjugated switch Switch peptides using the following procedure. The coupling solution was prepared by dissolving Cy3-CO_2H dye (100 mg, 0.103 mmol) 3.00 mL DMF with HATU (0.103 mmol) and DMF (0.309 mmol). Coupling using 2.00 equivalents of the dye relative to the resin occurred in a SPPS tube covered in aluminum foil over 24 hours before draining and rinsing the resin with NMP (7x), DMF (5x). The resulting pink resin was cleaved following the above procedure, and the precipitated peptide lyophilized after centrifugation and decanting of the ether supernatant. The lyophilized powder was analyzed using MALDI and carried on without HPLC purification. After dissolving in DMSO the dye-labeled epitopes were quantified at λ= 550 nm (ε= 150,000 $M^{-1}cm^{-1}$) and stored protected from light at 4 °C when not in use.

Measurement of Allosteric Ligand KRas EC_{50} Values with the B-RAP Technology

Buffers used:

Wash buffer: PBS + 0.05% Tween20 (PBST)

Blocking Buffer: PBS + 1% BSA

Protein Incubation Buffer: Tris-buffered saline (TBS) + 0.05% Tween20 (TBST)

1° ab buffer: PBS + 5% BSA

2° ab buffer: PBS + 1% BSA

***Wash steps used 50 $\mu L/_{well}$**

***Incubation steps used 30 $\mu L/_{well}$**

****The plate must be covered during incubation steps to protect the fluorescent blank****

****After loading the KRas protein change tips every time that solution is aspirated or added to a well to prevent cross-contamination****

A pre-fabricated PDMS template was aligned onto the DNA barcode microchip, and the microchip was taped into a 10 cm petri dish. The wells on the platform were wet with 50 μL PBST before filling with blocking buffer and placing into a 37 °C incubator for 1hr. Concurrently, 40 μL 1% BSA in PBS solutions containing 750 nM of a SAC-DNA conjugate and 3.75 μM of one biotinylated PCC ligand or biotin-A_{20}-Cy3 blank were prepared for each SAC-DNA conjugate. The biotinylated ligands were allowed to complex with the SAC protein for one hour before pooling the SAC-DNA-ligand solutions (final [SAC-DNA-ligand conjugates] = 50 nM). The blocking buffer was aspirated and each well was loaded with the SAC-DNA-ligand conjugates cocktail for hybridization with the DNA barcode at 37 °C for one hour. The wells were washed with PBST (3x) before loading serially diluted solutions of KRas protein in protein buffer (0 to 400 μM). After shaking at RT for one hour, the wells were rinsed with PBST (5x), making sure to pipet up/down with the first addition of PBST. A (100:1) dilution of CST rabbit anti-Ras Ab (#39655) in 1° ab buffer was added to each well before shaking at RT for one hour. After rinsing the wells with PBST (3x), the wells were loaded with a (200:1) dilution of Abcam goat anti-rabbit-Alexafluor 647 linked ab (ab150079) in 2° ab buffer before shaking at RT for one hour. A final rinse of the wells with PBST (3x) proceeded peeling off the PDMS slab from the barcode microchip and dipping the barcode into the following solutions: PBS, (1:1) PBS: MQ H_2O, MQ H_2O (2x). After being spun dry, the barcode was read on the Genepix (635 nm, PMT 600, PWR 40% (60 W); 532 nm, PMT 450, PWR 15% (23 W)). Data was extracted using 10 $^{\text{data blocks}}/_{\text{barcode lane}}$, double background corrected using the ab only

well fluorescence and dummy ligand fluorescence in each well, and graphed in Graphpad (Sigmoidal 4PL mode with the Hill coefficient set=1). The peeled off PDMS slab was rinsed under MQ H_2O and stored in MQ H_2O at RT until further use.

Measurement of Allosteric Ligand KRas EC_{50} Values using the Multi-Well ELISA Technology

Buffers used:

- Blocking Buffer: TBS + 5% milk + 0.05% Tween20
- Antibody (ab) Buffer: TBS, 5% BSA, 0.05% Tween20
- Binding Buffer: TBS, 0.1% BSA, 0.05% Tween20

***All steps were completed at room temperature**

***All wash steps used 200 $\mu L\ solution/well$**

***All incubations used 100 $\mu L\ solution/well$ except for the 5% milk blocking step, which used 200 $\mu L\ solution/well$**

A 96-well Pierce Neutravidin Plate was washed with binding buffer (3 x 5 minutes at RT) before loading plate with a 1μM solution of either blank (biotin-PEG_5-NHAc) (singly) or biotinylated PCC ligand (in triplicate). The plate incubated for two hours before washing with binding buffer (3 x 5 minutes). Blocking buffer was added to each well and the plate blocked for one hour before undergoing washing with binding buffer (3 x 5 minutes). Each well was loaded with either binding buffer or KRas solution (0→300 μM), and the plate was incubated for 30 minutes. Plate washing with binding buffer (3 x 5 minutes) preceded incubating the plate with a (1000:1) dilution of 1° antibody (ab) rabbit anti-Ras (CST #3965) in ab buffer for thirty minutes. The plate was washed with binding buffer (3 x 5 minutes), loaded with a (2000:1) dilution of 2° ab goat anti-rabbit, HRP-linked ab (CST #7074) in ab buffer, and incubated for an additional thirty minutes. The plate was rinsed with binding buffer (3 x 5 minutes), TBS (1 x 5 minutes),

loaded with a (1:1) mixture of TMB Peroxidase Solution and TMB Peroxidase Solution B, and developed with occasional agitation for 8-12 minutes. After quenching the enzymatic reaction with 1M $H_2SO_{4(aq)}$ (100 μL) the plate was read at λ= 450 nm within ten minutes. The data was double background corrected using the ab only absorbance and the dummy ligand absorbance, plotted using Prism GraphPad 7 (Sigmoidal 4PL mode with the Hill coefficient set=1), and an EC_{50} value was calculated.

Epitope Binding Study

Buffers used:

- Blocking Buffer: PBS + 5% milk + 0.05% Tween20
- Binding Buffer: PBS, 0.1% BSA, 0.05% Tween20

***All steps were completed at room temperature**

***All wash steps used 200 $^{\mu L\ solution}/_{well}$**

***All incubations used 100 $^{\mu L\ solution}/_{well}$ except for the 5% milk blocking step, which used 200 $^{\mu L\ solution}/_{well}$**

A black 96-well Pierce Neutravidin Plate was washed with binding buffer (3 x 5 minutes at RT) before loading plate with a 1 μM solution of either blank (biotin-PEG_5-NHAc) or ligand fractions **L1a**, **L2**, or **L8.** The plate incubated for two hours before washing with binding buffer (3 x 5 minutes). Blocking buffer was added to each well and the plate blocked overnight at 4 °C before undergoing washing with binding buffer (3 x 5 minutes). Incubation occurred in quadruplicate with either binding buffer or 25 μM of either Cy3-labeled switch 1 or Cy3-labeled switch 2, and the plate was incubated for one hour at RT. Plate washing with binding buffer (2 x 5 minutes) and PBS (1 x 200 μL) preceded loading the wells with 200 μL PBS and reading the plate on a Flexstation 3 platereader in fluorescence mode (excitation wavelength λ= 532 nm,

emission wavelength λ= 568 nm, filter cut-off λ= 550nm, 6 $^{reads}/_{well}$). The data was doubly background corrected and plotted using Prism GraphPad 7.

Probing the Selectivity of Lead Allosteric Ligands

Buffers used:

Wash buffer: PBS + 0.05% Tween20 (PBST)

Blocking Buffer: PBS + 1% BSA

Protein Incubation Buffer: Tris-buffered saline (TBS) + 0.05% Tween20 (TBST)

1° ab buffer: PBS + 5% BSA

2° ab buffer: PBS + 1% BSA

***Wash steps used 50 $^{\mu L}/_{well}$**

***Incubation steps used 30 $^{\mu L}/_{well}$**

****The plate must be covered during incubation steps to protect the fluorescent blank****

****After loading the protein solutions change tips every time that solution is aspirated or added to a well to prevent cross-contamination****

A pre-fabricated PDMS template was aligned onto the DNA barcode microchip, and the microchip was taped into a 10 cm petri dish. The wells on the platform were wet with 50 μL PBST before filling with blocking buffer and placing into a 37 °C incubator for 1hr. Concurrently, 1% BSA in PBS solutions containing a SAC-DNA conjugate and either **L1a, L1b, L2** or biotin-A_{20}-Cy3 blank were prepared for each SAC-DNA conjugate. The biotinylated ligands were allowed to complex with the SAC protein for one hour before pooling the SAC-DNA-ligand solutions (final [SAC-DNA-ligand conjugates] = 50 nM). The blocking buffer was aspirated and each well was loaded with the SAC-DNA-ligand conjugates cocktail for hybridization with the DNA barcode at 37 °C for one hour. The wells were washed with PBST

(3x) before loading either WT KRas protein, WT HRas protein, or WT MEK1 protein at 0 μM, 2.5 μM, 5 μM, or 10 μM in protein buffer. After shaking at RT for one hour, the wells were rinsed with PBST (5x), making sure to pipet up/down with the first addition of PBST. A (100:1) dilution of either CST rabbit anti-Ras Ab (#39655) or CST rabbit anti-MEK Ab (#9122S) in 1° ab buffer was added to each well before shaking at RT for one hour. After rinsing the wells with PBST (3x), the wells were loaded with a (200:1) dilution of Abcam goat anti-rabbit-Alexafluor 647 linked ab (ab150079) in 2° ab buffer before shaking at RT for one hour. A final rinse of the wells with PBST (3x) proceeded peeling off the PDMS slab from the barcode microchip and dipping the barcode into the following solutions: PBS, (1:1) PBS: MQ H_2O, MQ H_2O (2x). After being spun dry, the barcode was read on the Genepix (635 nm, PMT 600, PWR 40% (60 W); 532 nm, PMT 450, PWR 15% (23 W)). Data was extracted using 10 $^{\text{data blocks}}/_{\text{barcode lane}}$, singly background corrected using the ab only well fluorescence, and graphed in Graphpad. The peeled off PDMS slab was rinsed under MQ H_2O and stored in MQ H_2O at RT until further use.

Testing Lead Allosteric Ligands for Inhibition of Intrinsic KRas Protein GTPase Activity

The GTPase assays were run in triplicate on a multi-well plate using the GTPase Glo Assay kit from Promega with 10 μM KRas protein and varying concentrations of ligand (1 μM to 100 μM **L1a**, **L8** and 2.5 μM to 250 μM **L2**). All reagents were warmed to RT before use. A single opaque white 96-well plate was charged with 12.5 μL GTPase/GAP buffer (GTPase buffer w/ 1 mM DTT), 10 μM KRas in GTPase/GAP buffer, or 10 μM KRas protein with either **L1a**, **L2**, or **L8**. **Running the survey assays on the same plate allowed for direct comparison of the curves, but it necessitated the use of the first row/column on the multi-well plate which introduced some noise to the low ligand concentration points.** A 12.5 μL aliquot of a 2x GTP solution (10 μM rGTP in GTPase Buffer) was then added to each well before allowing the plate

to shake at RT for two hours (initial GTPase inhibition assay). The GTPase Glo reagent was reconstituted in the GTPase Glo Buffer (4 µL GTPase Glo (500x) reagent, 1996 µL GTPase Glo Buffer, 1 µL 10 mM ADP) immediately before adding 25 µL to each well. Shaking at RT for thirty minutes preceded the addition of 50 µL of the Detection reagent to each well. The plate was covered and incubated for a total of ten minutes before reading the luminescence with a Flexstation 3 plate reader (All wavelengths mode, 500 ms integration time), graphed using Graphpad Prism 7 (Sigmoidal 4PL mode), and an IC_{50} value was calculated.

For the full GTPase inhibition curve for **L2** shown in Figure 4, the above procedure was followed with the change that the incubation with KRas occurred over or four hours rather than two.

Supplemental Figures

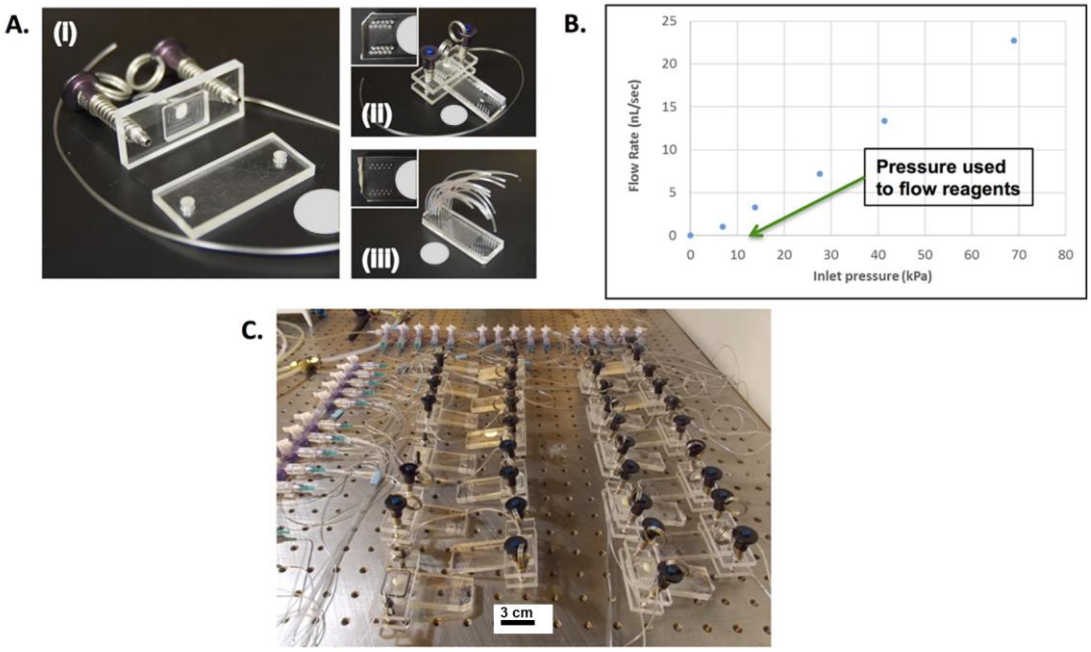


Figure S1. High throughput patterning of DNA barcode chips. A. (i) Solution loading device design and flow patterning set up with (ii) the solution loading device used in this work and (iii) the pins and tubing used for flow-patterning in previous work. Silver discs, the size of quarters, are shown for scale. B. Measured flow rates under various inlet pressures using the solution-loading device. C. Simultaneous flow patterning of 21 DNA barcode chips.

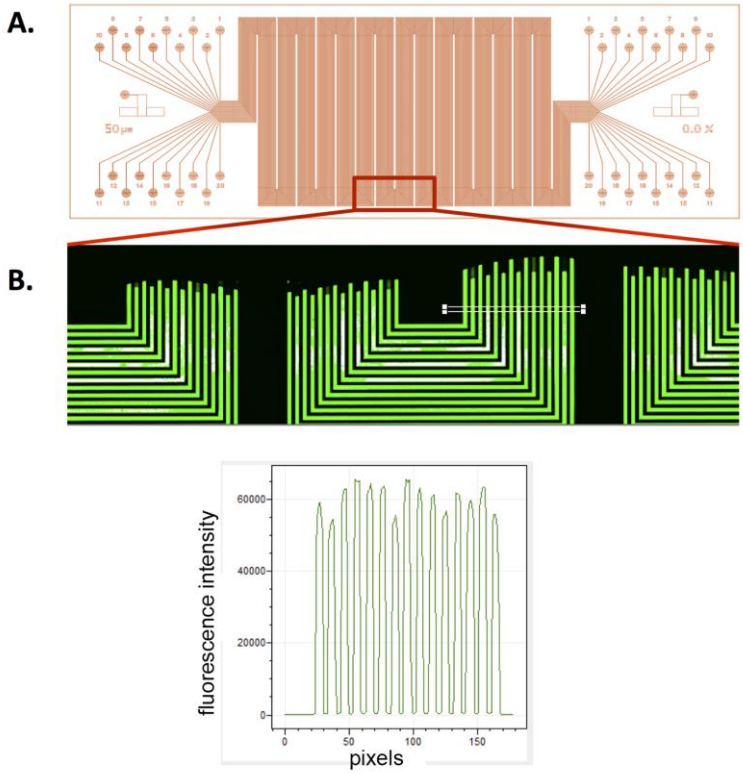


Figure S2. DNA barcode chip layout and validation. A. The 50 µm barcode chip layout, which encompasses the entire length of a 3" microscope slide. Input and outputs of the serpentine microchannels are at the right and left sides. B. An image and digitized line scan of a barcoded region of the slide. The white box in the image outlines a region that is approximately 50 µm x 2 mm wide.

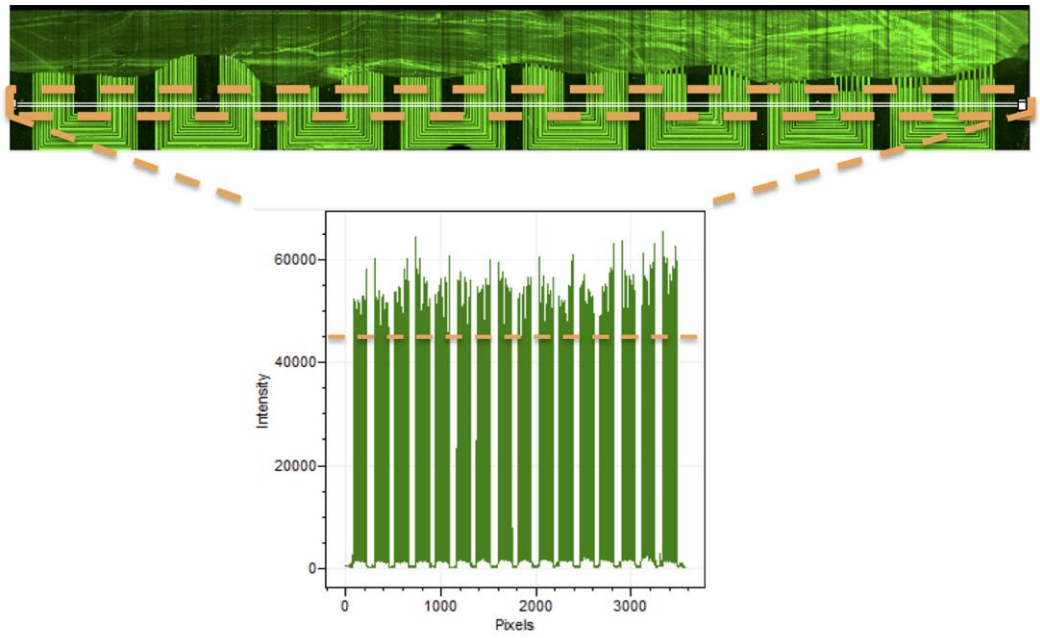


Figure S3. Comparison of barcode quality across an entire microchip. Measuring the barcode quality across the entire barcode region (about 3.8 cm) reveals that all of the barcode lanes meet the minimum F_{532} requirement for good levels of ssDNA patterning as highlighted by the dashed line on the graph.

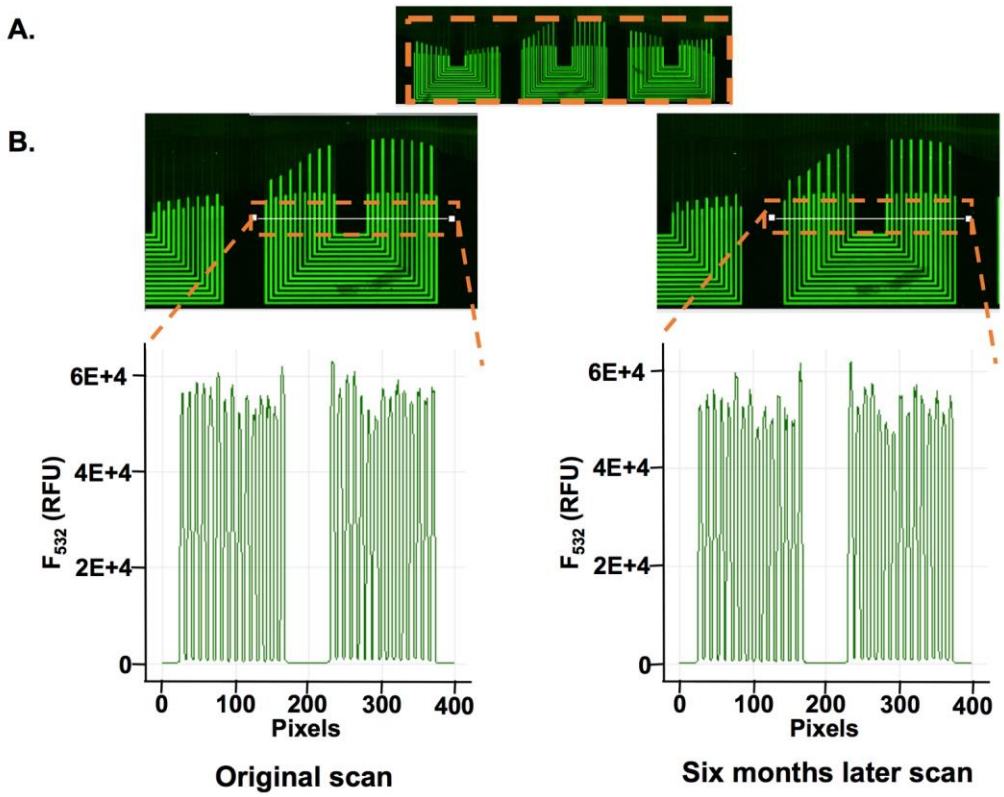


Figure S4. Probing barcode stability. A. The portion of the validation region used to generate Table A2C.4. B. A comparison of F_{532} output for two DNA barcode sets in the validation region with six months between each scan.

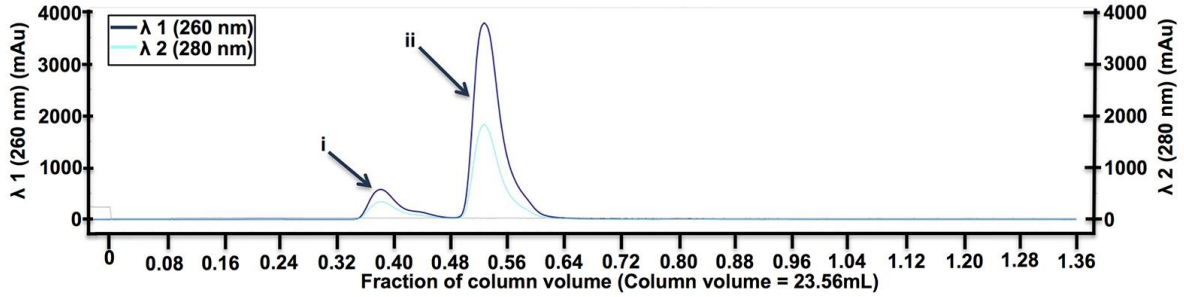


Figure S5. Representative SAC-DNA FPLC trace. The FPLC trace for B' SAC-DNA. The SAC-DNA elutes first (peak labeled "i") followed by the excess unconjugated B' ssDNA (peak labeled "ii").

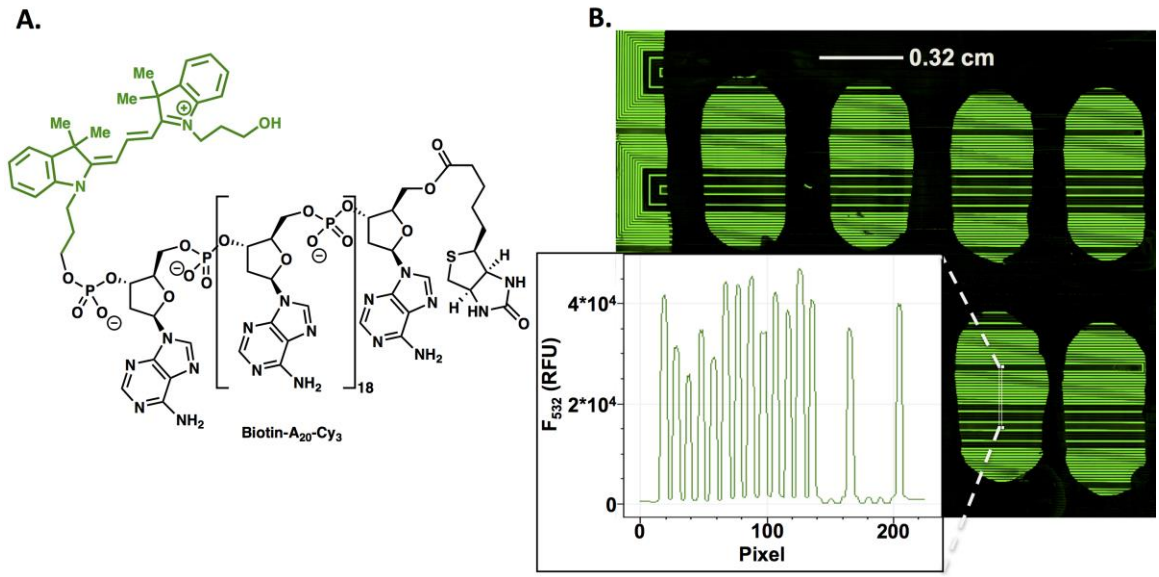


Figure S6. Biotin binding evaluation of the full SAC-DNA set with a Biotin* probe on the barcoded rapid assay platform. A. The Biotin* probe used to perform the biotin binding affinity test for the DESL set using the B-RAP technology. B. The output of the biotin-binding test on the DNA barcode.

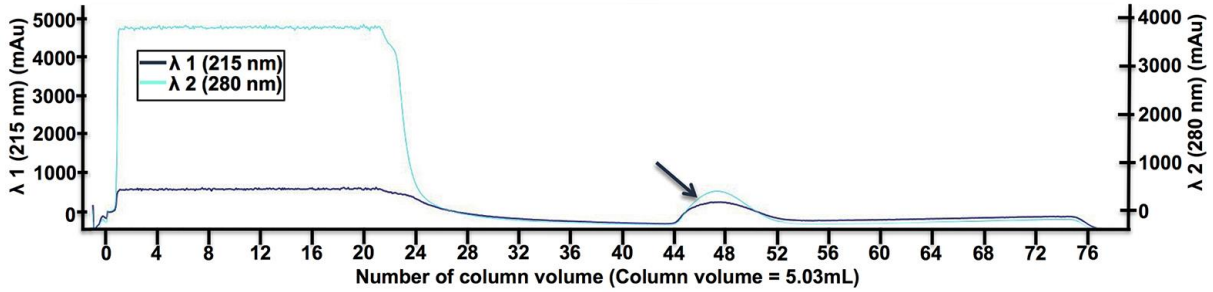


Figure S7. FPLC trace of wild-type KRas protein. The FPLC trace for wild-type KRas protein with the protein peak highlighted with a black arrow.

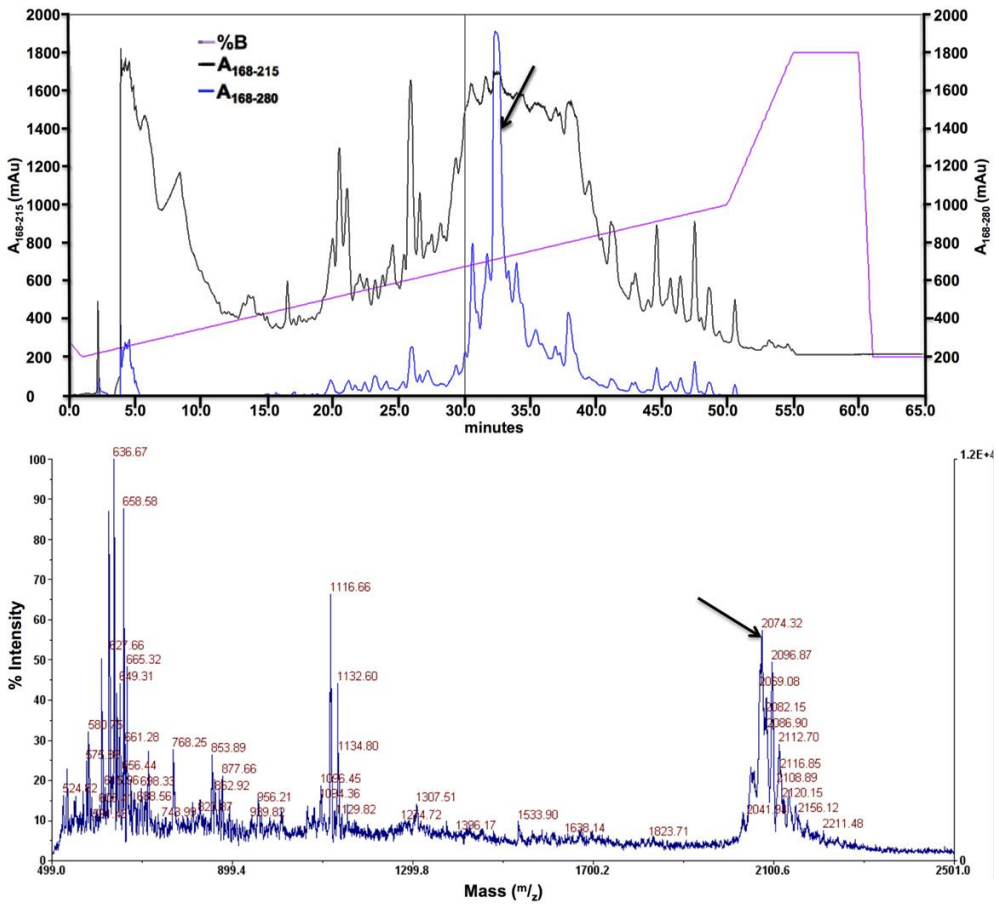


Figure S8. The characterization data for SynEp1. SynEp1 FDEYD[P→$^{4\text{-}N3}$P]TIEDSY-PEG$_4$-Biotin: $C_{90}H_{128}N_{20}O_{33}S$. (Top) The semi-preparative HPLC trace for **SynEp1** with the peak containing the correct mass indicated by an arrow. (Bottom) The MALDI-TOF spectra for **SynEp1** with the peak indicated with an arrow corresponding to [M+Na], expected [M+Na]=2071.188 amu.

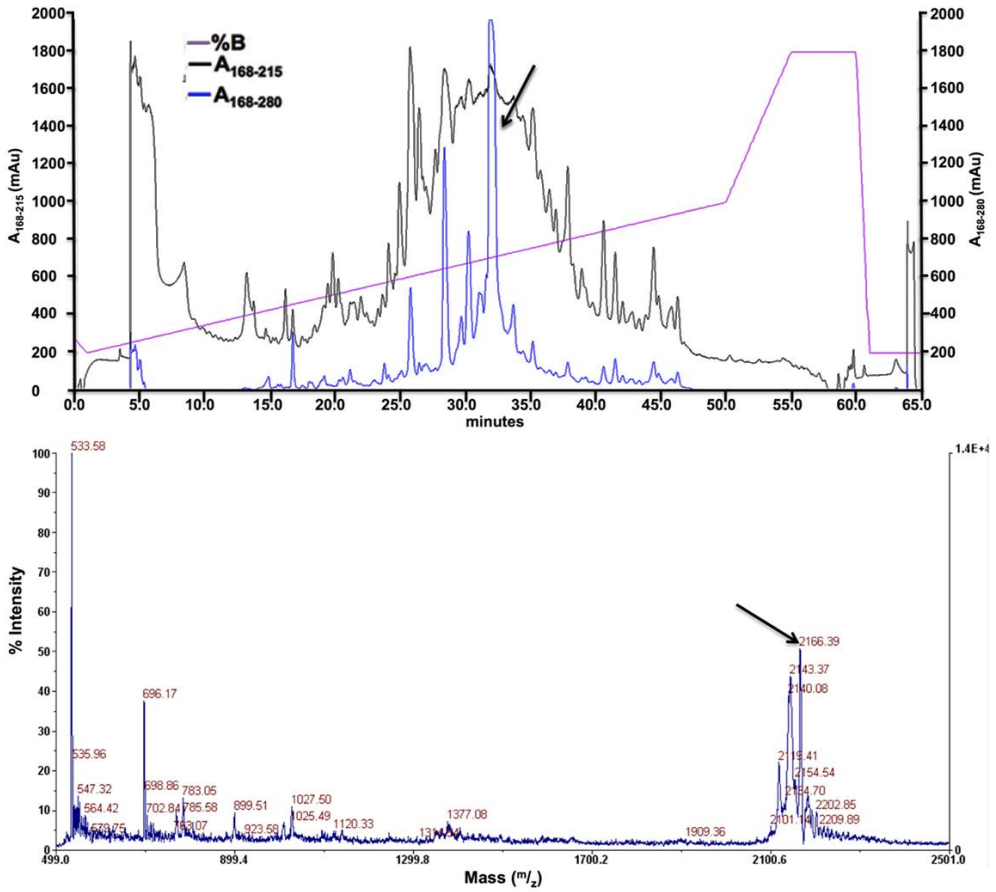


Figure S9. The characterization data for scrambled SynEp1. Scrambled SynEp1 [P→$^{4\text{-}N3}$P]EYDSIDDEFYT-PEG$_4$-Biotin: $C_{90}H_{128}N_{20}O_{33}S$. (Top) The semi-preparative HPLC trace for **scrambled SynEp1** with the peak containing the correct mass indicated with an arrow. (Bottom) The MADLI-TOF spectra for **scrambled SynEp1**. The peak indicated with an arrow corresponds to [M+2Pip-3 OH+H], expected [M+2Pip-3 OH+H]=2167.030 amu, which results from multiple piperidine and aspartamide adducts forming on the oligopeptide.

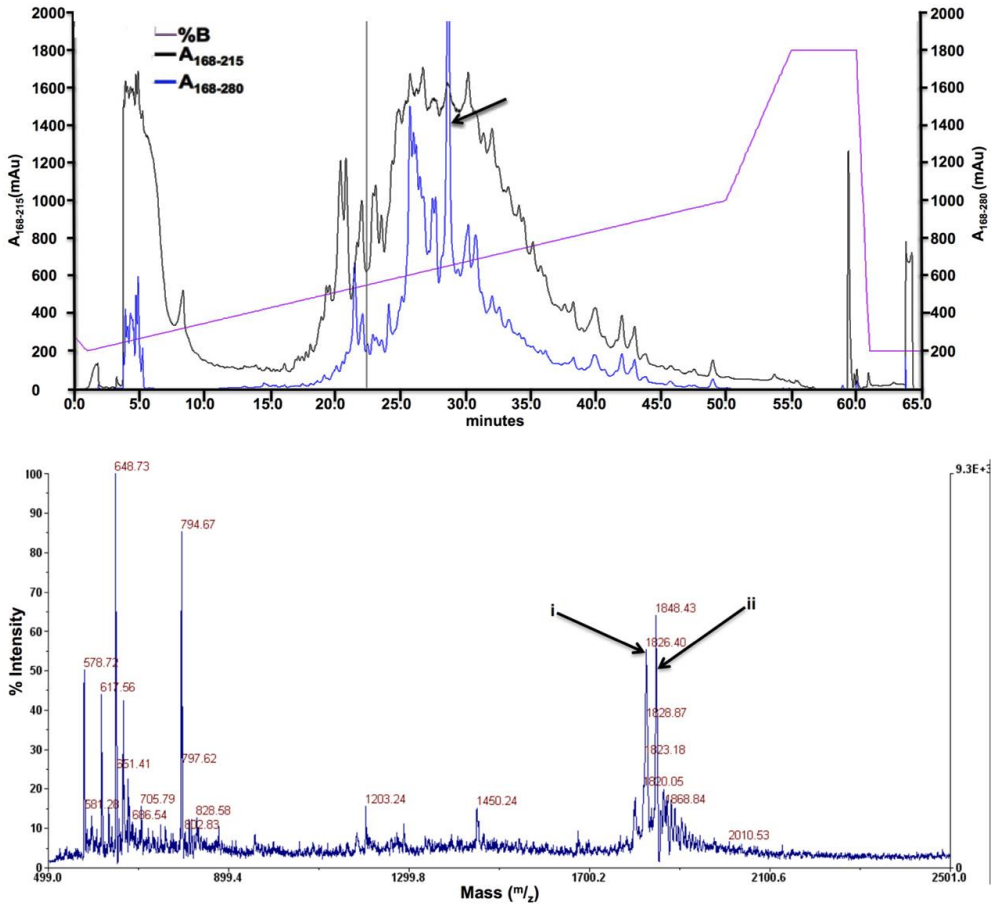


Figure S10. The characterization data for SynEp2. SynEp2 [I→$^{4\text{-}N3}$K]LDTAGQEEYS-PEG$_5$-Biotin: $C_{77}H_{124}N_{20}O_{29}S$. (Top) The semi-preparative HPLC trace for **SynEp2** with the peak containing the correct mass indicated by an arrow. (Bottom) The MALDI-TOF spectra for **SynEp2**. The peak labeled "i" corresponds to [M+H], expected [M+H]=1825.864 amu, and the peak labeled "ii" corresponds to [M+Na], expected [M+Na]=1847.846 amu.

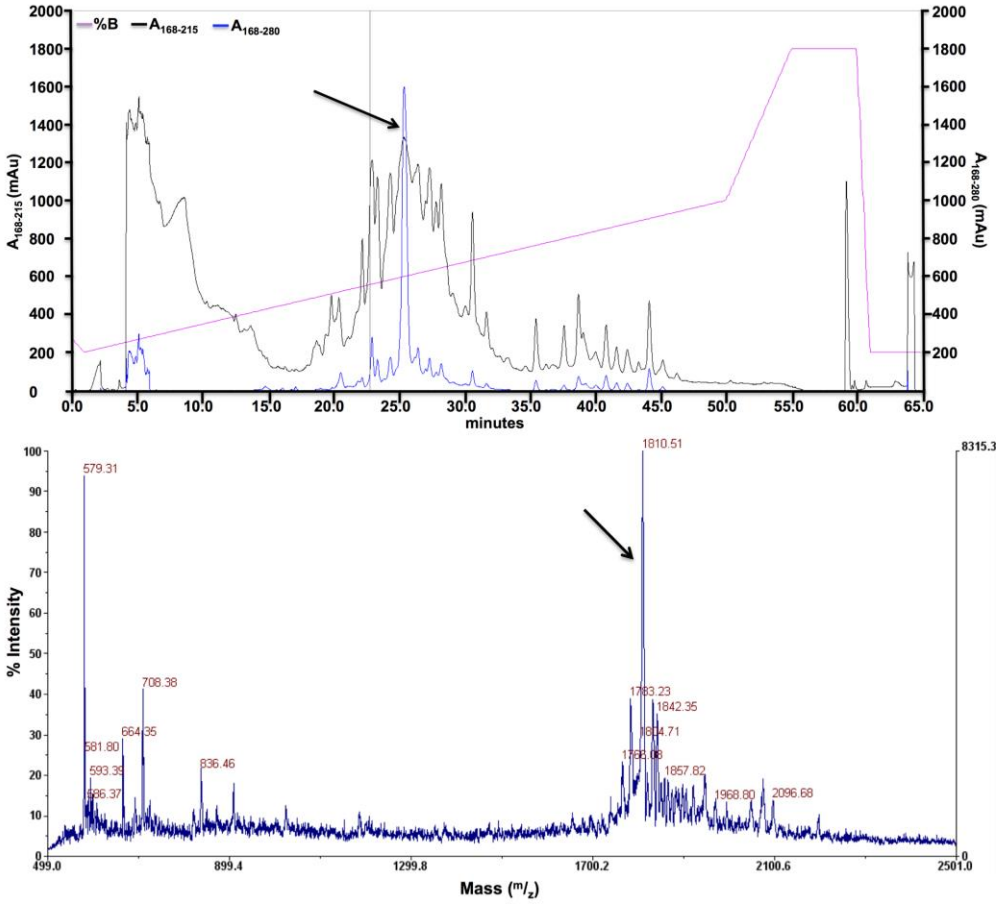


Figure S11. The characterization data for scrambled SynEp2. Scrambled SynEp2 [I→$^{4\text{-}N3}$K]LSTGEYDAQE-PEG$_5$-Biotin: $C_{77}H_{124}N_{20}O_{29}S$. (Top) The semi-preparative HPLC trace for **scrambled SynEp2** with the peak containing the correct mass indicated by an arrow. (Bottom) The MALDI-TOF spectra for **scrambled SynEp2** with the indicated peak corresponding to the epitope with a single aspartamide formation [M-OH+H], expected [M-OH+H]=1809.99 amu.

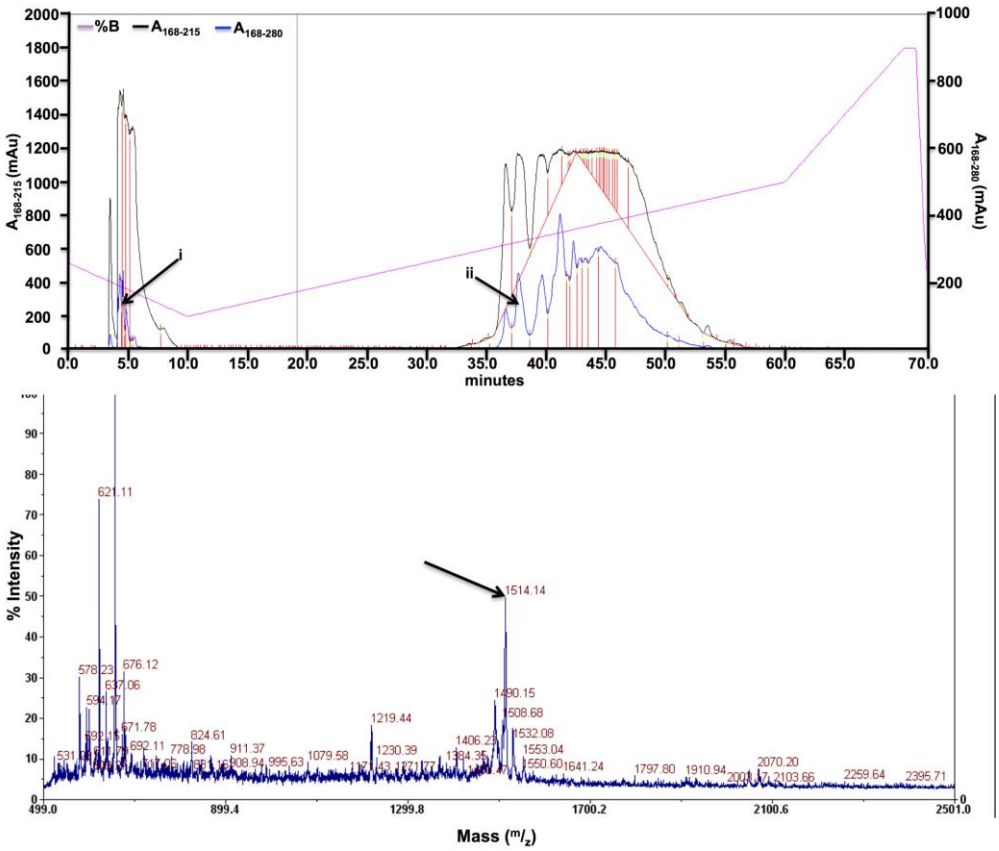


Figure S12. The characterization data for ligand L9. Ligand **L9** (Cyclic)-Pra-QDNFY-$^{4\text{-}N3}$K-PEG$_5$-biotin: $C_{67}H_{99}N_{17}O_{20}S$. (Top) The HPLC trace for Ligand **L9**. The peak labeled "i" corresponds to fraction **L9a**, and the peak labeled "ii" corresponds to fraction **L9b**. (Bottom) The MALDI-TOF spectra for ligand fraction **L9a**. The indicated peak corresponds to [M+Na], expected [M+Na] = 1516.687 amu.

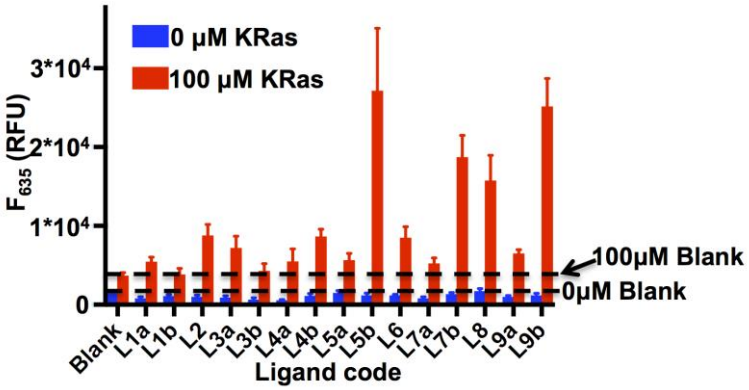


Figure S13. Single point Immunofluorescent Assay using the B-RAP technology. A single point Immunofluorescent assay on the rapid assay platform for the KRas allosteric switch ligands.

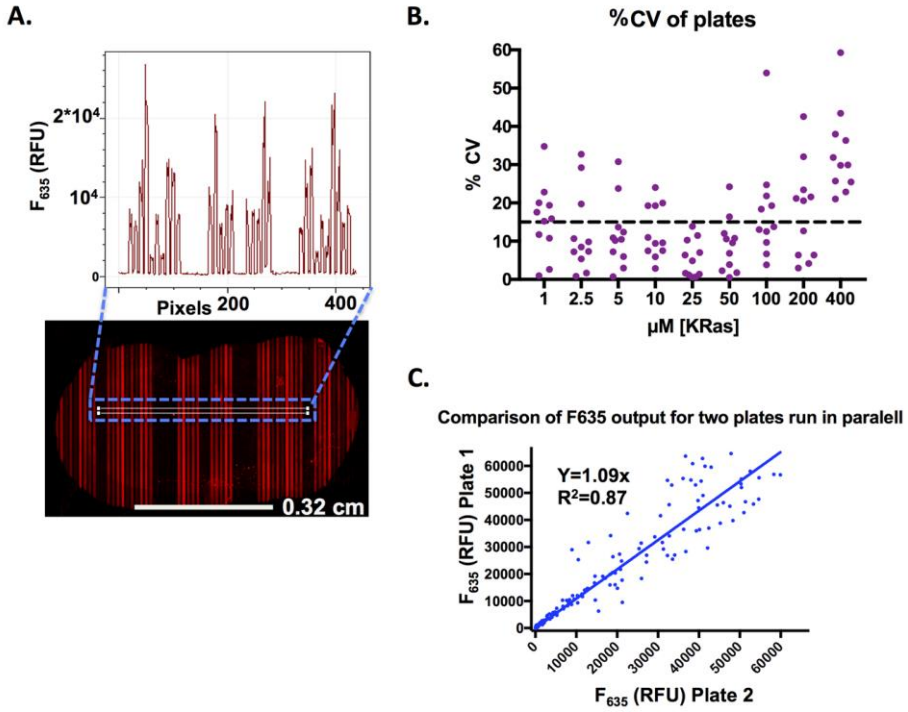


Figure S14. Statistical validation of the B-RAP Technology. A. Comparing the two full sets of DNA barcode in a given microwell at 5μM KRas reveals that extracting data from a single barcode repeat is sufficient. B. The %CV for 1 μM to 400 μM KRas with the average %CV for the entire concentration range (~18%) indicated by the black dashed line. C. A plot of the F_{635} values for both plates plotted against each other. The good correlation at low F_{635} values indicate that the variance at low [KRas protein] is satisfactory.

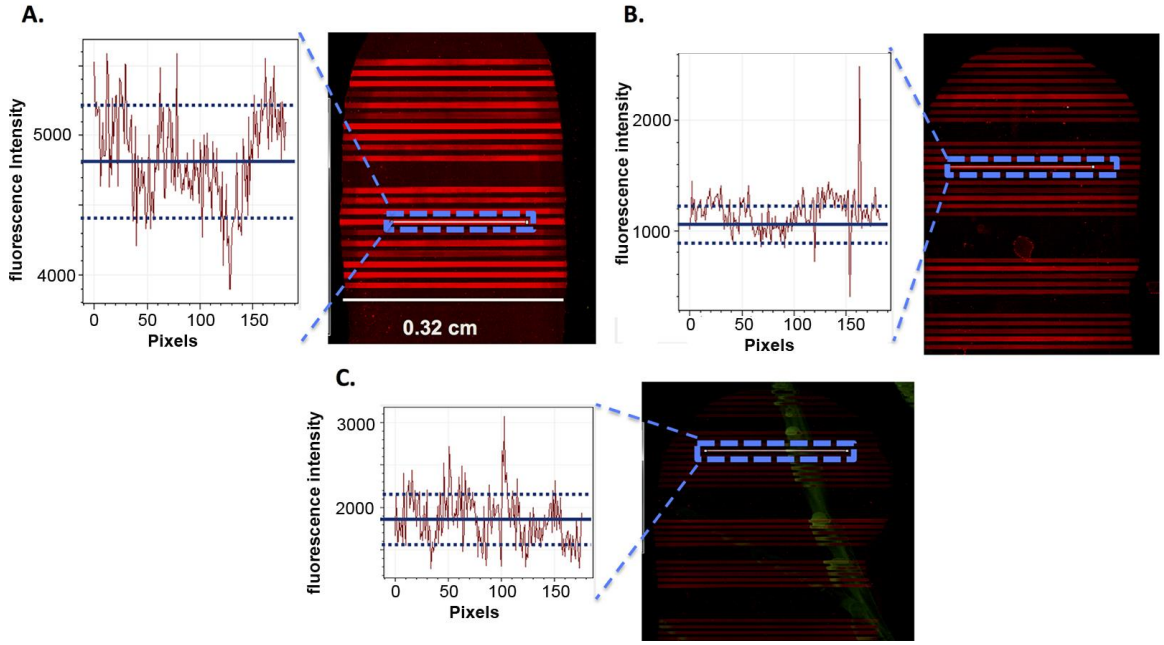


Figure S15. A comparison of the average line-scan with an averaged data-block extraction. A comparison of the full-line scan with the average F_{635} resulting from averaging ten discrete data block extractions and the corresponding standard deviations at A. 10 μM KRas protein: avgF_{635}=47,868.7, std-dev=4154.53. B. 1 μM KRas protein: avgF_{635}=11437.6, std-dev=1602.202. C. 100 nM KRas Protein, avgF_{635}=1780.6, std-dev=236.38.

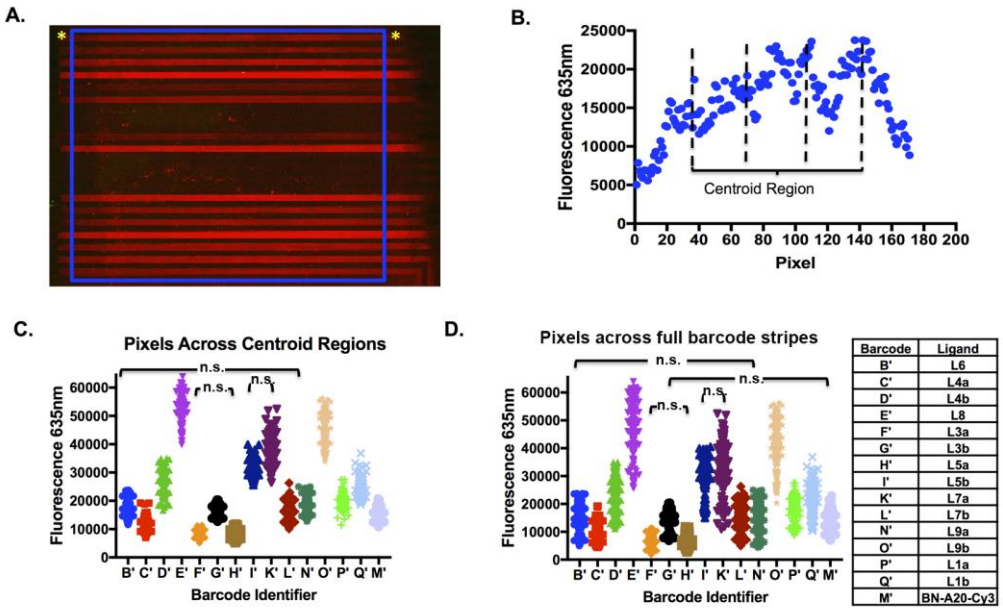
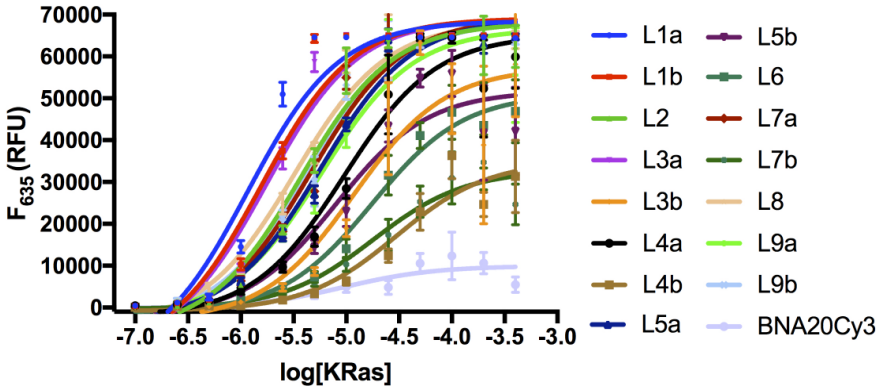


Figure S16. Pixel extraction of full barcode lanes in a given barcode. A. The region of the barcode lanes extracted with the B' lane graphed in a) set off by yellow asterisks. B. A close up of the scatterplot of barcode lane B' to highlight where the centroid region was defined and extracted (pixel 40-142) with the region divided into thirds for the left, middle, and right centroid respectively C. A scatterplot of the F_{635} extracted for individual pixels along the entire length of a barcode in the 10 μM well of plate #1 with lanes that were indistinguishable by a two-tailed student T test indicated by brackets, and an accompanying key for parts c) and d). D. A scatterplot of the F_{635} extracted for individual pixels in the centroid region of each barcode with lanes that were indistinguishable by a two-tailed student T-test (Table S5) indicated by brackets.

A.



B.

Ligand	L1a	L1b	L2	L3a	L3b	L4a	L4b	L5a	L5b	L6[a]	L7a	L7b	L8	L9a	L9b	Blank
EC_{50} (μM)	1.16± 0.20	1.56± 0.26	3.7±0. 53	1.75± 0.25	11.86 ±4.0	9.51± 1.9	29.80 ±8.7	5.63± 0.57	8.04± 2.2	17.62 ±3.5	4.63± 0.64	18.00 ±5.0	3.22± 0.39	5.84± 1.0	4.31± 0.62	9.49± 5.6

Figure S17. KRas binding curves after a single background correction. A. The KRas protein binding curves for the candidate PCC ligands run after correcting for the no-KRas well's fluorescence. The dummy ligand's (BN-A_{20}-Cy3) curve is shown in periwinkle. B. The EC_{50} values for **L1a** to **L9b** after a single background correction. [a]Ligand **L6** was run separately.

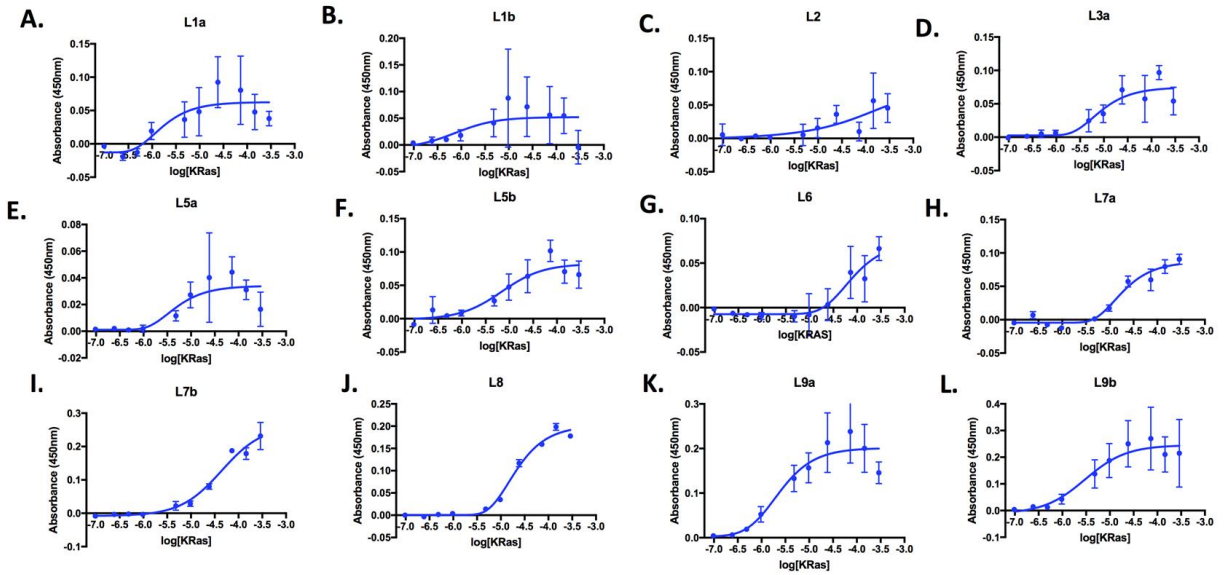


Figure S18. KRas protein binding curves using 96-well plate ELISAs.

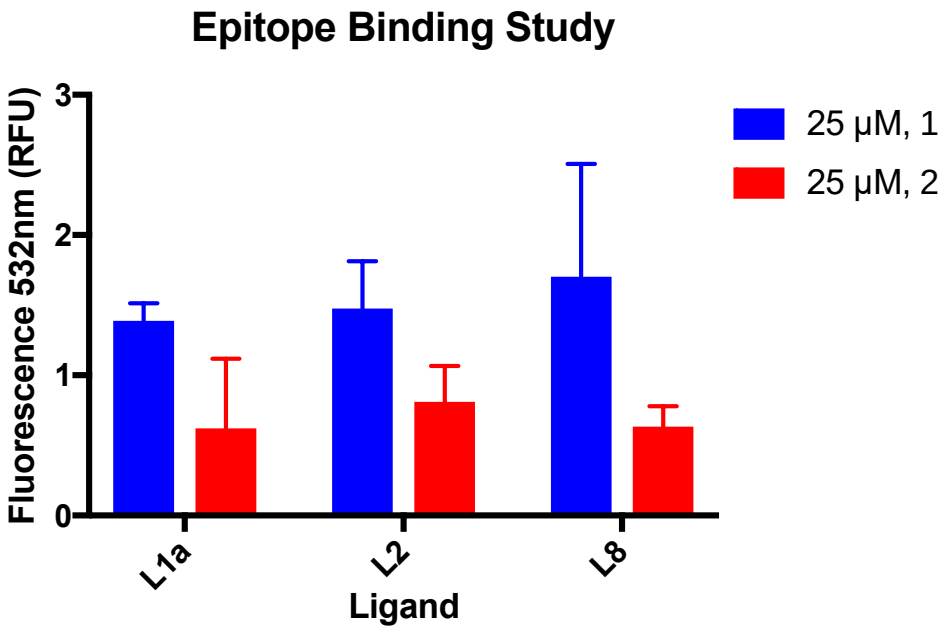


Figure S19. Epitope Binding study with the lead ligand fractions L1a, L2, and L8. The ligands showed enhanced affinity for the Cy3-labeled oligopeptide corresponding to the region of KRas protein used for **SynEp1**, which indicates that the lead ligands are switch 1 binders.

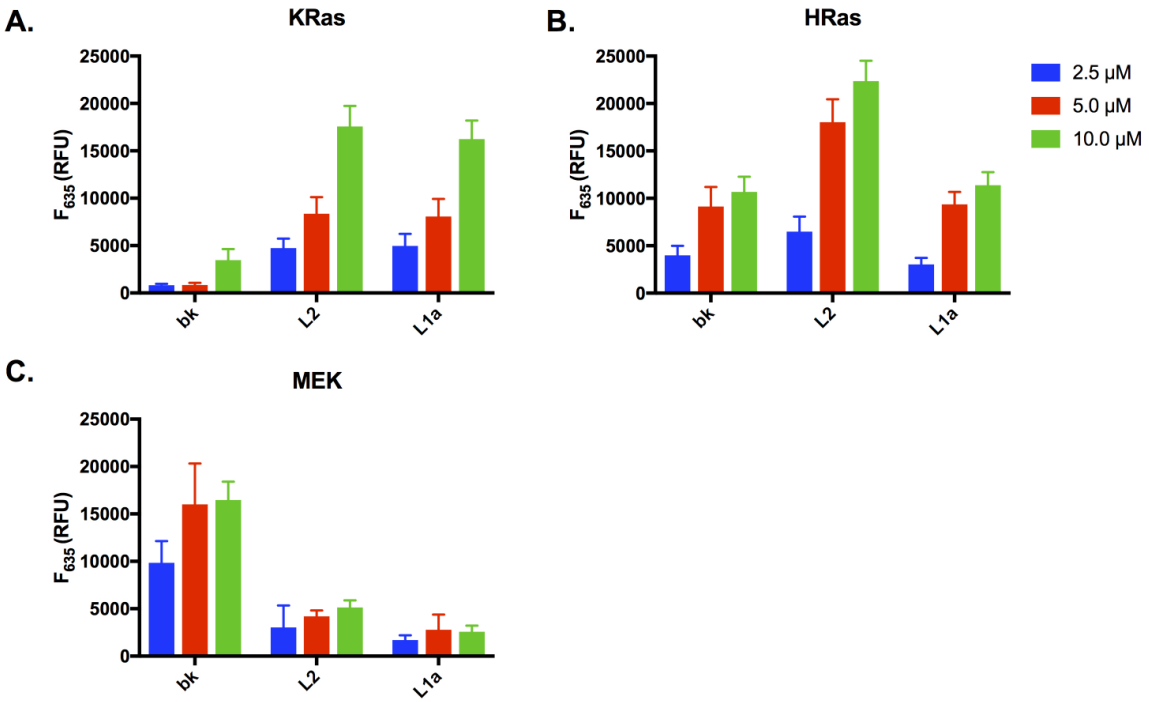


Figure S20. Evaluation of lead ligand selectivity for Ras proteins. A comparison of the selectivity of the lead ligands **L1a**, **L2** for binding to A. WT KRas protein, B. WT HRas protein, and C. MEK protein. The switch regions are 100% conserved in the Ras family, and as expected the ligands showed comparable selectivity for the two Ras proteins, and diminished selectivity for the unrelated MEK protein.

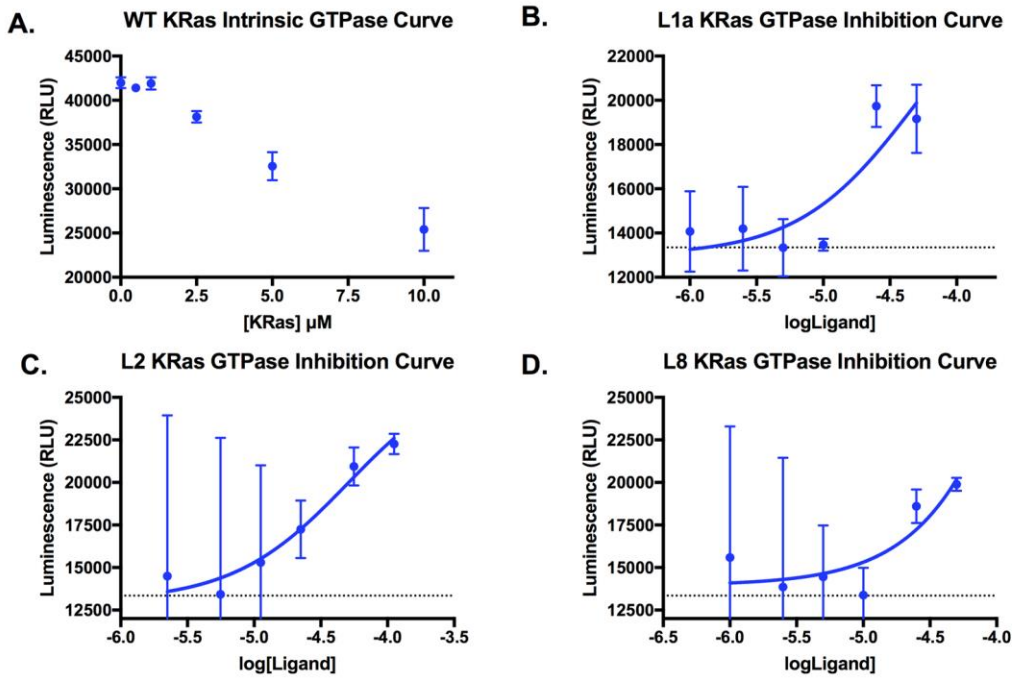


Figure S21. The wild-type KRas protein GTPase activity curves. A. The WT KRas standard curve generated with a one hour incubation with 5 µM rGTP. The inhibition curves generated for the best three ligand fractions with a two hour rGTP incubation period in B., C., and D. The dotted line represents the luminescence measurement (~13k RLU) when 10 µM KRas was incubated with 5 µM rGTP for two hours without ligand.

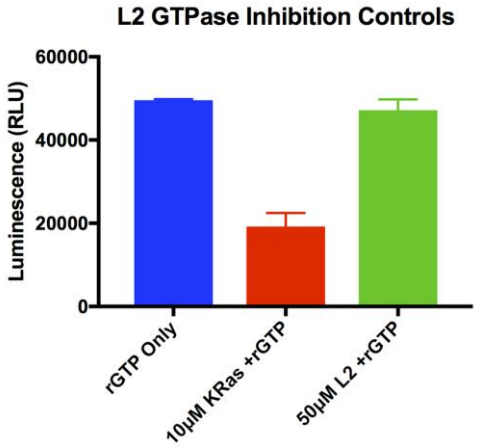


Figure S22. Controls for the L2 GTPase inhibition curve. Controls run for the **L2** inhibition curve. It is apparent that ligand **L2** lacks GTPase catalytic activity as less than 5% of the GTP was hydrolyzed when placed with just 50 µM of **L2**.

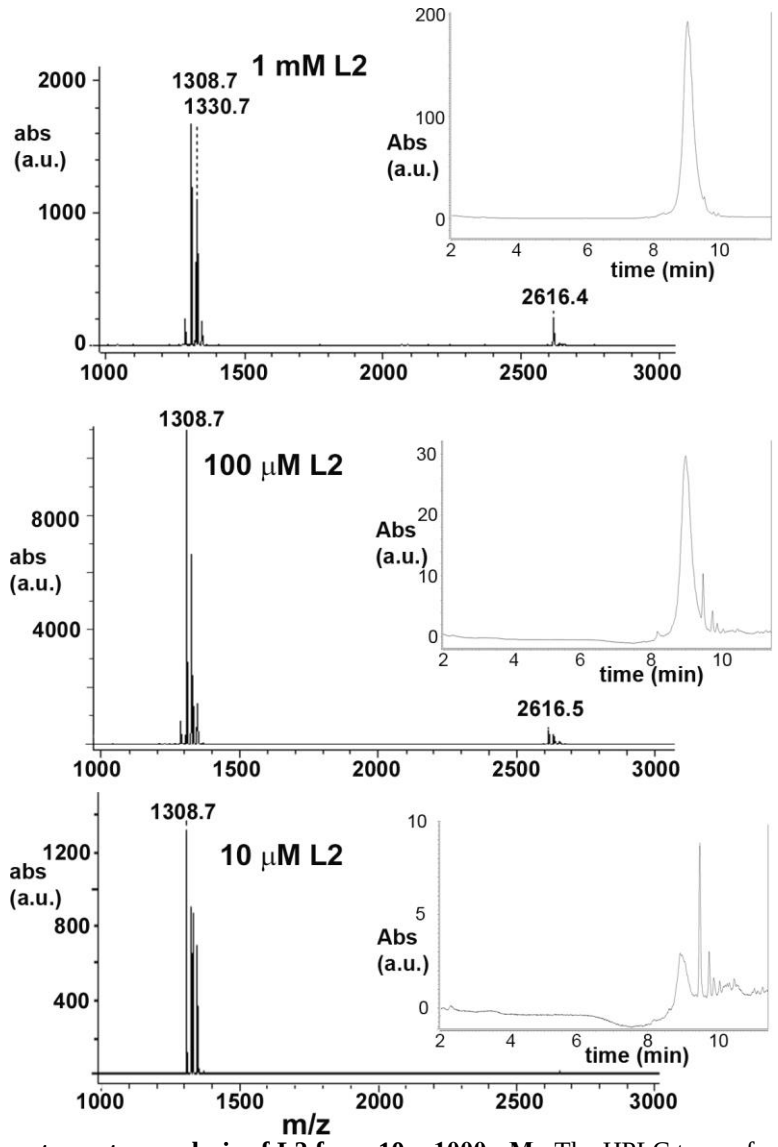


Figure S23. Mass spectrometry analysis of L2 from 10 – 1000 μM. The HPLC traces from which aliquots for mass analysis were collected are shown in the insets to each mass spectrum. Note that the dimer (near 2616 amu) is detected in both the 100 μM and 1 mM concentration samples. It is not clear whether the dimer is the major HPLC product under the dominant peak near t_r= 9 min.

Supplemental Tables

DNA Patterning step	Solution Loading Method (new)	Tubing Method (old)
Polylysine loading	12 min	> 24 min
Polylysine flow setting	10 min	20 min
DNA tubing preparation	n.a.	30 min
DNA loading	22 min	50 min

Table S1. A comparison of the various steps associated with the Solution Loading Method (reported here) and the previously reported Tubing Method for preparing microfluidic flow-patterned ssDNA barcodes. By using the solution loading method, 20-25 devices are handled within one hour as opposed to 8-10 devices with individual tubing for each amine DNA.

DNA i.d.	Sequence
B	5'-NH_2-C6-AAA AAA AAA AAA AGC CTC ATT GAA TCA TGC CTA-3'
B'	5'-NH_2-C6-AAA AAA AAA ATA GGC ATG ATT CAA TGA GGC-3'
C	5'-NH_2-C6-AAA AAA AAA AAA AGC ACT CGT CTA CTA TCG CTA-3'
C'	5'-NH_2-C6-AAA AAA AAA ATA GCG ATA GTA GAC GAG TGC-3'
D	5'-NH_2-C6-AAA AAA AAA AAA GGT CGA GAT GTC AGA GTA-3'
D'	5'-NH_2-C6-AAA AAA AAA ATA CTC TGA CAT CTC GAC CAT-3'
E	5'-NH_2-C6-AAA AAA AAA AAA AAT GTG AAG TGG CAG TAT CTA-3'
E'	5'-NH_2-C6-AAA AAA AAA ATA GAT ACT GCC ACT TCA CAT-3'
F	5'-NH_2-C6-AAA AAA AAA AAA AAT CAG GTA AGG TTC ACG GTA-3'
F'	5'-NH_2-C6-AAA AAA AAA ATA CCG TGA ACC TTA CCT GAT-3'
G	5'-NH_2-C6-AAA AAA AAA AAA AGA GTA GCC TTC CCG AGC ATT-3'
G'	5'-NH_2-C6-AAA AAA AAA AAA TGC TCG GGA AGG CTA CTC-3'
H	5'-NH_2-C6-AAA AAA AAA AAA AAT TGA CCA AAC TGC GGT GCG-3'
H'	5'-NH_2-C6-AAA AAA AAA ACG CAC CGC AGT TTG GTC AAT-3'
I	5'-NH_2-C6-AAA AAA AAA AAA ATG CCC TAT TGT TGC GTC GGA-3'
I'	5'-NH_2-C6-AAA AAA AAA ATC CGA CGC AAC AAT AGG GCA-3'
K	5'-NH_2-C6-AAA AAA AAA AAA ATA ATC TAA TTC TGG TCG CGG-3'
K'	5'-NH_2-C6-AAA AAA AAA ACC GCG ACC AGA ATT AGA TTA-3'
L	5'-NH_2-C6-AAA AAA AAA AAA AGT GAT TAA GTC TGC TTC GGC-3'
L'	5'-NH_2-C6-AAA AAA AAA AGC CGA AGC AGA CTT AAT CAC-3'
M	5'-NH_2-C6-AAA AAA AAA AAA AGT CGA GGA TTC GTA ACC TGT-3'
M'	5 '-NH_2-C6-AAA AAA AAA AAC AGG TTC AGA ATC CTC GAC-3'
N	5'-NH_2-C6-AAA AAA AAA AAA AGT CCT CGC TTC GTC TAT GAG-3'
N'	5'-NH_2-C6-AAA AAA AAA ACT CAT AGA CGA AGC GAG GAC-3'
O	5'-NH_2-C6-AAA AAA AAA AAA ACT TCG TGG CTA GTC TGT GAC-3'
O'	5'-NH_2-C6-AAA AAA AAA AGT CAC AGA CTA GCC ACG AAG-3'
P	5'-NH_2-C6-AAA AAA AAA AAA ATC GCC GTT GGT CTG TAT GCA-3'
P'	5'-NH_2-C6-AAA AAA AAA ATG CAT ACA GAC CAA CGG CGA-3'
Q	5'-NH_2-C6-AAA AAA AAA AAA ATA AGC CAG TGT GTC GTG TCT-3'
Q'	5'-NH_2-C6-AAA AAA AAA AGA CAC GAC ACA CTG GCT TAT-3'

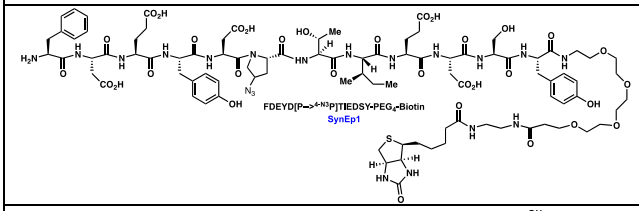
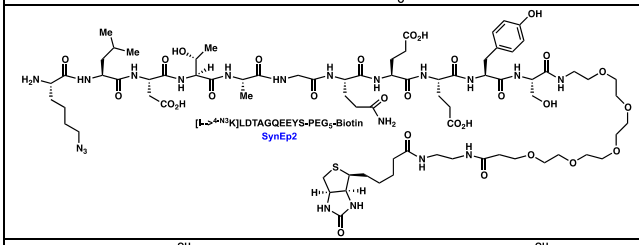
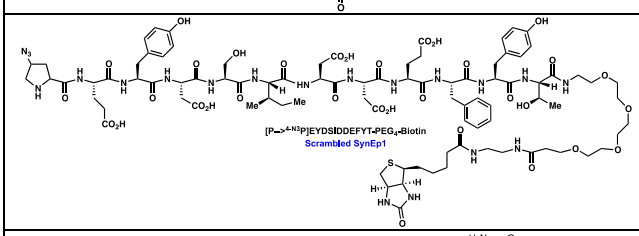
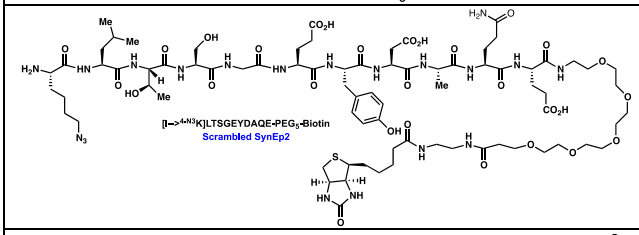
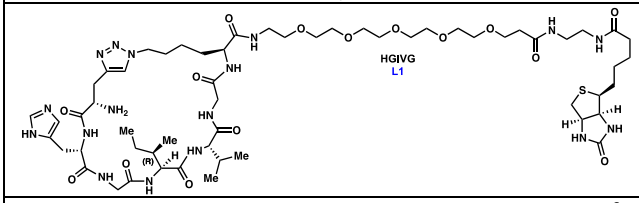
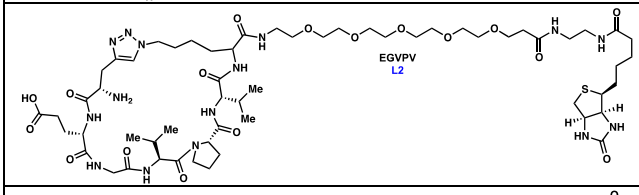
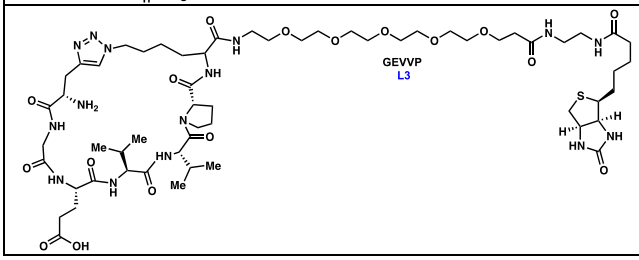
Table S2. Table of ssDNA sequences used for SAC-DNA conjugation and DNA barcode patterning. The rows shaded in grey are used for conjugation to SAC protein.

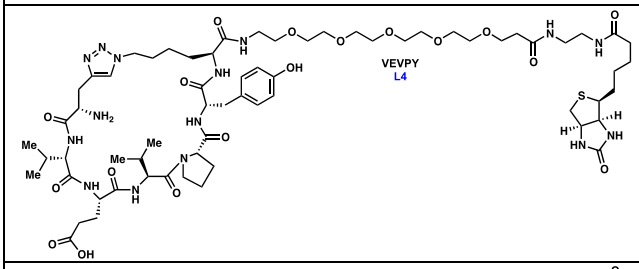
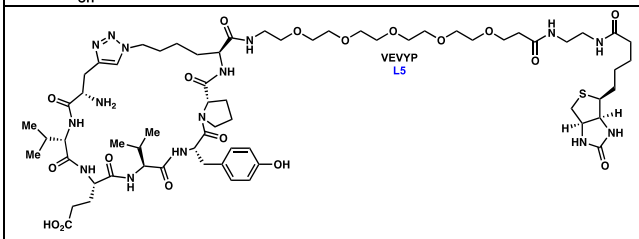
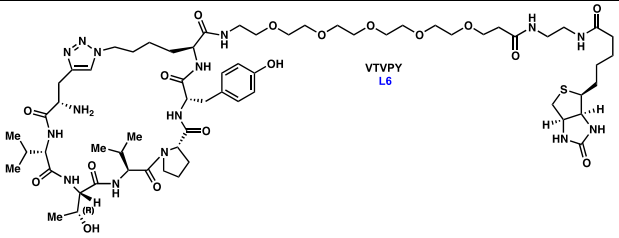
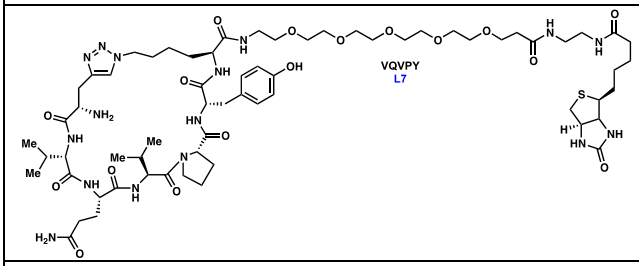
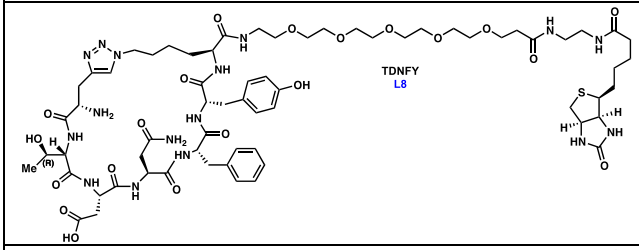
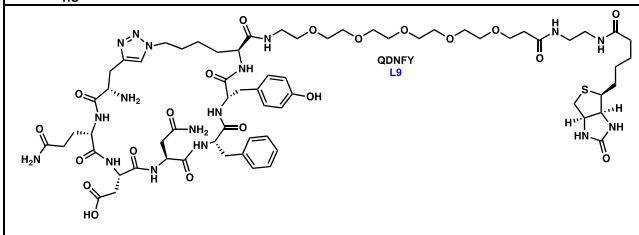
Lane	B'	C'	D'	E'	F'	G'	H'	I'
original scan (F_{532})	59024.03	53476.07	54155.37	56759.83	52075.97	51375.13	49365.43	55336.00
six month rescan (F_{532})	55831.77	51549.57	50095.27	52715.00	49412.37	48684.57	45213.60	51664.53
Difference (F_{532})	3192.27	1926.50	4060.10	4044.83	2663.60	2690.57	4151.83	3671.47
% Difference	5.41	3.60	7.50	7.13	5.11	5.24	8.41	6.63

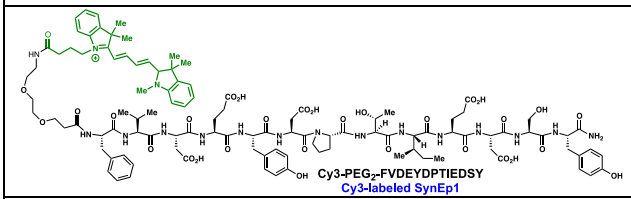
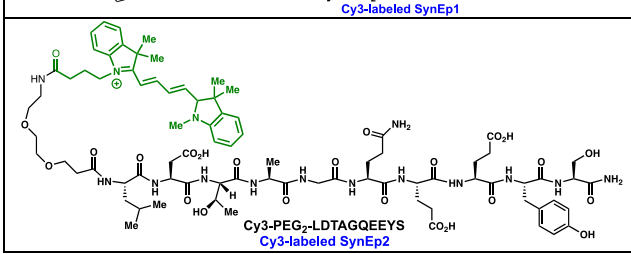
Lane	K'	L'	M'	N'	O'	P'	Q'
original scan (F_{532})	52048.67	56120.87	52744.73	53185.87	52884.73	52943.00	52287.07
six month rescan (F_{532})	48675.67	53388.87	48104.17	50050.87	49501.07	48818.27	49845.17
Difference (F_{532})	3373.00	2732.00	4640.57	3135.00	3383.67	4124.73	2441.90
% Difference	6.48	4.87	8.80	5.89	6.40	7.79	4.67

Table S3. Analysis of the degradation in barcode quality during extended storage. The data above is from a barcode validated two months after its preparation and a subsequent re-scan of the validation region six months later. The average difference in F_{532} output was 3348.80, and the average % difference was 6.26%.

Table S4. Synthetic epitopes and PCC ligands characterization table.

Structure	Chemical Formula	HPLC Retention Time (min)	Expected mass (amu)	Observed mass (amu)
 FDEYD[P-->4-N3P]TIEDSY-PEG4-Biotin SynEp1	$C_{90}H_{128}N_{20}O_{33}S$	32:30-33:30	[M+H]= 2049.88 [M+Na]= 2071.188	2074.32
 [L-->4-N3K]LDTAGQEEYS-PEG3-Biotin SynEp2	$C_{77}H_{124}N_{20}O_{29}S$	28:30-29:30	[M+H]= 1825.864	1826.222
 [P-->4-N3P]EYDSIDDEFYT-PEG4-Biotin Scrambled SynEp1	$C_{90}H_{128}N_{20}O_{33}S$	32:00-33:00	[M+H]= 2049.875 [M+2 Pip-3 OH+H]= 2167.030	2166.39
 [I-->4-N3K]LTSGEYDAQE-PEG3-Biotin Scrambled SynEp2	$C_{77}H_{124}N_{20}O_{29}S$	25:00-26:00	[M+H]= 1825.649, [M-OH+H] =1809.991	1810.51
 HGIVG L1	$C_{57}H_{95}N_{17}O_{15}S$	3:30-4:30 (f1), 36:00-38:00 (f2)	[M+H]= 1290.699 [M+Na]= 1312.681	1310.81 (f1), 1289.31 (f2)
 EGVPV L2	$C_{58}H_{97}N_{15}O_{17}S$	2:30-5:00	[M+H]= 1306.699 [M+Na]= 1330.682	1329.9
 GEVVP L3	$C_{58}H_{97}N_{15}O_{17}S$	2:30-5:00 (f1), 41:30-43:00 (f2)	[M+H]= 1308.699 [M+Na]= 1330.681	1330.16 (f1), 1312.34 (f2)

Structure	Chemical Formula	HPLC Retention Time (min)	Expected mass (amu)	Observed mass (amu)
 VEVPY L4	$C_{65}H_{103}N_{15}O_{18}S$	36:30-39:30 (f1), 40:00-41:00 (f2)	[M+H]= 1414.740 [M+Na]= 1436.722	1414.38 (f1), 1435.89 (f2)
 VEVYP L5	$C_{65}H_{103}N_{15}O_{18}S$	37:00-39:00 (f1), 39:00-40:00 (f2)	[M+H]= 1414.740 [M+Na]= 1436.721	1413.47 (f1), 1435.88 (f2)
 VTVPY L6	$C_{64}H_{103}N_{15}O_{17}S$	37:30-39:00	[M+H]= 1386.746 [M+Na]= 1408.727	1387.36
 VQVPY L7	$C_{65}H_{104}N_{16}O_{17}S$	36:00-38:30 (f1), 39:00-41:30 (f2)	[M+H]= 1413.722 [M+Na]= 1435.738	1412.29 (f1), 1434.80 (f2)
 TDNFY L8	$C_{66}H_{98}N_{16}O_{20}S$	36:30-38:00	[M+H]= 1467.694 [M+Na]= 1489.676	1488.37
 QDNFY L9	$C_{67}H_{99}N_{17}O_{20}S$	4:30-5:30 (f1), 37:00-38:30 (f2)	[M+H]= 1494.705 [M+Na]= 1516.687	1514.14 (f1), 1494.04 (f2)

Structure	Chemical Formula	HPLC Retention Time (min)	Expected mass (amu)	Observed mass (amu)
 Cy3-PEG$_2$-FVDEYDPTIEDSY Cy3-labeled SynEp1	$C_{108}H_{144}N_{17}O_{31}^+$	*a*	[M+]= 2175.021; [M-H_2O+]= 2157.011	2157.284; 2176.275
 Cy3-PEG$_2$-LDTAGQEEYS Cy3-labeled SynEp2	$C_{82}H_{116}N_{15}O_{24}^+$	*a*	[M+]= 1694.832	1695.163

[a] *Used without HPLC purification.*

	Full Lane			Full Centroid			Left Part Centroid			Middle Part Centroid			Right Part Centroid		
Ligand	Avg F_{635}	Std-dev	%CV	Avg F_{635}	Std-dev	%CV	avg F635	Std-dev	%CV	avg F635	Std-dev	%CV	avg F635	Std-dev	%CV
L6	15968	4436	27.8	17836	3037	17.0	15645	2058	13.2	19382	2578	13.3	18546	3072	16.6
L4a	10434	3093	29.6	11363	2491	21.9	10179	1444	14.2	11181	2208	19.8	12764	2935	23.0
L4b	25079	5502	21.9	27245	4173	15.3	23307	3555	15.3	27398	2128	7.8	31147	2030	6.5
L7	48537	9262	19.1	53496	5310	9.9	48599	4548	9.4	54620	3690	6.8	57411	3113	5.4
L3a	7151	2185	30.6	8117	1291	15.9	7137	1106	15.5	8430	952	11.3	8811	1171	13.3
L3b	15157	3057	20.2	16158	2040	12.6	15070	1916	12.7	16390	2085	12.7	17046	1615	9.5
L5a	7851	2139	27.2	8341	1748	21.0	7918	1268	16.0	8920	1975	22.1	8196	1826	22.3
L5b	31581	5350	16.9	32390	2859	8.8	31947	2750	8.6	32789	2700	8.2	32448	3133	9.7
L7a	32992	7831	23.7	36651	5433	14.8	32020	2774	8.7	37152	4709	12.7	40918	4399	10.8
L7b	14791	4605	31.1	16195	3322	20.5	12956	1819	14.0	16944	1899	11.2	18781	2914	15.5
L9a	16612	4630	27.9	18391	3310	18.0	14966	1107	7.4	20262	2145	10.6	20045	3023	15.1
L9b	41812	7828	18.7	45500	5838	12.8	39380	2637	6.7	47791	5200	10.9	49508	3078	6.2
L1a	18768	3903	20.8	20134	2815	14.0	17838	2497	14.0	20725	2225	10.7	21906	1983	9.1
L1b	22519	4786	21.3	24340	3430	14.1	21889	1763	8.1	24245	2629	10.8	26959	3580	13.3
Blank	14498	3225	22.2	15102	2350	15.6	12609	891	7.1	15486	1525	9.8	17285	1505	8.7

Region	average %CV
Full Lane	23.9
Full centroid	15.5
left part centroid	11.4
middle centroid	11.9
right part centroid	12.3

Table S5. A pixel by pixel analysis of variance along a barcode lane. The %CV values for the entire barcode set shown in Figure S15 using the full lane, full centroid, left part of the centroid, middle part of the centroid, and right part of the centroid are displayed in the top table. The average %CV values for each set are displayed in the bottom table.

centroid	L6	L4a	L4b	L7	L3a	L3b	L5a	L5b	L7a	L7b	L9a	L9b	L1a	L1b	Blank
L6	–	***	***	***	***	***	***	***	***	***	n.s.	***	***	***	***
L4a		–	***	***	***	***	***	***	***	***	***	***	***	***	***
L4b			–	***	***	***	***	***	***	***	***	***	***	***	***
L7				–	***	***	***	***	***	***	***	***	***	***	***
L3a					–	***	n.s.	***	***	***	***	***	***	***	***
L3b						–	***	***	***	n.s.	***	***	***	***	**
L5a							–	***	***	***	***	***	***	***	***
L5b								–	***	***	***	***	***	***	***
L7a									–	***	***	***	***	***	***
L7b										–	***	***	***	***	**
L9a											–	***	***	***	***
L9b												–	***	***	***
L1a													–	***	***
L1b														–	***
blank															–

Full lane	L6	L4a	L4b	L7	L3a	L3b	L5a	L5b	L7a	L7b	L9a	L9b	L1a	L1b	Blank
L6	–	***	***	***	***	*	***	***	***	*	n.s.	***	***	***	**
L4a		–	***	***	***	***	***	***	***	***	***	***	***	***	***
L4b			–	***	***	***	***	***	***	***	***	***	***	***	***
L7				–	***	***	***	***	***	***	***	***	***	***	***
L3a					–	***	**	***	***	***	***	***	***	***	***
L3b						–	***	***	***	n.s.	**	***	***	***	n.s.
L5a							–	***	***	***	***	***	***	***	***
L5b								–	n.s.	***	***	***	***	***	***
L7a									–	***	***	***	***	***	***
L7b										–	***	***	***	***	n.s.
L9a											–	***	***	***	***
L9b												–	***	***	***
L1a													–	***	***
L1b														–	***
Blank															–

Table S6. Calculated p-values for the pixel by pixel full-lane and centroid scatterplots. Symmetric 2-tail, unequal variance p-value matrixes for the centroid and full-lane scatterplots in Figure S15. The p-values are dentoted: n.s. $p>0.05$, * $0.05<p<0.005$, ** $0.005<p<0.0005$, *** $p<0.0005$.

	L1a	L1b	L2	L3a	L3b	L4a	L4b	L5a	L5b	L6	L7a	L7b	L8	L9a	L9b	BN-A_{20}-Cy3
Fig. 3	0.91	0.92	0.95	0.94	0.81	0.91	0.8	0.97	0.85	0.92	0.95	0.85	0.96	0.94	0.96	N/A
Fig. S16	0.95	0.95	0.96	0.96	0.83	0.93	0.87	0.98	0.88	0.94	0.97	0.89	0.98	0.95	0.97	0.66
Fig. S17	0.56	0.18	0.45	0.73	N/A	N/A	N/A	0.53	0.78	0.73	0.93	0.96	0.97	0.81	0.72	N/A

Table S7. The goodness of fit measurements for the allosteric KRas binding curves. The r-squared values for the curves after a double background correction in Figures 3 and S17, and a single background correction in Figure S16.

log[KRAS]	L1a	L1b	L2	L3a	L4a	L4b	L5a
-3.54	28.57	736.24	47.37	38.32	78.34	–	78.36
-3.84	55.65	61.13	73.45	10.80	–	–	23.71
-4.14	63.67	95.71	135.76	61.12	–	129.09	25.89
-4.62	41.32	78.02	37.31	29.92	113.59	18.39	83.24
-5.01	75.01	104.74	91.75	37.41	129.43	126.70	35.44
-5.32	72.72	62.92	311.26	68.47	–	75.72	33.16
-6.01	66.87	58.07	116.58	68.77	129.48	45.88	–
-6.32	43.25	34.28	74.47	117.95	94.72	21.05	114.95
-6.62	27.30	83.53	383.23	219.70	208.89	51.85	93.22
-7.01	91.85	79.90	298.37	112.63	59.01	182.28	88.78
avg %CV	56.62	139.45	156.95	76.51	116.21	81.37	64.08
log[KRAS]	L5b	L6	L7a	L7b	L8	L9a	L9b
-3.54	30.85	20.29	8.43	17.55	2.82	16.76	58.89
-3.84	24.60	80.02	12.77	9.85	3.79	26.82	31.45
-4.14	15.86	73.89	27.32	2.45	2.70	29.67	43.65
-4.62	38.87	–	14.52	10.08	6.82	31.29	34.73
-5.01	41.45	294.01	26.54	28.29	4.31	21.32	34.12
-5.32	29.12	65.36	179.54	59.14	23.74	22.19	38.54
-6.01	52.54	41.04	13.50	87.28	51.17	32.95	42.65
-6.32	69.24	29.65	22.00	178.75	125.33	29.51	70.74
-6.62	152.61	36.53	76.64	79.07	66.46	61.52	36.05
-7.01	16.40	134.31	69.97	16.02	–	30.45	48.13
avg %CV	47.15	86.12	45.12	48.85	31.90	30.25	43.89

Table S8. The %CV data for the ELISAs. The %CV measurements are given as the |%CV|. Any %CV>=300% has been marked with an "–". The global average %CV for the assays was 73.2%.

References

(1) Sano, T.; Cantor, C. R. *Proc. Natl. Acad. Sci.* **1990**, *87* (1), 142.

(2) *The extinction coefficients for the proteins, peptides, and epitopes were calculated using the peptide properties calulator found at http://biotools.nubic.northwestern.edu/proteincalc.html.*

(3) *The exctinction coefficients for the ssDNA strands were calulated using the IDT oligo analyzer at https://www.idtdna.com/calc/analyzer.*

(4) Boriack-Sjodin, P. A.; Margarit, S. M.; Bar-Sagi, D.; Kuriyan, J. *Nature* **1998**, *394* (6691), 337.

(5) *Expected masses were calculated using the mass calculator at the following: http://www.lfd.uci.edu/~gohlke/molmass/?q=C152H224N32O38S2Na*.