Chapter 2

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

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Protein catalyzed capture (PCC) agents are synthetic antibody surrogates that can target a wide variety of biologically relevant proteins. As a step towards developing a high-throughput PCC pipeline we report on the preparation of a barcoded rapid assay platform, used here for the analysis of hits from an in situ click screen of a macrocycle peptide library against allosteric epitopes of the KRas protein. The platform utilizes patterned, micrometer scale barcodes composed of orthogonal ssDNA strands on a glass slide. The slide is partitioned into microwells, each of which contains multiple copies of the full barcode. Biotinylated candidate PCCs from the screen are assembled onto designated barcode stripes using a complementary ssDNA-encoded cysteine-modified streptavidin library. A single microchip was utilized for the simultaneous evaluation of fifteen PCC candidate fractions under more than a dozen different assay conditions. The platform permitted a more than a 10-fold savings in time and a more than 100-fold reduction in biological and chemical reagents, relative to traditional multi-well plate assays. The platform was utilized to identify a KRas ligand that exhibits an *in vitro* inhibition constant (IC₅₀) of ~24 µM, which is an excellent starting point drugging this challenging target.

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Section 2.1-Introduction

Protein-catalyzed capture agents (PCCs) have been demonstrated to mimic the epitope targeting ability and high avidity of monoclonal antibodies for a number of protein targets.¹ PCCs can be engineered to have combined properties that are difficult to achieve for biologics, such as combinations of physical and biological stability, or, in one example, cell penetration.² State-ofthe-art PCCs are identified by carrying out an *in situ* click screen³ of a synthetic, strategically modified polypeptide fragment (the synthetic epitope, or SynEp) of the protein target against a synthetic one-bead-one compound (OBOC) library of macrocyclic peptides. The comprehensive OBOC library typically contains the roughly two million sequences that result from using all combinations of an 18-20 amino acid basis set to construct the variable 5-mer portion of the peptide.

PCC lead compounds are identified through a multi-step process, much of which is highly efficient. The OBOC library is first cleared of non-selective binders by screening against designated interferents. Candidate binders are then identified via a single generation *in situ* click screen against one or more SynEps of the targeted protein. That screen typically yields five to ten hits per SynEp. Once identified, those hit peptides are cleaved from the bead and sequenced using Edman degradation or mass spectrometry, prepared in ~1 mg quantities, and then chromatographically purified. These steps are relatively efficient, and, with commercial robotics, can be accomplished in a few days. However, each PCC candidate must then be tested for binding to the full-length

protein, often in various levels of serum background and under different blocking conditions. These assays are carried out on 96-well plates using a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) format, and represent a limiting factor in PCC production. Consider an *in situ* click screen in which a single OBOC library is screened against two SynEps to yield 15 PCC candidates. Each candidate is tested in, for example, a 10-point binding assay (run in triplicate) against the target protein. This yields $15 \times 3 \times 10 = 450$ data points, which might be repeated for various background serum concentrations. In addition to being laborious, these assays also consume significant amounts of chemical and biological reagents. Finding a more efficient solution for carrying out such assays should be useful for the production of other artificial antibody-type ligands, such as other classes of



Figure 2.1. Overview of the B-RAP Technology. A. The individual SAC-DNA conjugates. B. The biotinylated PCC ligands are added to the SAC-DNAs and allowed to complex. C. The individual SAC-DNA-ligand solutions are pooled and the cocktail is added to individual microwells on the DNA barcode. D. The SAC-DNA-ligand conjugates self-assemble with the DNA barcode to produce a fully assembled B-RAP assay.

peptides or aptamers.^{4–7}

We report here on the barcoded rapid assay platform (B-RAP) (Figure 2.1), which is a microchip platform designed so that an entire set of candidate PCC

ligands may be rapidly evaluated in parallel, using minimal quantities of reagents. Simultaneous testing of all PCCs under identical environments means that all assays are subject to the same uncertainties, which permits ready comparison of the EC₅₀ values for the entire set of hit peptides. The B-RAP technology draws from the Nucleic Acid Cell Sorting (NACS)⁸ and DNA-Encoded Antibody Library (DEAL) methods.^{9–12} The B-RAP process starts with a microscope slide that is patterned, using microfluidic flow channels, with a distinct set of orthogonal ssDNA oligomers. The PCC candidates are prepared with a biotin label, and then assembled onto cysteine-modified streptavidin (SAC) scaffolds that have been labelled with complementary ssDNA oligomers.^{13–15} Once assembled, these reagents are combined into a cocktail, and assembled onto specific stripes of the barcode pattern using DNA hybridization.¹⁶ The microchip surface itself is partitioned into microliter volume wells, each of which contains multiple copies of the full barcode. The B-RAP technology can simultaneously assay a full panel of candidate PCCs over a range of target protein concentrations (or other conditions), such that the EC₅₀ binding values for each candidate PCC are concurrently measured.

We used the B-RAP technology to analyze the resulting hits from an epitope targeted *in situ* click screen against the Kirsten rat sarcoma (KRas) protein.¹⁷ Oncoprotein variants of KRas are implicated in driving ~20-25% of all human cancers including almost all pancreatic cancers.¹⁸ Oncogenic Ras proteins have largely evaded targeting by traditional therapeutic techniques,^{19–22} but recent work has shown that specific mutant isoforms may be targetable.^{23,24} We targeted conserved epitopes denoted Switch I (aa 25-40) and Switch II (aa 56-75), which are known to allosterically influence KRas activity.²⁵ To our

knowledge, these allosteric regions have not previously been targeted, perhaps because there is no obvious hydrophobic binding pocket. After screening, we tested the resultant hit compounds for their relative binding strengths. The strongest binders were then tested in a functional assay for *in vitro* KRas GTPase activity inhibition.

Section 2.2-Methods²⁶

2.2.1-Preparation of the Barcode Rapid Assay Platform

DNA flow-patterned barcode chips, biotinylated peptides, and SAC-DNA were all used to assemble a miniaturized barcode of candidate PCCs for testing in a surface Immunofluorescent assay (IFA). Microfluidic flow patterning of 50 µm wide, 100 µm pitch ssDNA barcodes starts with adhering a polydimethylsiloxane (PDMS) microchannel mold onto a poly-L-lysine (PLL) coated glass microscope slide (Figure A2B.1, Appendix 2B). Reagents were flowed through the microchannels using a "pins-and-tubing-free" system that greatly simplified the preparation of barcoded microchips relative to the previous protocols (Figure A2B.2 and Table A2C.1, Appendix 2B and Appendix 2C).^{27,28} The PDMS mold was patterned with microwells at each microchannel inlet (Figure A2B.1A (i, ii), Appendix 2B). Reagents (3-5 µL) are micropipetted into the wells, and two machined acrylic plates are clamped across the top and bottom of the inlet region. The top acrylic plate contains a cavity that encompasses all of the inlet microwells. This cavity is pressurized to fill the microchannels in about 20 minutes (Figure A2B.2B, Appendix 2B). The increased pressure tolerance of the design can enable the use of microchannels of widths as small as 10 μ m. Initially 3 μ L of poly-l-lysine (0.1% (^w/_w) in H₂O) is flow patterned and dried overnight before flowing 5μ L of 300 μ M of each ssDNA (Table A2C.2, Appendix 2C) with 2mM bis(sulfosuccinimidyl)suberate (BS3) crosslinker. Approximately twenty to twenty-five DNA barcoded chips may be prepared in parallel (Figure A2B.2C, Appendix 2B). The bottom edge of the barcode is used to validate the coverage density and uniformity of the molecular patterns using fluorophore-labelled complementary ssDNA (Figures A2B.2 and A2B.3, Appendix 2B). Once validated, the barcoded slides may be vacuum-sealed for up to six months storage before use (Figure A2B.4, Appendix 2B; Table A2C.3, Appendix 2C).

The second component of the B-RAP technology, which is also independent of the specific identities of any PCC candidates to be tested, is the library of DNA-bound SAC (SAC-DNA) conjugates used to assemble individual biotinylated PCC candidates onto specific barcode lanes. The SAC protein (see Supplementary Methods in Appendix 2A) was conjugated with ssDNA strands complementary to the barcode DNA oligomers. This was done with Nsuccinimidly-4-formylbenzaldehyde (S-4FB) and maleimide 6-hydrazinonicotinamide (MHPH), followed by fast protein liquid chromatography (FPLC) purification (Figure A2B.4, Appendix 2B).

The performance of the library of fifteen SAC-DNAs 16,29 was evaluated by hybridizing library elements onto the flow patterned ssDNA barcodes. The barcodes were then incubated with varying amounts of the fluorophore probe biotin-A₂₀-Cy3 (Biotin*, 50-400nM) (Figure A2B.5, Appendix 2B). The resulting surface fluorescence was measured and compared to the fluorescence signal from the bottom edge barcode validation region. The fluorescent output with 532 nm excitation (F₅₃₂) of the captured biotinylated probe was lower than

that of the validation region (45 to 65k fluorescence units (f.u.)), likely reflecting the size of the SAC protein relative to the Cy3 fluorophore.

2.2.2-KRas Protein Expression and Purification

The KRas protein isoform 4B was expressed from transformed BL21(D3) *E. coli* cells as a His₆-tagged protein³⁰ and purified by FPLC using a Ni-NTA resin (Figure A2B.6, Appendix 2B). The fractions with pure KRas protein were dialyzed into tris-buffered saline (TBS, pH=7.4), aliquotted, and stored at -80.0 °C until needed.

2.2.3-Preparation of Switch I and Switch II SynEps and Scrambled SynEps

The synthetic epitopes (**SynEp1** and **SynEp2**) were 11-12 amino acid polypeptides with sequences extracted from the allosteric switch regions of KRas (Figure 2.2 and Table A2C.3, Appendix 2C). The **SynEp1** differs from the wild-type sequence as it is missing a valine residue. An azido click handle was added by substituting residue-similar azido-amino acids, as shown in Figure 2.2A. Rearranged versions of the SynEps were also prepared, and used in a pre-screen step to remove promiscuous binders. All epitopes were synthesized on biotin Novatag resin and purified using semi-preparative high performance liquid chromatography (semi-prep HPLC). The appropriate fractions were identified using matrix-assisted laser desorption ionization timeof-flight mass spectrometry (MALDI-TOF MS) (Figures A2B.7-A2B.19, Appendix 2B). Each SynEp was dissolved in dimethyl sulfoxide (DMSO), quantified using a Nanodrop 2000 spectrophotometer, and stored at 4 °C until use.

2.2.4-Library Preparation and In-situ Library Click Screen

A comprehensive OBOC library of 5-mer variable peptide macrocycles, using an 18 amino acid basis set, was prepared as previously reported.¹ The macrocyclic peptides were closed with a 1,4 triazole using Cu(I)-catalyzed click chemistry. These macrocycles were designed to present a propargylglycine click handle. The in situ library click screen was a dual SynEp version of a previously reported protocol.¹ After removing the beads that bound to the scrambled SynEps during a pre-clear screen the remaining library was incubated with both SynEp1 and SynEp2 (Supplementary Experimental Methods in Appendix 2A). After incubating with an anti-biotin capture antibody and an alkaline-phosphatase conjugated secondary antibody, the hit beads were identified by their deep purple color. The isolated hit beads were stored at RT in 0.1 M hydrochloric acid. Just prior to sequencing by Edman degradation, the beads were decolorized in N-methyl 2-pyrrolidone (NMP) (Table A2C.3, Appendix 2C). The hit compounds were then scaled up on biotin following previously established protocols,¹ purified, Novatag resin lyophilized, reconstituted in DMSO, quantified, and then stored at 4 °C until ready for use.

2.2.5-Surface Immunofluorescent Assays on the Barcoded Rapid Assay Platform

The barcode patterned microchip surface was partitioned into 16 individual microwells using a pre-fabricated PDMS slab. Individual biotinylated PCC candidates were complexed to specific SAC-DNA conjugates, combined into a cocktail, and then self-assembled, via DNA hybridization, onto designated barcode stripes (Figure 2.1). Incubation with a specific concentration of the target protein preceded incubation with a primary capture antibody and then a fluorophore-conjugated secondary detection antibody. During assay execution,

each well represents a different target concentration or assay condition. Once developed, the fluorescence of the barcodes is digitized using a GenePix 4400A array scanner, with an excitation laser power optimized to a power level of 40% (60 W), which maximizes detection sensitivity while also minimizing signal saturation. Data extraction occurs using 10 µm radius circles, taken along the length of a barcode stripe. A fluorescence signal representing the average of all the pixels within a given circle is collected. A total of ten circles (data-blocks) are measured along a 180 µm span of the middle portion for each individual barcode lane in a given well (Figure A2B.22, Appendix 2B – this illustrates the intensity across a stripe compared to the intensity from the data-block extraction). After extraction the data is background corrected. The background signal arises from (a) non-specific binding of the primary and secondary antibodies (independent of [KRas]), but can vary across different barcode stripes), and (b) non-specific binding of KRas protein ([KRas] dependent). Background (a) was assessed by measuring the average signal in the null protein well for each stripe. Background (b) was assessed by measuring the average fluorescence for the dummy ligand (Biotin* probe) that was in each well. The background-subtracted data was then graphed in Graphpad Prism 7 and fitted to a sigmoidal curve (Hill coefficient=1).

2.2.6-Measuring the Effect of the Allosteric Ligands on the Intrinsic KRas GTPase Activity

KRas inhibition assays were carried out using a GTPase Glo Assay kit from Promega (Figure A2B.26). Each candidate inhibitor PCC was initially tested by combining a concentration series of the ligand with 10 μ M KRas protein in an opaque white 96-well plate and incubated with 5 μ M 5'-guanosine triphosphate (GTP) for two hours The remaining GTP was converted to 5'-adenosine triphosphate (ATP) over 30 minutes using the reconstituted GTPase Glo reagent before a ten-minute incubation with the detection reagent. Chemiluminescence was measured using a Flexstation 3 plate reader (All wavelengths mode, 500 ms integration), and plotted using Graphpad Prism 7. A full inhibition curve of the most potent inhibitor was then generated using a four-hour incubation with GTP and a 2.5 μ M to 100 μ M concentration range. All measurements were done in triplicate.

Section 2.3-Results and Discussion

2.3.1-Optimizing B-RAP Technology Assay Conditions



(Figure 2.2A) yielded five beads from which nine candidate sequences were determined (Figure 2.2B). Biotinylated candidate ligands were then tested using a single-point IFA with the B-RAP technology (Figure A2B.20, Appendix 2B) to identify appropriate blocking conditions. Modification of the protein incubation solution to include the nonionic surfactant Polysorbate 20 (Tween20) was found to minimize non-specific binding between the KRas protein and the unmodified PLL surface.

2.3.2-Validation of the B-RAP Technology

Following optimization of the assay conditions the B-RAP technology was subjected to statistical tests to assess the variance in assay results measured within an individual microwell, between microwells on the same chip, and between different microchips. The average percent coefficient of variation (%CV) seen along an individual barcode stripe in the wells above background (500 nM to 400 µM KRas) using the values from the data-block extraction method was ~15%. Each microwell contains between two and three full copies of the DNA barcode. For the same barcode lane in the different full barcode sets in the same microwell, the fluorescence output was measured to have an average %CV of ~14% (Figure A2B.21A, Appendix 2B). The %CV between wells on the same microchip run under identical conditions was ~9%. The average %CV for identical barcode lanes between two separate platforms run in parallel by different users was ~18% with an average %CV of ~15% for the 1 µM to 400 µM range of KRas protein (Figure A2B.21B, Appendix 2B). Additionally, to validate that our data-block extraction method of a portion of the barcode lane captured the F_{635} for the entire barcode lane, the average F_{635} from a full-line line scan of the barcode lane was compared to the average F_{635} resulting from our data-block extraction method. The values from the full-line scan were contained within two standard deviations of the data-block extraction's average F_{635} (Figure A2B.22, Appendix 2B). This was compared to taking the measurement of individual pixels along the entirety of the barcode lanes in one full set of the 10 µM well for one plate (Figure A2B.23A, Appendix 2B) then graphing to find the centroid region (Figure A2B.23B, Appendix 2B), which is the region that is roughly stable in fluorescent

intensity. The average F_{635} , the standard deviation, and the %CV for each lane was calculated for the full lane, the centroid region of the lane, and the different parts of the centroid region (Table A2C.4, Appendix 2C). The full lane %CVs were in the 20-30% range, while the % CVs of the centroid regions were 10-20%. This arises from edge effects near the microwell walls. Assays of individual PCC candidates (different barcode stripes) collected within a single microwell, and so representing a single point of a binding curve, could be readily distinguished (Table A2C.5, Appendix 2C). These results indicated that the centroid region of a barcode stripe yielded the most reliable data, but also that assay results from different microwells, or different B-RAP chips, could be readily compared.



2.3.3-Measuring the EC₅₀ of the Allosteric Binding PCC Ligands

After characterizing the B-RAP technology, we used the platform to generate complete binding curves for 15 PCC ligand fractions simultaneously, (Figure 2.3B, for the binding curves without dummy ligand correction see Figure A2B.24, Appendix 2B) and determined the EC₅₀ values for each (Figure 2.3C) (for goodness of fit measurements for the curves see Table A2C.6, Appendix 2C).

These measurements were comprised of a 13-point concentration series, with each point collected in decaplicate. The EC₅₀ values enabled the ranking of the ligands, and the best binders were identified to be L1, L2, and L8. The true amino acid sequences for each hit peptide were also distinguished from the artifact sequences that arose from sequencing uncertainties. The true on-bead sequences for the hit beads are identified as L1, L2, L5, L7, and L8.

We also provide a comparison of the EC_{50} values from multi-well ELISA assays (triplicate measurements). While both assays identify **L1a** and **L1b** as the strongest binders, the ELISA assays are significantly noisier, with binding saturation not achieved for several ligands (for the ELISA curves see Figure A2B.25, Appendix 2B). The poor relative performance of the ELISAs arises from a few factors. First, the ligands tested are relatively weak (μ M-level) binders, and this exacerbates certain issues associated with the ELISAs. ELISAs are absorbance measurements, and thus have a significantly smaller dynamic range than the B-RAP fluorescence assays. Second, ELISA signal arises from enzymatic amplification, while the B-RAP assays are not amplified. For weak binders, amplified assays tend to be noisy, as both signal and noise are amplified. The improved relative sensitivity and statistics afforded by the B- RAP technology readily enables the comparative evaluation of these relatively weak KRas binders.

2.3.4-Testing the Allosteric Ligands as Inhibitors of KRas GTPase Activity

The ligands identified here were screened for binding to epitopes that exhibit structural fluctuations as the KRas protein switches between its *inactive* 5'-guanosine diphosphate (GDP)-bound form and its *active* GTP-bound form.²⁵ Consequently, the best three ligand fractions **L1a**, **L2**, and **L8** were probed in a functional, solution phase assay for their ability to disrupt the intrinsic GTPase



enzymatic activity of KRas protein (Figure 2.4A).

This assay measures the enzymatic conversion of GTP to GDP by KRas – a process that can potentially be inhibited. After incubation, an added GTPase Glo^{TM} reagent converts the remaining GTP to ATP, and the ATP is converted into a chemiluminescent signal. Thus, higher chemiluminescence translates to lower KRas enzymatic activity. For the measurements, a fixed [KRas protein] is incubated with varying ligand concentrations. A concentration of 10 μ M KRas protein was selected after generating a standard curve for the intrinsic

KRas GTPase activity (Figure A2B.26A, Appendix 2B). KRas is a slow acting enzyme, so a KRas/PCC incubation time of two hours was used for the initial survey scans. However, four-hour incubation times were used for the higher resolution data (Figure 2.4B). The survey assays indicated that all three ligands exhibited an inhibitory effect on the KRas protein's GTPase activity, but L2 was the most potent (Figure A2B.26, Appendix 2B). Thus, the modulation of KRas activity by L2 was recorded with an expanded concentration range (Figure 2.4B). We found that L2 switches from weakly *activating* to strongly inhibiting above 20 µM. Less than 5% of the rGTP was hydrolyzed in the L2only (no KRas) wells, and ~61% was hydrolyzed in the KRas-only wells. This result confirms that L2 lacks any innate GTPase enzymatic activity (Figure A2B.27, Appendix 2B). The sharp transition in the titration curve fits to a Hill coefficient of ~10, and suggests that upon full occupancy of the allosteric switch region, KRas flips into an inactive conformation. An IC₅₀ value was $24.0 \pm 1.2 \ \mu M$ for L2. This is an excellent starting point for a first generation allosteric inhibitor against this challenging target.

Section 2.4-Conclusions

Table 2.1. Comparison of the capacity, reagent quantities used, and assay times for multi-well ELISA plates relative to the barcoded rapid assay platform.		
Criteria	ELISA Platform	B-RAP Chip
Full binding curves per Assay	1	15
Relative amount PCC per binding curve (nmol)	7	0.15
Relative amount protein per binding curve (nmol)	300	2.7
Protein concentration points per assay	12	16
Assay run time (h)	10-36	8-10
# Data Points per platform	96	2400

We report on the development and use of a barcoded rapid assay microchip, which allows for the simultaneous evaluation of fifteen PCC candidate ligands in up to sixteen unique assay conditions, with significant associated savings in terms of both time and reagent use (Table 2.1). In a single day the B-RAP technology was applied to identify the best allosteric KRas binders from a pool of 15 ligands identified from a dual SynEp PCC library *in situ* click screen. The B-RAP technology is designed to yield an equilibrium-based EC₅₀ value for assessing relative binding strengths. For a number of PCCs, the EC₅₀ value provides an upper limit for the dissociation constant (K_D).¹ Importantly, relative binding affinities can provide guidance for selecting ligands for further quantitative characterizations, such as the solution phase KRas activity assay explored here. To this end, the B-RAP technology works well. A comparison of the B-RAP assay metrics relative to standard 96-well plate ELISAs is presented (Table 2.1). Extending this platform to evaluating PCC binders, or other ligand classes, against new protein targets should work well, requiring only an optimization of both concentration ranges (determined by the candidate ligands) and blocking conditions (typically determined by the protein target).³¹

Using the B-RAP platform coupled with the epitope-targeted *in situ* click screening approach, we identified a PCC ligand lead (**L2**) that serves as an allosteric inhibitor of the intrinsic GTPase enzymatic activity of KRas, with an IC₅₀ value of around 20 μ M. **L2** is a first generation ligand, and, as such, can surely be optimized, via medicinal chemistry methods, for increased potency and selectivity. Thus, given the well-known challenging nature of KRas as a drug target, **L2** provides an excellent starting point for developing a more potent inhibitor.

Section 2.5-References

Das, S.; Nag, A.; Liang, J.; Bunck, D. N.; Umeda, A.; Farrow, B.; Coppock, M.
 B.; Sarkes, D. A.; Finch, A. S.; Agnew, H. D.; Pitram, S.; Lai, B.; Yu, M. B.;

Museth, A. K.; Deyle, K. M.; Lepe, B.; Rodriguez-Rivera, F. P.; McCarthy, A.; Alvarez-Villalonga, B.; Chen, A.; Heath, J.; Stratis-Cullum, D. N.; Heath, J. R. *Angew. Chemie Int. Ed.* **2015**, *54* (45), 13219.

- (2) Farrow, B.; Wong, M.; Malette, J.; Lai, B.; Deyle, K. M.; Das, S.; Nag, A.;
 Agnew, H. D.; Heath, J. R. *Angew. Chemie Int. Ed.* 2015, *54* (24), 7114.
- (3) Agnew, H. D.; Rohde, R. D.; Millward, S. W.; Nag, A.; Yeo, W. S.; Hein, J. E.; Pitram, S. M.; Abdul Ahad Tariq, V.; Burns, A. M.; Krom, R. J.; Fokin, V. V.; Barry Sharpless, K.; Heath, J. R. *Angew. Chemie Int. Ed.* 2009, *48* (27), 4944.
- (4) Yüce, M.; Ullah, N.; Budak, H. Analyst **2015**, *140* (16), 5379.
- (5) Jost, C.; Plückthun, A. *Current Opinion in Structural Biology* **2014**, 102.
- (6) Mascini, M.; Palchetti, I.; Tombelli, S. Angew. Chemie Int. Ed. 2012, 51 (6), 1316.
- (7) Csordas, A. T.; Jørgensen, A.; Wang, J.; Gruber, E.; Gong, Q.; Bagley, E. R.;
 Nakamoto, M. A.; Eisenstein, M.; Soh, H. T. *Anal. Chem.* 2016, 88 (22), 10842.
- (8) Kwong, G. A.; Radu, C. G.; Hwang, K.; Shu, C. J.; Chao, M.; Koya, R. C.;
 (8) Comin-Anduix, B.; Hadrup, S. R.; Bailey, R. C.; Witte, O. N.; Schumacher, T. N.; Ribas, A.; Heath, J. R. *J. Am. Chem. Soc.* 2009, *131* (28), 9695.
- Bailey, R. C.; Kwong, G. A.; Radu, C. G.; Witte, O. N.; Heath, J. R. J. Am.
 Chem. Soc. 2007, 129 (7), 1959.
- (10) Boozer, C.; Ladd, J.; Chen, S.; Yu, Q.; Homola, J.; Jiang, S. Anal. Chem. 2004, 76 (23), 6967.
- Kozlov, I. A.; Melnyk, P. C.; Stromsborg, K. E.; Chee, M. S.; Barker, D. L.;
 Zhao, C. *Biopolymers* 2004, 73 (5), 621.
- (12) Adler, M.; Wacker, R.; Bootltink, E.; Manz, B.; Niemeyer, C. M. Nat. Methods

2005, *2* (2), 147.

- (13) Sano, T.; Cantor, C. R. Proc. Natl. Acad. Sci. 1990, 87 (1), 142.
- (14) Reznik, G. O.; Vajda, S.; Cantor, C. R.; Sano, T. *Bioconjug. Chem.* 2001, *12*(6), 1000.
- (15) Ramachandiran, V.; Grigoriev, V.; Lan, L.; Ravkov, E.; Mertens, S. A.; Altman, J. D. J. Immunol. Methods 2007, 319 (1–2), 13.
- (16) Shin, Y. S.; Ahmad, H.; Shi, Q.; Kim, H.; Pascal, T. A.; Fan, R.; Goddard, W. A.; Heath, J. R. *ChemPhysChem* **2010**, *11* (14), 3063.
- (17) Cooper, G. M. Science 1982, 217 (4562), 801.
- (18) Cox, A. D.; Fesik, S. W.; Kimmelman, A. C.; Luo, J.; Der, C. J. *Nature Reviews Drug Discovery*. 2014, pp 828–851.
- Whitehead, R. P.; Mccoy, S.; Macdonald, J. S.; Rivkin, S. E.; Neubauer, M. A.;
 Dakhil, S. R.; Lenz, H.-J.; Tanaka, M. S.; Abbruzzese, J. L. *Invest. New Drugs*2006, 24, 335.
- Macdonald, J. S.; Mccoy, S.; Whitehead, R. P.; Iqbal, S.; Wade Iii, J. L.;Giguere, J. K.; Abbruzzese, J. L. *Invest. New Drugs* 2005, *23*, 485.
- (21) Winquist, E.; Moore, M. J.; Chi, K. N.; Ernst, D. S.; Hirte, H.; North, S.;
 Powers, J.; Walsh, W.; Boucher, T.; Patton, R.; Seymour, L. *Urol. Oncol.* 2003, 23 (3), 143.
- (22) Sharma, S.; Kemeny, N.; Kelsen, D. P.; Ilson, D.; O 'reilly, E.; Zaknoen, S.;
 Baum, C.; Statkevich, P.; Hollywood, E.; Zhu, Y.; Saltz, L. B. Ann. Oncol.
 2002, 13, 1067.
- (23) Ostrem, J. M.; Peters, U.; Sos, M. L.; Wells, J. A.; Shokat, K. M. *Nature* 2013, 503.
- (24) Sakamoto, K.; Kamada, Y.; Sameshima, T.; Yaguchi, M.; Niida, A.; Sasaki, S.;

Miwa, M.; Ohkubo, S.; Sakamoto, J. ichi; Kamaura, M.; Cho, N.; Tani, A. Biochem. Biophys. Res. Commun. **2017**, 484 (3), 605.

- (25) Hall, B. E.; Yang, S. S.; Boriack-Sjodin, P. A.; Kuriyan, J.; Bar-Sagi, D. J. Biol.
 Chem. 2001, 276 (29), 27629.
- (26) For detailed protocols of all experiments see Appendix 2A.
- (27) Yu, J.; Zhou, J.; Sutherland, A.; Wei, W.; Shin, Y. S.; Xue, M.; Heath, J. R.
 Annu. Rev. Anal. Chem. 2014, 7 (1), 275.
- Wei, W.; Shin, Y. S.; Xue, M.; Matsutani, T.; Masui, K.; Yang, H.; Ikegami,
 S.; Gu, Y.; Herrmann, K.; Johnson, D.; Ding, X.; Hwang, K.; Kim, J.; Zhou, J.;
 Su, Y.; Li, X.; Bonetti, B.; Chopra, R.; James, C. D.; Cavenee, W. K.;
 Cloughesy, T. F.; Mischel, P. S.; Heath, J. R.; Gini, B. *Cancer Cell* 2016, 29
 (4), 563.
- (29) Xue, M.; Wei, W.; Su, Y.; Kim, J.; Shin, Y. S.; Mai, W. X.; Nathanson, D. A.;
 Heath, J. R. J. Am. Chem. Soc. 2015, 137 (12), 4066.
- (30) Boriack-Sjodin, P. A.; Margarit, S. M.; Bar-Sagi, D.; Kuriyan, J. *Nature* 1998, *394* (6691), 337.
- (31) Gibbs, J. ELISA Tech. Bull. Corning Inc. Life Sci. Kennebunk, ME 2001, No. 3,
 1.