Supporting Information for

Off-DNA DNA-Encoded Library Affinity Screening

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Materials Sources. All reagents were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise specified. 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethansesulfonic acid (HEPES, TCI, Tokyo, Japan), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS, GBiosciences), 1,4-dithiothreitol (DTT), 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis-Tris), copper (II) sulfate ($CuSO_4$), ascorbic acid (Across Organic), tris(benzyltriazolylmethyl)amine (TBTA), Ethylenediaminetetraacetic acid (EDTA, BioRad), Triethylammonium acetate (TEAA), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), α-cyano-4-hydroxycinnamic acid (HCCA) (Life Technologies, Carlsbad, CA), N,N'-diisopropylcarbodiimide (DIC, Acrōs Organics, Fair Lawn, NJ), 1- hydroxy-7-azabenzotriazole (HOAt, Accela ChemBio Inc., San Diego, CA), 2,4,6- trimethylpyridine (TMP), ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma), dimethylformamide (DMF, Thermo Fisher Scientific, Waltham, MA), dichloromethane (DCM, Thermo Fisher Scientific), N,N-dimethylacetamide (DMA, Acros Organics), N,N-diisopropylethylamine (DIEA, Thermo Fisher Scientific), acetonitrile (ACN, Thermo Fisher Scientific), dimethyl sulfoxide (DMSO, AMRESCO Inc., Solon, OH), 40% acrylamide/bis solution 19:1 (AAM/Bis, BioRad), ammonium persulfate (APS, BioRad), tetramethylethylenediamine (TEMED, BioRad), phosphate buffered saline (PBS), bovine serum albumin (BSA), magnesium chloride ($MgCl_2$), poly- (dimethylsiloxane) (PDMS, Dow Corning, Midland, MI), 1-(4-methoxyphenyl)-3-(4-tert-butylphenyl)-1,3-propanedione (Avobenzone, Spectrum, Gardena, CA), 4',6-diamidino-2-phenylindole (DAPI, Anaspec), (4- Fmoc-2-methoxy-5-nitrophenoxy)butanoic acid (Fmoc-PC-OH, Santa Cruz Biotechnology Inc., Dallas, TX), N-α-Fmoc-N-ε-7-methoxycoumarin-4-acetyl-L-lysine (*N*-α-Fmoc-K(Mca)-OH), N_α-Fmoc-N_ω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine (N-α-Fmoc-R(Pbf)-OH, Thermo Fisher Scientific), CAFmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-PEG_2, ChemPep, Inc, Wellington, FL), sodium acetate, calcium chloride, Taq DNA polymerase (Taq, New England Biolabs, Ipswich, MA), and 2′-

deoxyribonucleotide triphosphate (dNTP, set of dATP, dTTP, dGTP, dCTP, Promega Corp., Milwaukee, WI) were used as provided. Solvents used in solid-phase synthesis were dried over molecular sieves (3 Å, 3.2 mm pellets).

Synthesis on mixed-scale resin was performed on TentaGel rink amide resin (160 μm, 0.43 mmol/g, Rapp-Polymere, Tuebingen, Germany) and TentaGel amino functionalized resin (10 μm, 0.29 mmol/g, Rapp-Polymere, Tuebingen, Germany). DDR1 FP probe synthesis was perform on Fmoc-Rink amide MBHA resin (MBHA resin, 0.44 mmol/g, AnaSpec) with 5(6)-carboxylfluorescein (5(6)-FAM, Chem Impex International Inc, Wood Dale, IL).

Oligonucleotides (Integrated DNA Technologies, Inc. Coralville, IA) were purchased as desalted lyophilate and used without further purification. Oligonucleotide ligation substrates were 5′-phosphorylated (/5Phos/). Amino-modified headpiece DNA (NH_2-HDNA, /5Phos/GAGTCA/iSp9//iUniAmM//iSp9/TGACTCCC) was HPLC purified at the manufacturer and used without further purification.

Fmoc-amino acid and carboxylic acid building blocks were selected from the Roche Pharma Research and Early Development (pRED) compound collection.

Buffers. Bis-Tris propane wash buffer (BTPWB, 50 mM NaCl, 0.04% Tween-20, 10 mM Bis-Tris, pH 7.6), Bis-Tris propane breaking buffer (BTPBB, 10 mM NaCl, 1% SDS, 1% Tween-20, 10 mM Bis-Tris, pH 7.6), 1× PCR master mix (2 mM dATP, 2 mM dGTP, 2 mM dCTP, 2 mM dTTP, 15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris, pH 8.3), saline-Tris-EDTA buffer (STE) (75 mM NaCl, 50 mM Tris pH 7.5, 5 mM EDTA), saline Tris-EDTA with ammonium persulfate buffer (15 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA with 0.2% APS, flooded with argon for 5 minutes, then sonicated for 10 minutes), acrylamide solution (8% AAM/Bis, 15 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA , 0.2% APS, sparged with argon for 10 minutes), and DDR1 binding assay buffer (50 mM Hepes pH 7.5, 10 mM $MgCl_2$, 0.03% CHAPS, 1 mM DTT) were prepared in DI H_2O.

DDR1 ligand FP probe synthesis. All reaction and wash volumes were constant (1 mL) unless otherwise specified. Fmoc-Rink amide MBHA resin (0.44 mmol/g, 23 mg, 10 μmol) was transferred to a fritted syringe, swelled in DMF (1 h, 8 rpm, RT), washed (DMF, 3x; DCM, 3x; DMF, 3x) and Fmoc was removed (20% piperidine in DMF, 2 x 5 min, 8 rpm, RT). The resin was washed (DMF, 3x; DCM, 3 x; DMF, 3x). Azido-L-lysine (50 μmol, Fmoc-L-Lys(N3)-OH, AstaTech) was activated (2 min) with COMU/DIEA (50 μmol/100 μmol) in DMF (1 mL), added to resin, and incubated (15 min, 37°C, 8 rpm, 2 x). Resin was washed (DMF, 3x;DCM, 3 x; DMF, 3x) and capped (20% acetic anhydride in DMF, 37°C, 8 rpm, 15 min). Resin was washed (DMF, 6x; DCM, 3 x; DMF, 3x). Fmoc was removed (20% piperidine in DMF, 2 x 5 min, 8 rpm, RT) and resin was washed (DMF, 3 x; DCM, 3 x; DMF, 3x). The remaining probe synthesis proceeded via iterative cycles of solid-phase peptide synthesis. Each cycle included (1) 9-fluorenylmethoxycarbonyl (Fmoc) deprotection (20% piperidine in DMF, 2 x 5 min, 8 rpm, RT); (2) N-α-Fmoc-amino acid (30 μmol) activation with COMU/DIEA (30 μmol/ 60 μmol) in DMF and incubation (2 minutes); (3) N-α-Fmoc amino acid coupling to resin by transferring activated acid (1 mL) to resin and incubating (5 min x 2, 37 °C, 8 rpm). After each deprotection and monomer coupling step, reactants were expelled and the resin washed (DMF, 3x; DCM, 3x; DMF, 3x). The following N-α-Fmoc amino acid couplings were performed in order: (1) Fmoc-PEG_2 (Oakwood Chemical); (2) Fmoc-3-aminomethyl-phenylacetic acid (ChemImpex, Wood Dale, IL) ; (3) Fmoc-3-carboxymethyl-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one (CombiBlocks, San Diego, CA); (4) 1H-indazole-5-carboxylic acid (ArkPharm, Arlington Heights, IL) . Resin was then washed (DMF, 3 x; DCM, 3 x; DMF, 3x), and dried under vacuum overnight.

The synthesized DDR1 ligand azide intermediate was purified before coupling to alkyne fluorescein. Resin was transferred to a clean fritted spin column (Mobicol, large filter, 10 μm pore size, MoBiTec GmbH, Goettingen, Germany), washed (DCM, 3 x), and dried under vacuum. Cleavage cocktail (90/5/5 TFA/TIPS/DCM, 300 μL) was added to resin and incubated

(1 h, RT). Cleaved linker was expelled, dried down under vacuum, resuspended (20% DMSO and 0.1% TFA in H_2O, 100 µL), and purified by reversed-phase HPLC (XBridge BEH130 C18, 4.6 x 100 mm, 130 Å, 3.5 µm, Waters, Milford, MA) with gradient elution (mobile phase A: ACN; mobile phase B: 0.1% TFA in H_2O; 5% - 75% A, 21 min). HPLC fraction aliquots (1 µL) were spotted on a MALDI-TOF MS plate, dried, and covered with HCCA matrix solution (1.5 mg/mL HCCA in 1:2 ACN:0.1% TFA in H_2O) and analyzed with MALDI-TOF MS (Microflex, Bruker Daltonics, Inc., Billerica, MA). After purification, DDR1 ligand azide product fractions were dried under vacuum and resuspended in DMSO (10 mM, 50 µL).

5(6)-FAM (10 µmol) and DIEA (60 µmol, 10.7 µL) were dissolved in DMF (39.3 µL), combined with COMU (30 µmol in 48.1 µL DMF), and incubated (2 minutes, RT). Propargylamine (30 µmol, 1.9 µL) was added and the reaction was incubated with shaking (50 °C, 1 hr, 300 rpm). Propargylated FAM product was directly injected (4 x 25 µL) into reverse-phase for purification (XBridge Prep C18 5 µm 10 x150 mm, Waters, Milford, MA) with gradient elution (mobile phase A: ACN; mobile phase B: 0.1% TFA in H_2O; 35% - 40% A, 21 min). HPLC fraction aliquots (1 µL) were spotted on a MALDI-TOF MS plate, dried, and covered with HCCA matrix solution (1.5 mg/mL HCCA in 1:2 ACN:0.1% TFA in H_2O) and analyzed with MALDI-TOF MS (Microflex, Bruker Daltonics, Inc., Billerica, MA). After purification, propargylated FAM fractions were dried under vacuum and resuspended in DMSO (100 µL).

Azido DDR1 ligand (10 mM, 4 µL) was combined with the propargylated FAM (25 mM, 0.4 µL) in H_2O (4.4 µL). CuAAC catalyst (4.2 mM $CuSO_4$, 20.6 mM ascorbic acid, 5 mM TBTA, and 333.3 mM TEAA pH 7 in H_2O, 30 µL) was added to the azide-alkyne mix, and the reaction was incubated with shaking (1.5 h, RT, 300 rpm). EDTA (0.5 M pH 8, 1 µL) was added and the reaction was incubated (2 min, 60 °C). The FAM-labeled DDR1 ligand product was resuspended in DMSO (100 µL) and purified via HPLC. Fractions containing product were collected, analyzed via MALDI-TOF MS, dried down via vacuum, and resuspended in DMSO (100 µL).

The FAM-labeled DDR1 ligand product resin was transferred to a clean fritted spin

column (Mobicol, large filter, 10 μm pore size, MoBiTec GmbH, Goettingen, Germany), washed (DCM, 3 x), and dried under vacuum. Cleavage cocktail (90/5/5 TFA/TIPS/DCM, 300 μL) was added to resin and incubated (1 h, RT). Cleaved linker was expelled, dried down under vacuum, resuspended (20% DMSO and 0.1% TFA in H_2O, 100 μL), and purified by reversed-phase HPLC (XBridge BEH130 C18, 4.6 x 100 mm, 130 Å, 3.5 μm, Waters) with gradient elution (mobile phase A: ACN; mobile phase B: 0.1% TFA in H_2O; 5% - 75% A, 21 min). HPLC fraction aliquots (1 μL) were spotted on a MALDI-TOF MS plate, dried, and covered with HCCA matrix solution (1.5 mg/mL HCCA in 1:2 ACN:0.1% TFA in H_2O) and analyzed with MALDI-TOF MS (Microflex, Bruker Daltonics, Inc., Billerica MA). After purification, FAM-labeled DDR1 ligand product fractions were dried under vacuum and resuspended in DMSO.

Autotaxin ligand FP probe synthesis. All reaction and wash volumes were constant (400 μL) unless otherwise specified. Fmoc-Rink amide MBHA resin (0.44 mmol/g, 11.5 mg, 5 μmol) was transferred to a fritted syringe, swelled in DMF (overnight, 8 rpm, RT), washed (DMF, 3x; DCM, 3x; DMF, 3x). Probe synthesis proceeded via iterative cycles of solid-phase peptide synthesis. Each cycle included (1) 9-fluorenylmethoxycarbonyl (Fmoc) deprotection (20% piperidine in DMF, 2 x 5 min, 8 rpm, RT); (2) N-α-Fmoc-amino acid (15 μmol) activation with COMU/DIEA (15 μmol/ 30 μmol) in DMF and incubation (2 minutes); (3) N-α-Fmoc amino acid coupling to resin by transferring activated acid (400 μL) to resin and incubating (5 min x 2, 37 °C, 8 rpm). After each deprotection and monomer coupling step, reactants were expelled and the resin washed (DMF, 3x; DCM, 3x; DMF, 3x). The following N-α-Fmoc amino acid couplings were performed in order: (1) Fmoc-Lys(Mtt)-OH (CombiBlocks, San Diego, CA); (2) Fmoc-PEG_2 (Oakwood Chemical); (3) Fmoc-(R)-3-amino-4-(3,4-dichloro-phenyl)-butyric acid (CombiBlocks); (4) 2-benzyl-1,3-thiazole-4-carboxylic acid (Enamine). Resin was then washed (DMF, 3 x; DCM, 3 x; DMF, 3x). The Mtt protecting group on the lysine was deprotected (1% TFA, 5% TIPS in DCM, 3 x 5 min, 8 rpm, RT). Resin was washed with Mtt deprotection solution

(1% TFA, 5% TIPS in DCM, 2x). Resin was washed (DMF, 8x; DCM, 8x; DMF, 8x). 5(6)-FAM (25 μmol in 150 μL DMF) and DIEA (50 μmol in 100 μL DMF) were combined with COMU (50 μmol in 150 μL DMF), and incubated (2 minutes, RT). Activated FAM solution was added to resin displaying ATX ligand with deprotected lysine (90 min, 50° C, 8 rpm). Resin was washed (DMF, 8x; DCM, 8x; DMF, 8x).

Resin was washed (DCM, 3 x), and dried under vacuum. Cleavage cocktail (90/5/5 TFA/TIPS/DCM, 450 μL) was added to resin and incubated (1 h, RT). Cleaved ATX ligand FP probe product was expelled, dried down under vacuum, resuspended (20% DMSO and 0.1% TFA in H_2O, 100 μL), and purified by reversed-phase HPLC (XBridge BEH130 C18, 4.6 x 100 mm, 130 Å, 3.5 μm, Waters, Milford, MA) with gradient elution (mobile phase A: ACN; mobile phase B: 0.1% TFA in H_2O; 5% - 75% A, 21 min). HPLC fraction aliquots (1 μL) were spotted on a MALDI-TOF MS plate, dried, and covered with HCCA matrix solution (1.5 mg/mL HCCA in 1:2 ACN:0.1% TFA in H_2O) and analyzed with MALDI-TOF MS (Microflex, Bruker Daltonics, Inc., Billerica, MA). After purification, ATX-FAM FP probe product fractions were dried under vacuum and resuspended in DMSO (25 μL).

Microplate fluorescence polarization assays. Biotin or DDR1 FP probe (10 nM, 5 μL) was added to increasing concentrations of streptavidin or DDR1 target (0 — 1000 nM, 5 μL) in 384-well black microplates (Greiner Bio-One, Frickenhausen, Germany). After incubation (15 minutes, RT), fluorescence polarization of the probe (λ_{ex} = 470 nm and λ_{em} = 520 nm) was measured using a multi-mode plate reader (Infinite M1000, Tecan, Zurich, Switzerland). The ΔmP for probe binding was calculated by subtracting the FP of probe-only wells from the FP of target-containing wells.

Microfluidic device fabrication. Channel structures were fabricated in PDMS using soft lithography (1). Two-tone PDMS devices were fabricated as previously described (2), (3).

Avobenzone (34 mg) was dissolved in toluene (200 μL) and mixed into PDMS prepolymer (5.5 g, 10:1 elastomer base/curing agent). Degassed avobenzone- PDMS prepolymer was loaded into a syringe (3 mL, BD Medical, Franklin Lakes, NJ), and applied over the incubator and bead introduction regions of the circuit master. After partial curing (12 min, 80 °C), degassed PDMS prepolymer (44 g, 10:1 base:curing agent) was poured over the master, cured (1 h, 80 °C), cooled (10 min, RT), and peeled from the master. Fluidic ports were punched with a biopsy punch (0.75 mm dia., World Precision Instruments, Inc., Sarasota, FL). PDMS molds and glass slides were cleaned with Safe-Soap (Gold Biotechnologies Inc., St. Louis, MO), then rinsed with DI water and isopropyl alcohol prior to immersion in acid hydrolysis solution (5:1:1 DI H2O/HCl/H2O2, 30 min). Glass slides and PDMS molds were again rinsed with DI water and isopropyl alcohol, and then immediately bonded (16 h, 80 °C) {Sui:tm}. Microfluidic devices were fitted with integrated waveguides (2). Waveguide illumination intensity was calibrated by pumping (0.5 μL/min) a solution of DAPI (500 μM, 20 mM Tris-HCl, 150 mM NaOAc, 2.5 mM CaCl2, pH 8.3) through the waveguide calibration channel while varying the LED current (5.00, 2.81, 1.58, 0.89 V). Calibrant solution fluorescence emission was measured in the $I_{\parallel}$ channel (100 Hz). Afterward, the calibration channel was rinsed with water, dried with air, and filled with trimethylsiloxy-terminated PDMS (DMS-T22, Gelest, Inc., Tullytown, PA).

Mixed-scale photocleavable linker synthesis. TentaGel Rink-amide resin (160 μm dia., 0.40 mmol/g, 50 mg, Rapp-Polymere, Tuebingen, Germany) was transferred to a synthesis spin column (Mobicol classic, 1 mL, Boca Scientific, Westwood, MA) fitted with a polyethylene filter (10 μm pore size, Boca Scientific, Westwood, MA) and swelled in DMF (400 μL, 1 h, RT, 8 rpm). Resin was washed (DMF, 3 x 400 μL) and Fmoc was removed (20% piperidine in DMF, 400 μL, 1 x 5 min, 1 x 15 min, RT, 8 rpm). Photocleavable linker synthesis proceeded via iterative cycles of solid-phase peptide synthesis. Each cycle included (1) 9-fluorenylmethoxycarbonyl (Fmoc) deprotection (20% piperidine in DMF, 2 x 5 min, RT, 8 rpm); (2) N-α-Fmoc-amino acid (60

μmol) activation with COMU/DIEA (30 μmol/ 120 μmol) in DMF and incubation (2 minutes); (3) N-α-Fmoc amino acid coupling to resin by transferring activated acid (400 μL) to resin and incubating (2 x 30 min, 50°C, 8 rpm). After each deprotection and monomer coupling step, reactants were expelled and the resin washed (DMF, 3x 400 μL ; DCM, 3x 400 μL ; DMF, 3x 400 μL). The following N-α-Fmoc amino acid couplings were performed in order: (1) Fmoc-Lys(Mca)-OH ; (2) Fmoc-R(Pbf)-OH ; (3) . Resin was then washed (DMF, 3 x; DCM, 3 x; DMF, 3x, 400 μL.

TentaGel resin (10 μm dia., 0.29 mmol/g, 120 mg, Rapp-Polymere) was transferred to a fritted syringe (6 mL, Torviq, Tucson, AZ) along with the above functionalized 160 μm diameter TentaGel resin and swelled in DMF (1 h, RT, 8 rpm), washed (DMF, 3x, 2 mL). Fmoc was removed (20% piperidine in DMF, 1 x 5 min, 1 x 15 min, RT, 8 rpm) and resin was washed (DMF, 3 x 2 mL ; DCM, 3 x 2 mL ; DMF, 3 x 2mL). Fmoc-Gly-OH (400 μmol) was combined with DIC/Oxyma/DIEA (400 μmol/400 μmol/800 μmol) in DMF (2 mL), added to resin, and incubated (1 h, 8rpm, 50 °C). Resin was washed (DMF, 3 x 2 mL ; DCM, 3 x 2 mL ; DMF, 3 x 2mL) and capped (20% acetic anhydride in DMF, 15 min, 37 °C, 8 rpm). Resin was washed (DMF, 3 x 2 mL ; DCM, 3 x 2 mL ; DMF, 3 x 2mL). Fmoc was removed (20% piperidine in DMF, 2 x 5 min, RT, 8 rpm) and resin was washed (DMF, 3 x 2 mL ; DCM, 3 x 2 mL ; DMF, 3 x 2mL). Bromoacetic acid (400 μmol) was activated with DIC (800 μmol) in DMF (2 mL), added to resin, and incubated (1 h, 50 °C, 8 rpm). Resin was washed (DMF, 3 x 2 mL ; DCM, 3 x 2 mL ; DMF, 3 x 2mL). Propargylamine (1 M) was prepared in DMF (1 mL), added to resin, and incubated (3 h, 50 °C, 8 rpm). Resin was washed (DMF, 3 x 2 mL ; DCM, 3 x 2 mL ; DMF, 3 x 2mL). Fmoc-Gly-OH (400 μmol) was combined with DIC/Oxyma/DIEA (400 μmol/400 μmol/800 μmol) in DMF (2 mL), added to resin, and incubated (1 h, 50 °C, 8 rpm). Resin was washed (DMF, 3 x 2 mL ; DCM, 3 x 2 mL ; DMF, 3 x 2mL). Fmoc was removed (20% piperidine in DMF, 1 x 5 min., 1 x 15 min., 8 rpm, RT), and the resin was washed (DMF, 3 x 2 mL ; DCM, 3 x 2 mL ; DMF, 3 x 2mL). Fmoc-PC-OH (4- Fmoc-2-methoxy-5-nitrophenoxy)butanoic acid,

Sant Cruz Biotechnology Inc., 200 µmol) was combined with DIC/Oxyma/TMP (300 µmol/200 µmol/200 µmol) in DMF (2 mL), added to resin, and incubated (2 x 2 h, 50°C, 8 rpm). The resin was washed (DMF, 3 x 2 mL ; DCM, 3 x 2 mL ; DMF, 3 x 2mL) to yield finished mixed-scale photocleavable liner resin (165 mg).

MALDI-TOF MS analysis of mixed-scale compound synthesis. After solid-phase synthesis, 160-µm TentaGel beads were separated from 10-µm beads by filtration (150 µm, CellTrics, Sysmex, Goerlitz, Germany). The 160-µm resin was transferred to a new fritted spin column (10 µm pore size, MoBiTec GmbH, Goettingen, Germany), washed (DCM, 3x, 400 µL), and dried under vacuum. Cleavage cocktail (90/5/5 TFA/TIPS/DCM, 300 µL) was added to resin and incubated (1 h, RT). Cleavage supernatant was expelled, dried under vacuum, and resuspended (20% DMSO, 50:50 ACN:0.1% TFA in H_2O, 100 µL). A dilution of the product (1:10 in 50:50 ACN: 0.1% TFA in H_2O, 100 µL) was spotted (1 µL) on a MALDI-TOF MS plate, dried, covered with HCCA matrix solution (1.5 mg/mL HCCA in 1:2 ACN:0.1% TFA in H_2O) and analyzed with MALDI-TOF MS (Microflex, Bruker Daltonics, Inc.).

DDR1 ligand positive control synthesis. DDR1 ligand positive control resin was synthesized on mixed-scale photocleavable linker resin (3 mg). Resin was loaded into a fritted synthesis spin column (10 µm pore size, Mobicol) and washed (DMF, 3 x; DCM, 3 x; DMF, 3x, 400 µL). Synthesis of hits proceeded as described previously (3). Briefly, Fmoc was removed (20% piperidine in DMF, 1 x 5 min, 1 x 15 min, RT, 8 rpm) and resin was washed (DMF, 3 x; DCM, 3 x; DMA, 3x, 400 µL). Fmoc-3-aminomethyl-phenylacetic acid (40 mM) was combined with HOAt/DIC (40 mM/57 mM) in DMA (0.4 mL), added to resin, and incubated (1 h, 37 °C, 8 rpm). Resin was washed (DMA, 3 x; DCM, 3 x; DMF, 3x, 400 µL). Fmoc was removed (20% piperidine in DMF, 1 x 5 min, 1 x 15 min, RT, 8 rpm) and resin was washed (DMF, 3 x; DCM, 3 x; DMA, 3x, 400 µL). Fmoc-3-carboxymethyl-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one (40 mM)

was combined with HOAt/DIC (40 mM/57 mM) in DMA (0.4 mL), added to resin, and incubated (1 h, 37 °C, 8 rpm). Resin was washed (DMA, 3 x; DCM, 3 x; DMF, 3x, 400 µL). Fmoc was removed (20% piperidine in DMF, 1 x 5 min, 1 x 15 min, RT, 8 rpm) and resin was washed (DMF, 3 x; DCM, 3 x; DMA, 3x, 400 µL). 1H-indazole-5-carboxylic acid (40 mM) was combined with HOAt/DIC (40 mM/57 mM) in DMA (0.4 mL), added to resin, and incubated (1 h, 37 °C, 8 rpm). Resin was washed (DMA, 3 x; DCM, 3 x; DMF, 3x, 400 µL).

DEL preparation for screening. Library beads (10^6) were washed (4% Tween-80, 3 x 500 µL), resuspended (4% Tween-80, 500 µL), and sonicated (30 min). After sonication, beads were washed (4% Tween-80, 500 µL; 8% acrylamide, 2 x 500 µL), and resuspended (8% acrylamide, 500 µL). Beads were sonicated (30 min) and centrifuged (30 s, 6,000 rpm). Supernatant was and the beads were resuspended (STE, 0.2% APS, 100 µL). Oil (4% w/w Span-80 in mineral oil, 500 µL) was added to bead solution and the suspension was emulsified (TissueLyser, Qiagen, 2 min, 20 Hz). Immediately after first emulsification, oil with TEMED (0.4% TEMED in 4% Span-80/mineral oil) was added to bead and oil solution and emulsified (2 min, 20 Hz). Emulsions were incubated (1h, 60 °C, 300 rpm), centrifuged (5min, 14,000 x g), and oil supernatant. BTPBB (1 mL) was added to emulsions, beads were centrifuged (2 min, 14,000 x g), and oil supernatant removed. BTPBB wash (500 µL) was repeated six times. BTPWB (500 µL) was added to the beads, beads were centrifuged (30 s, 6,000 rpm), and supernatant was removed. This BTPWB wash was repeated three times. Beads were washed (3x, 0.4% Tween-80 DDR1 assay buffer without DTT) and stored for filtering.

Acrylamide-treated beads (10^6) were filtered before screening (CellTrics, 20 µm, 3 x), loaded into a syringe (1 mL, 0.4% Tween-80 DDR1 assay buffer without DTT, BD Medical), fitted with a blunt-tip Luer-Lok needle (30 gauge, Small Parts, Inc.), connected to a microfluidic filter device (3) via Microbore Tygon tubing (0.01 in. i.d. X 0.03 in. o.d., Saint Gobain), and manually driven through the microfluidic filter. The syringe was refilled (1 mL, 0.4% Tween-80

DDR1 assay buffer without DTT) and syringe contents were filtered twice.
The filtered bead solution was pelleted and exchanged to fresh DDR1 assay buffer for quantitation with a hemocytometer.

Hit bead preparation and DNA amplification for sequencing. Water-in-oil emulsion hit collections were resuspended in emulsion-breaking wash buffer (1% SDS, 1% Tween-20 in BTPWB) and vortexed (O/N). The broken emulsion was filtered (10 μm, Mobicol) and hit bead retenate collected. The hit collection tube was washed (1% SDS, 1% Tween-20 in BTPWB, 5 x 1 mL) and the wash was filtered to collect any remaining beads from the hit collection tube. The filter was rinsed (BTPWB, 6 x 1 mL). All filter washes were collected into a clean flask (125 mL). The hit beads were transferred from the filter (BTPWB, 180 μL) into a clean PCR tube (200 μL). To collect any beads stuck to the filter, the filter was moved to a clean 1.5 mL eppendorf tube, resuspended (BTPWB, 500 μL), centrifuged (30 s, 6,000 rpm), and beads were added to the bead collection tube. Finally, the filter wash was was centrifuged (5 min, 14,000 rcf) and collected beads were added to the bead collection PCR tube. The volume in the PCR tube was reduced (~10 μL), beads were washed (BTPWB, 2 x 200 μL), and volume was reduced again (~ 10 μL).

DNA sequencing sample preparation for this DEL was previously described (3-5). Briefly, qPCR mix containing *Taq* DNA polymerase (0.05 U/μL), oligonucleotide primers 5′-GCCGCCCAGTCCTGCTCGCTTCGCTAC-3′ and 5′-/5AmMC6/ GTGGCACAACAACTGGCGGGCAAAC-3′ (0.3 μM each), SYBR Green (0.2x, Life Technologies), betaine (1 M), $MgCl_2$ (1 mM), DMSO (8%), and PCR master mix was added to the bead-containing PCR tube (40 μL). Template standard solutions (100 amol, 10 amol, 1 amol, 100 zmol, 10 zmol, 1 zmol, 100 ymol, and 10 ymol, each in 1 μL BTPWB) were added to separate amplification reaction wells (40 μL). Reactions were thermally cycled (96 °C, 10 s; [95 °C, 8s; 72 °C, 24 s] × 32 cycles; 72 °C, 2 min). Samples were centrifuged (30 s, 6,000 rpm) and diluted

10,000-fold. Barcoding qPCR mix contained *Taq* DNA Polymerase (0.05 U/ μL), oligonucleotide primer 5′-CCTCTCTATGGGCAGTCGGTGATGTGGCACAACAACTGGCGGGCAAAC-3′ (0.3 μM), SYBR Green (0.2×, Life Technologies), DMSO (6%), betaine (1 M), MgCl2 (1 mM) and PCR master mix (1×). Initial PCR amplification supernatant (2 μL) and a sequencing barcode oligonucleotide primer (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNGATGCCGCCCAGTCCTGCTCGCTTCGCTAC-3′, 0.3 μM) were added to separate barcoding amplification wells to encode sample identity (40 μL). Reactions were thermally cycled ([95 °C, 8 s; 72 °C, 24 s] × 20 cycles). Barcoded amplification products were pooled, resolved by native PAGE (6%, 1 x TBE, 8 W, 30 min), and visualized with by SYBR Gold staining (Life Technologies, Inc.). Gel slices that contained 251-bp DNA product were excised, eluted, and used for standard DNA sequencing library preparation and analysis (Ion Proton, Life Technologies, Inc.).

Data processing of hit DNA sequences. DNA sequence pattern matching, trimming, aggregation by bead barcode, and unique molecular identifier (UMI) filtering were previously described. (3,5). Sequences with a UMI < 10 were discarded. Of the remaining sequences, compound replicates (*k* class) were determined by taking the sum of the sequences having the same synthesis encoding region but distinct bead barcodes. (4)

Hit resynthesis on photocleavable linker resin. Hits were resynthesized on mixed-scale photocleavable linker resin (3 mg). An aliquot of linker resin (3 mg) was removed into a fritted synthesis column (10 μm, Mobicol) and washed (DMF, 3 x; DCM, 3 x; DMF, 3 x, 400 μL). Synthesis of hits followed the same synthetic protocol as DEL synthesis. (3) Briefly, Fmoc was removed (20% piperidine in DMF, 1 x 5 min, 1 x 15 min, RT, 8 rpm) and resin was washed (DMF, 3 x; DCM, 3 x; DMA, 3 x, 400 μL). The amino acid building block (40 mM) was combined

with HOAt/DIC (40 mM/57 mM) in DMA (0.4 mL), added to resin, and incubated (1 h, 37 °C, 8 rpm). Resin was washed (DMA, 3 x; DCM, 3 x; DMF, 3 x, 400 µL). Fmoc was removed (20% piperidine in DMF, 1 x 5 min, 1 x 15 min, RT, 8 rpm) and resin was washed (DMF, 3x; DCM, 3x; DMA, 3x, 400 µL). The carboxylic acid building block (40 mM) was combined with HOAt/DIC (40 mM/57 mM) in DMA (0.4 mL), added to resin, and incubated (1 h, 37 °C, 8 rpm). Resin was washed (DMA, 3 x; DCM, 3 x; DMF, 3 x, 400 µL).

Scaled synthesis of select hits. Hits **4**, **5**, **9**, and **12** were synthesized at milligram scale on Fmoc-Rink amide MBHA resin (Novabiochem, 0.78 mmol/g, 83 mg, 65 µmol). All reaction and wash volumes were constant (1 mL) unless otherwise specified. Resin was transferred to a fritted syringe (3 mL, Torviq, Tucson, AZ) and swelled in DMF (overnight, 8 rpm, RT). The resin was washed (DMF, 3x; DCM, 3 x; DMF, 3x). Probe synthesis proceeded via iterative cycles of solid-phase peptide synthesis. Fmoc was removed (20% piperidine in DMF, 1 x 5 min, 1 x 15 min, RT, 8 rpm) and resin was washed (DMF, 3 x; DCM, 3 x; DMF, 3 x). The amino acid building block (195 µmol) was combined with Oxyma/TMP/DIC (195 µmol/195 µmol/260 µmol) in DMF, added to resin, and incubated (3 h, 37 °C, 8 rpm). Resin was washed (DMF, 3 x; DCM, 3 x; DMF, 3 x). Fmoc was removed (20% piperidine in DMF, 1 x 5 min, 1 x 15 min, RT, 8 rpm) and resin was washed (DMF, 3x; DCM, 3x; DMF, 3x, 400 µL). The carboxylic acid building block (195 µmol) was combined with Oxyma/TMP/DIC (195 µmol/195 µmol/260 µmol) in DMF, added to resin, and incubated (3 h, 37 °C, 8 rpm). Resin was washed (DMF, 3 x; DCM, 3 x; DMF, 3 x). Resin was washed (DCM, 3 x) and dried. Cleavage cocktail (90/5/5 TFA/TIPS/DCM) was added to resin and incubated (1 h, RT, 8 rpm). Cleaved products were expelled, washed (DCM, 3x), and dried down under vacuum.

Compound **4** and **5** were protected with Boc anhydride after acid cleavage from resin. Di-tert-butyl dicarbonate (325 µmole) and triethylamine (325 µmol) were added to cleaved compound and DCM (5 mL) and stirred (overnight, RT). Boc protected compounds were

diluted in DCM (40 mL), extracted with saturated NaCl, dried with magnesium sulfate, and concentrated under vacuum. Crude compounds **4, 5, 9** and **12** were purified by flash chromatography to yield pure compound **4** (6.8 mg, 13.6 µmol, 21% yield), compound **5** (7.0 mg, 12.7 µmol, 20% yield), compound **9** (1.7 mg, 5.6 µmol, 9% yield), and compound **12** (12.0 mg, 27.6 µmol, 42% yield).

Compound **4** 1H-NMR (400 MHz, (CD3)2SO):

δ 8.11 (s, 1H); 7.74 (s, 1H); 7.64 - 7.58 (m, 2H); 7.44 (s, 1H); 7.29 (s, 1H); 4.80 (d, 1H, *J*=11.4 Hz); 4.60 (s, 2H); 4.11 (q, 1H, *J* = 5.0 Hz), 3.99-3.86 (m, 2H); 3.67 (d, 4H, *J* = 11.7 Hz); 3.51 - 3.41 (m, 4H); 3.17 (d, 3H, *J* = 5.1); 1.36 (d, 9H, *J* = 29.0 Hz)

Compound **5** 1H-NMR (400 MHz, (CD3)2SO): δ 8.11 (s, 1H); 7.74 (s, 1H); 7.59 (s, 2H); 7.44 - 7.41 (m, 3H); 7.29 (s, 1H); 7.06 - 6.99 (m, 3H); 4.60 (s, 2H); 4.02 (t, 2H, *J* = 5.7 Hz); 3.67 (s, 4H); 3.31 (d, 6H, *J* = 5.5 Hz); 3.17 (s, 1H); 1.39 (s, 9H)

Compound **9** 1H-NMR (400 MHz, (CD3)2SO): δ 8.63 (d, 2H, *J* = 5.0 Hz); 7.22 (s, 1H); 6.89 (s, 1H); 6.59 (s, 1H); 2.80 (s, 3H); 2.47 (s, 3H); 2.14 - 2.09 (m, 2H); 2.01 - 1.97 (m, 2H); 1.68 (d, 4H, *J* = 5.4 Hz)

Compound **12** 1H-NMR (400 MHz, (CD3)2SO): δ 8.24 (d, 1H, *J* = 8.6 Hz); 7.65 (d, 2H, *J* = 7.2 Hz); 7.58 - 7.55 (m, 3H); 7.46 (t, 2H, *J* = 7.4 Hz); 7.34 (q, 3H, *J* = 8.2 Hz); 7.12 (s, 1H); 4.53 - 4.47 (m, 1H); 3.96 - 3.82 (m, 3H); 3.43 (t, 1H, *J* = 9.1 Hz); 3.31 - 3.28 (m, 3H); 3.12 - 3.08 (m, 2H); 2.79 (q, 1H, *J* = 10.4 Hz); 2.36 (q, 1H, *J* = 9.6 Hz); 2.08 (q, 1H, *J* = 7.2 Hz); 1.69 - 1.54 (m, 2H); 1.46 - 1.38 (m, 2H)

References

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2. Price, A.K.; MacConnell, A.B.; Paegel, B.M. hvSABR: photochemical dose-response bead screening in droplets. *Anal. Chem.* **2016**, *88(5)*, 2904–11.

3. Cochrane, W.G.; Malone, M.L.; Dang, V.Q.; Cavett, V.; Satz, A.L.; Paegel, B.M. Activity-based DNA-encoded library screening. *ACS Comb. Sci.* **2019**, *21(5)*, 425–35.

4. Mendes, K.R.; Malone, M.L.; Ndungu, J.M.; Suponitsky-Kroyter, I.; Cavett, V.J.; McEnaney, P.J.; MacConnell, A.B.; Doran, T.M.; Ronacher, K.; Stanley, K.; Utset, O.; Walzl, G.; Paegel, B.M.; Thomas Kodadek, T. High-throughput identification of DNA- encoded IgG ligands that distinguish active and latent mycobacterium tuberculosis infections. *ACS Chem. Biol.* **2017**, *12(1)*, 234–43.

5. MacConnell, A.B.; Price, A.K.; Paegel, B.M. An integrated microfluidic processor for DNA- encoded combinatorial library functional screening. *ACS Comb. Sci.* **2017**, *19*, 181–92.

Figure S1. FP-based detection of model macromolecular binding in microfluidic droplets. (**A**) Droplet-scale FP binding assays were developed with fluorescent probes and targets. The streptavidin FP probe contains a fluorescein label (FAM, green) coupled to biotin by a PEG_2 spacer. (**B**) Biotin FP probe (20 nM) emission polarization increases upon titration of streptavidin. (**C**) Example raw transient droplet fluorescence data illustrate four droplet detection events ($I_{\parallel}$, black trace; $I_{\perp}$, cyan trace) and their associated FP values (purple bars). FP probe droplets (*top*) contained FP probe (5 nM) and no target. FP probe + target droplets (*bottom*) contained FP probe (5 nM) and streptavidin (200 nM). (**D**) Droplet FP populations for either probe-only droplets (gray histograms) or probe + target droplets (green histograms) were used to calculate Z'. Droplet assay quality score was Z' = 0.43 for biotin FP probe binding to streptavidin.

Figure S1

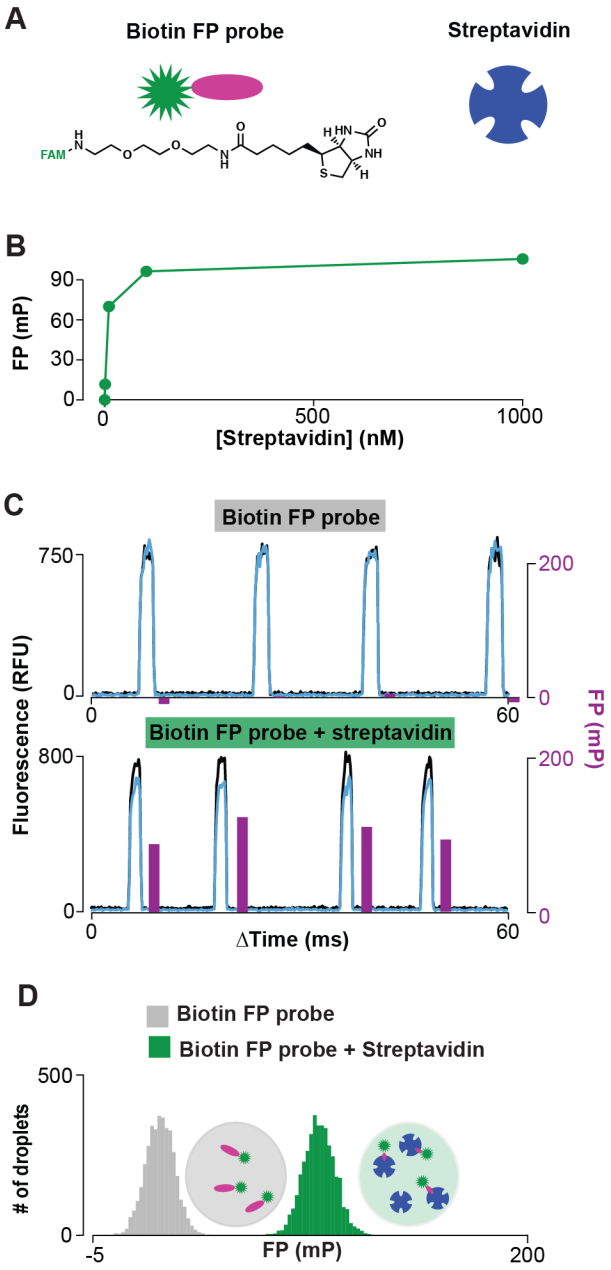

A
Biotin FP probe
Streptavidin
FAM
B
FP (mP)
90
60
30
0
0
500
1000
[Streptavidin] (nM)
C
Biotin FP probe
750
0
200
0
0
60
FP (mP)
Biotin FP probe + streptavidin
800
0
200
0
0
60
Fluorescence (RFU)
ΔTime (ms)
D
Biotin FP probe
Biotin FP probe + Streptavidin
500
0
# of droplets
-5
FP (mP)
200

Figure S2. Synthesis of DDR1 ligand FP probe. (**A**) The HPLC chromatogram contained a major peak corresponding to the DDR1 FP probe by (**B**) MALDI-TOF MS analysis. The mass spectrum contains a peak with the observed mass (black) that agrees with the theoretical exact mass (cyan).

Figure S2

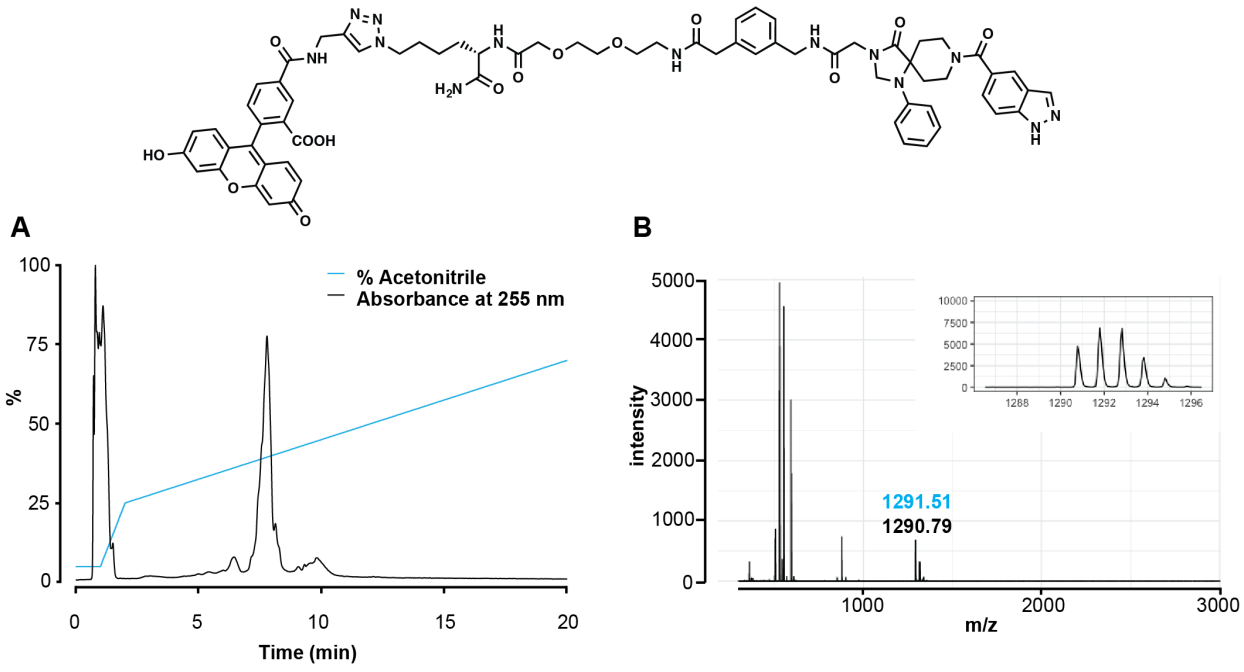

A
100
75
50
25
0
%
% Acetonitrile
Absorbance at 255 nm
0
5
10
15
20
Time (min)
B
5000
4000
3000
2000
1000
0
intensity
1291.51
1290.79
1000
2000
3000
m/z
10000
7500
5000
2500
0
1288
1290
1292
1294
1296

Figure S3. Synthesis of ATX ligand FP probe. (**A**) The HPLC chromatogram contained a major peak corresponding to the ATX FP probe by (**B**) MALDI-TOF MS analysis. The mass spectrum contains a peak with the observed mass (black) that agrees with the theoretical exact mass (cyan).

Figure S3

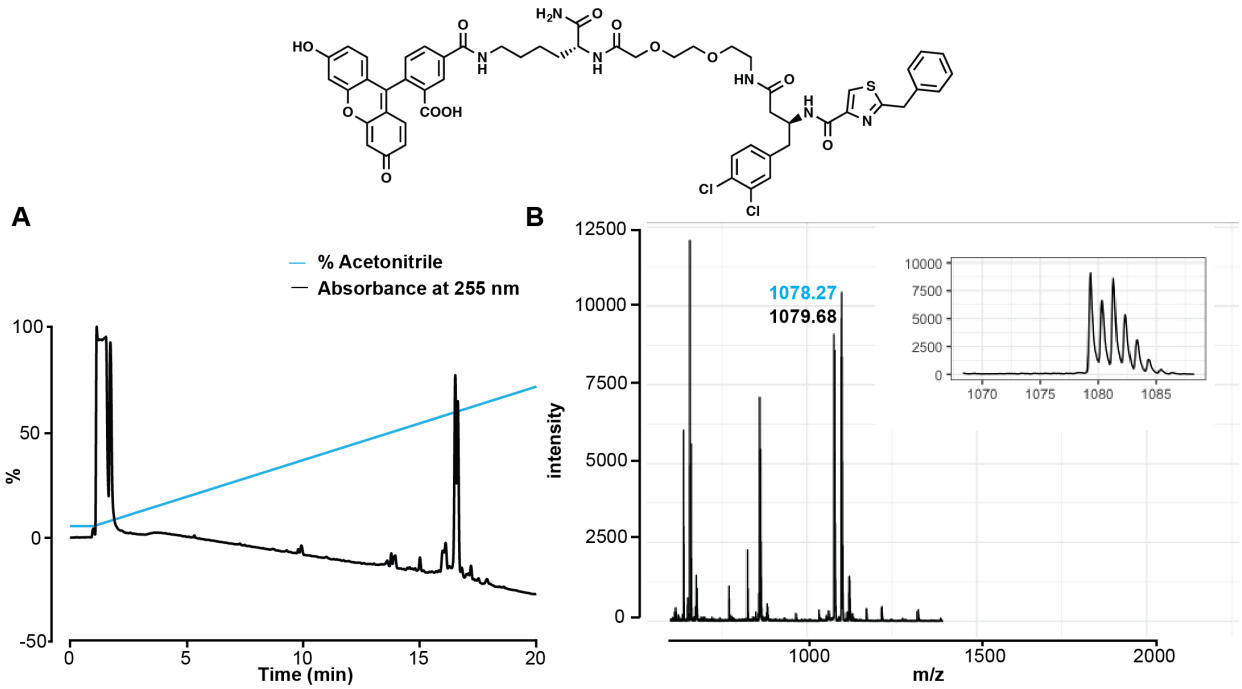

A
% Acetonitrile
Absorbance at 255 nm
100
50
0
-50
%
0
5
10
15
20
Time (min)
B
12500
10000
7500
5000
2500
0
intensity
1078.27
1079.68
1000
1500
2000
m/z
10000
7500
5000
2500
0
1070
1075
1080
1085

Figure S4. Integrated microfluidic screening device. Library beads enter the circuit with the FP probe (LIB) and mix with target (TAR) immediately upstream of the water-in-oil (OIL1) flow-focusing junction. Droplets are illuminated with a fiber optic LED (λ = 365 nm) as they pass through the dosing serpentine, photochemically releasing compound from the bead. Droplets are packed into the incubator as oil drains from the droplet flow. After incubation, the droplets are spaced (OIL2) and focused (OIL3) for analysis. Laser-induced fluorescence assay detection occurs upstream of the droplet sorting junction. A high-voltage AC pulse is applied to the working electrode (V_{AC}), electrokinetically forcing the hit droplet toward the electrode and into the hit collection flow.

Figure S4

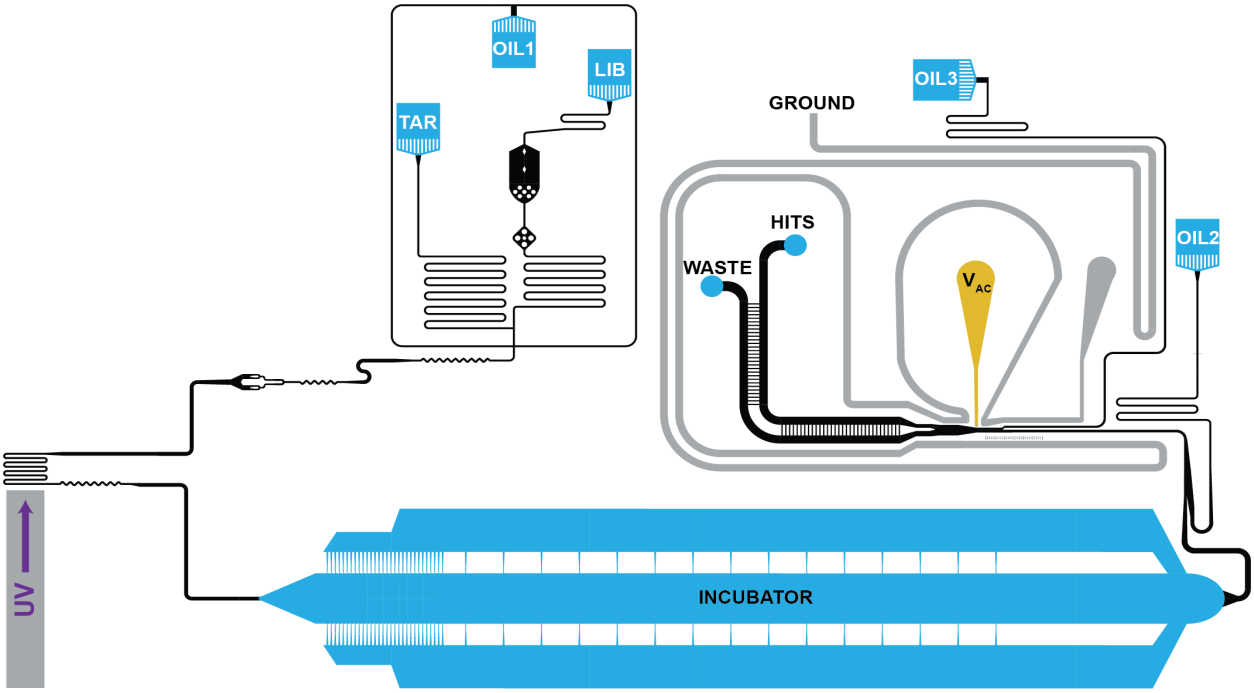


Figure S5. Droplet FP detection via confocal laser-induced fluorescence.

Fluorescence polarization detection is performed using a custom-built confocal LIF detection system. Emission from a diode laser (λ = 488 nm, CW) is vertically polarized (VP) and focused on the microfluidic channel through a 20× objective (OB). Fluorescence is collected with the objective, transmitted through a long-pass dichroic mirror (DC1), and reflected off a silver mirror (M). Long-wavelength emission (λ_{em} > 600 nm) is reflected off a short-pass dichroic mirror (DC2) to the high-speed camera. Short-wavelength emission (λ_{em} < 600 nm) is transmitted through DC2 for spectral filtering with a 520-nm bandpass filter (BP). Filtered fluorescence impinges a polarizing beam splitter (PB), which directs p-polarized ($I_{\parallel}$) light into one photomultiplier tube (PMT1) and s-polarized light ($I_{\perp}$) is directed into another photomultiplier tube detector (PMT2). Both PMT optical trains contain a 30-mm plano-convex lens (L) and a 50-μm pinhole (P) for spatial filtering.

Figure S5

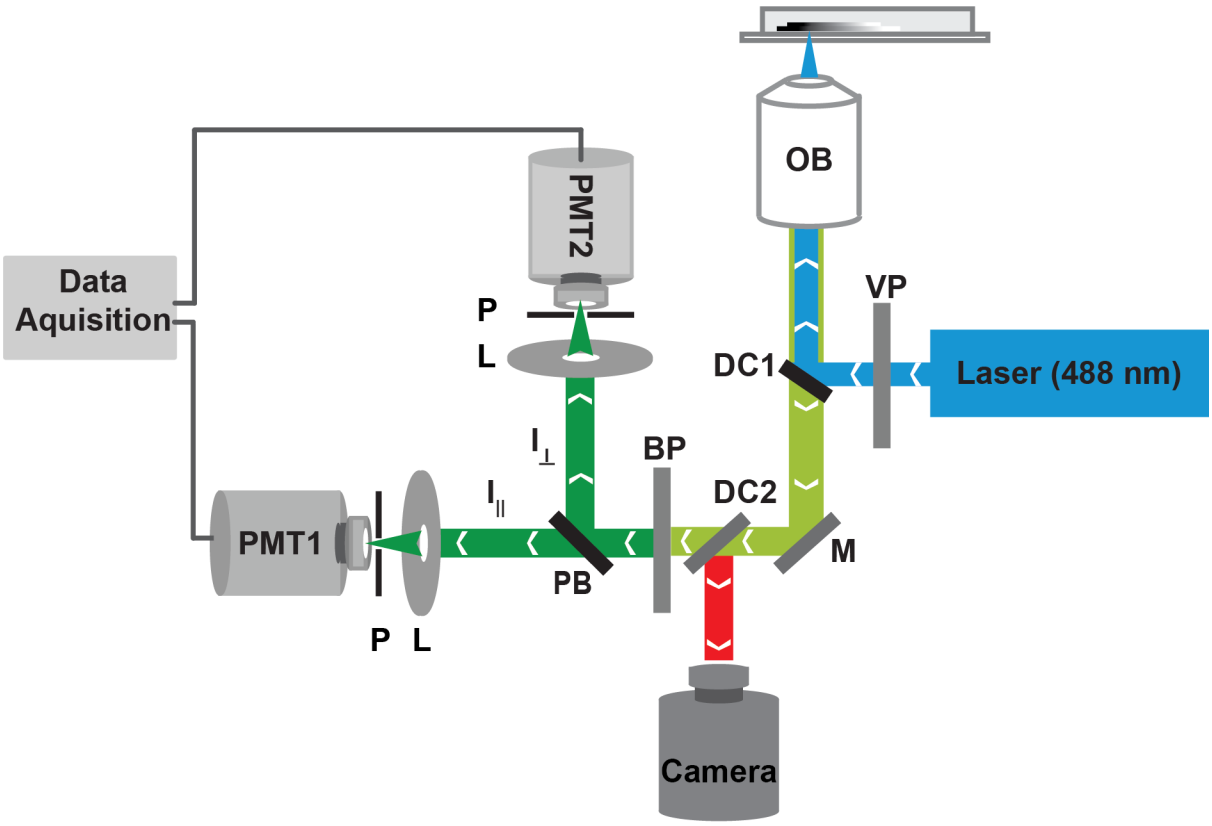
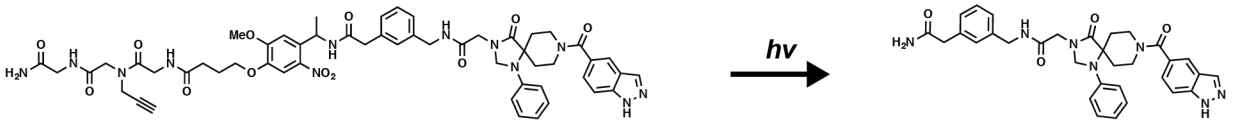

Data
Aquisition
PMT2
P
L
OB
VP
DC1
Laser (488 nm)
$I_{\perp}$
BP
$I_{\parallel}$
DC2
PMT1
PB
M
P
L
Camera

Figure S6. Positive control competitor ligand. The DDR1 ligand was synthesized on 10-μm TentaGel resin with an *o*-nitrobenzyl photolabile linker, yielding **2**. Irradiation with UV liberates the amide-terminated positive control competitor ligand, **S1**. The mass spectrum of **S1** contains a peak with observed mass (black) that agrees with the theoretical exact mass (cyan).

Figure S6


hv

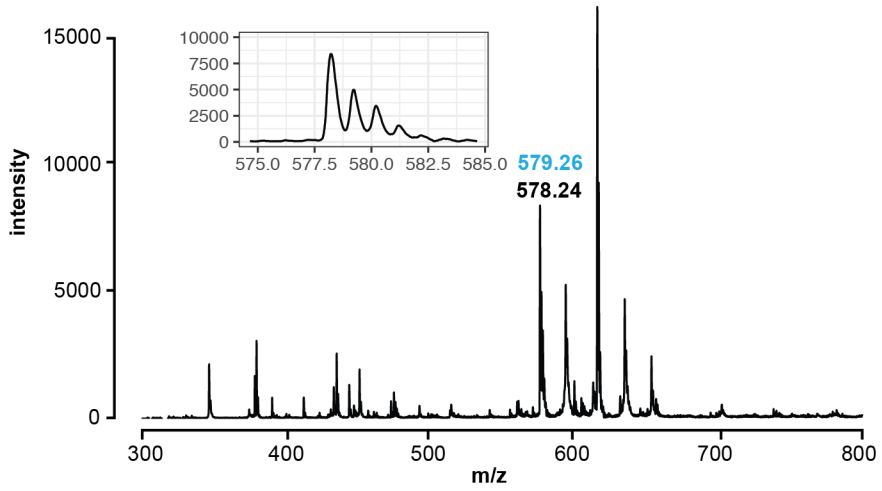

intensity
15000
10000
5000
0
300
400
500
600
700
800
m/z
579.26
578.24
10000
7500
5000
2500
0
575.0
577.5
580.0
582.5
585.0

Figure S7. Displacement versus competition FP assay. The photocleaved competitor ligand was combined with DDR1 FP probe (5 nM) and target (500 nM) in various orders of addition. In competition binding assays (blue), the three reagents were combined simultaneously. In displacement binding assays (purple), probe and target were combined and incubated until binding equilibrium was achieved. Then, competitor was added.

Figure S7

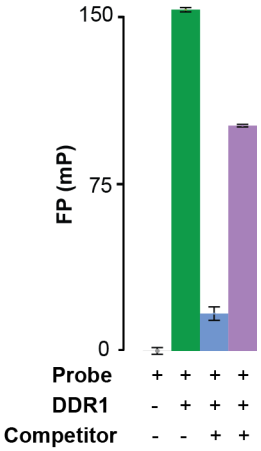


Figure S8. Off-bead droplet FP assay using a positive control competitor ligand - no dose control. (**A**) Transient histogram visualization of the entire control screen at maximum UV dose (5 V) clearly reveals the population of bead-occupied droplets observed below the dynamically calculated sort threshold (green, 4σ). Droplet FP values were binned (1 mP per bin) in 30-s windows. (**B**) Transient histogram visualization of the control screen at no UV dose (0 V) does not reveal a population of bead-occupied droplets observed below the dynamically calculated sort threshold (green, 4σ). Droplet FP values were binned (1 mP per bin) in 30-s windows.

Figure S8

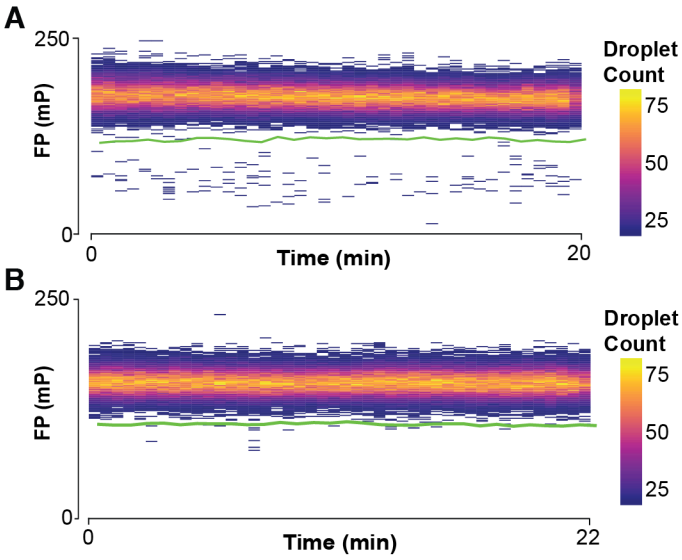

A
250
FP (mP)
0
0
Time (min)
20
Droplet Count
75
50
25
B
250
FP (mP)
0
0
Time (min)
22
Droplet Count
75
50
25

Figure S9. DDR1 Hit Collection Monomer Representation, *k* class $\geq$ 2. Hit bead sequences (*k* class $\geq$ 2) were decoded to determine the synthesis cycle 1 and 2 monomer identities of each hit. Dashed lines indicate monomer use conservation (> 8) in synthesis cycles 1 (vertical) and 2 (horizontal). The most conserved monomers for *k* class $\geq$ 2 hits contained at least one *k* class $\geq$ 3 hit.

Figure S9

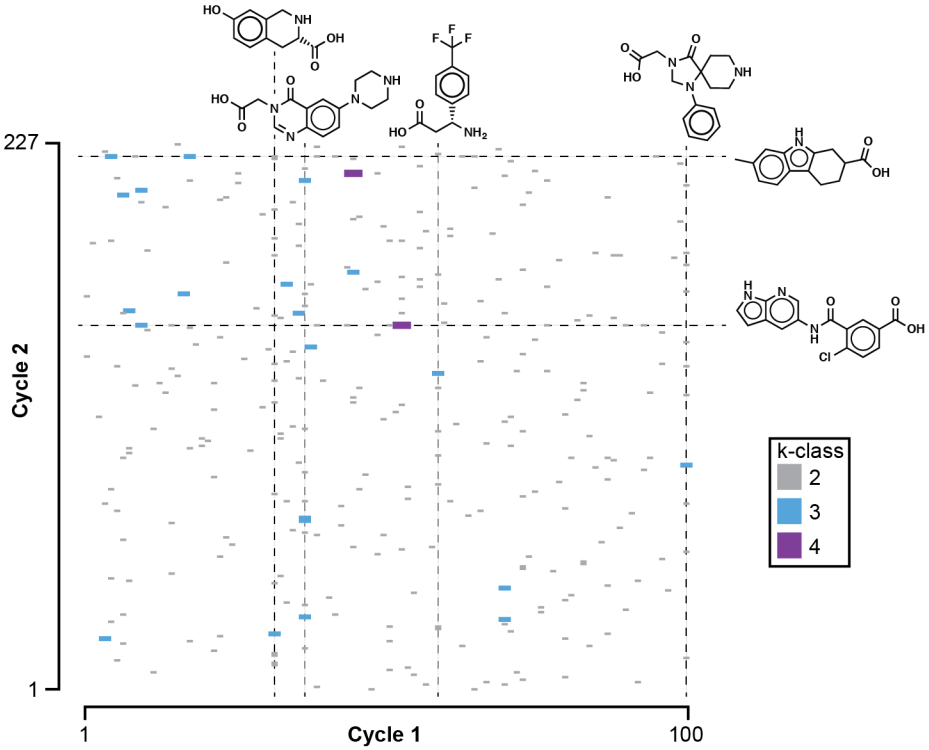


Figure S10. Off-bead droplet FP assays for hit validation. Bead release of various control and hit compounds into droplets of the DDR1 competition binding assay yielded populations of bead-occupied droplets exhibiting varying degrees of probe emission depolarization. Population analysis of unoccupied (green histogram) and bead-occupied droplets was conducted for (**A**) the DDR1 ligand (positive control), (**B**) an inactive compound (negative control), (**C**) hit **2**, (**D**) hit **4**, (**E**) hit **5,** (**F**) hit **8,** (**G**) hit **9,** (**H**) hit **12**

Figure S10

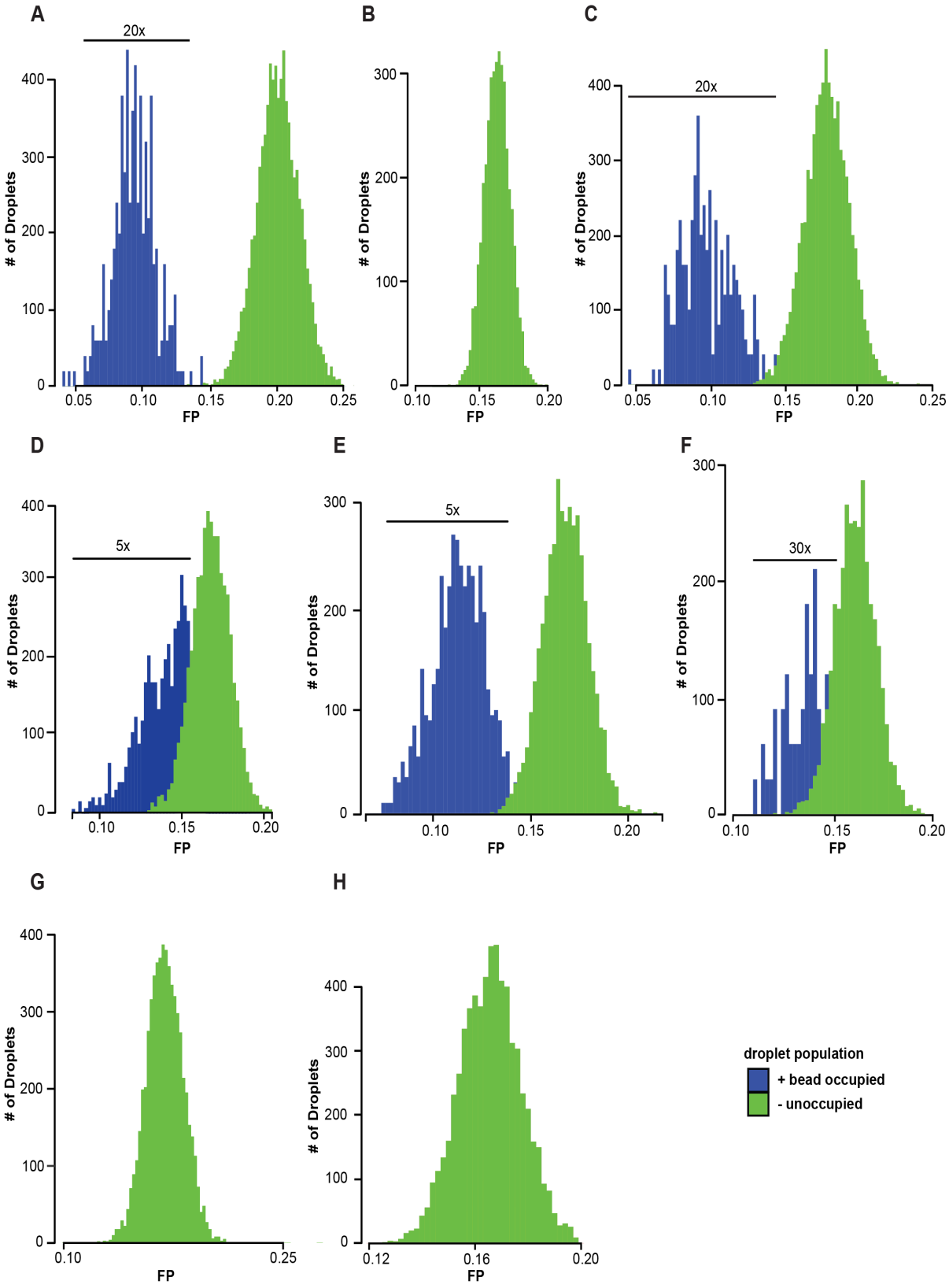
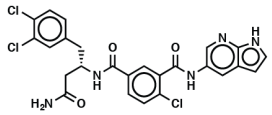
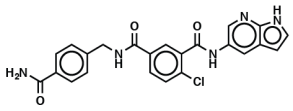
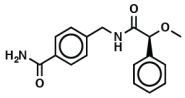
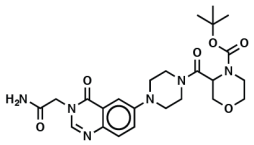
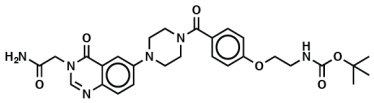
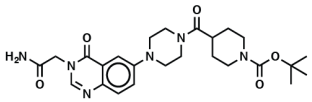
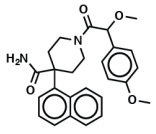
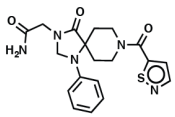
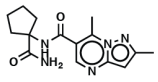
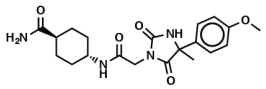
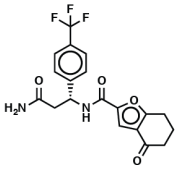
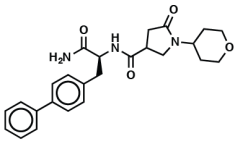
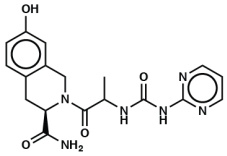
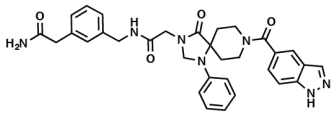
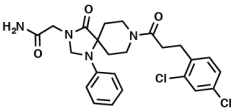

A
20x
# of Droplets
FP
B
# of Droplets
FP
C
20x
# of Droplets
FP
D
5x
# of Droplets
FP
E
5x
# of Droplets
FP
F
30x
# of Droplets
FP
G
# of Droplets
FP
H
# of Droplets
FP
droplet population
+ bead occupied
- unoccupied

Table S1. Hit resynthesis on photocleavable resin

Hit ID	Hit Structure	Observed Mass Theoretical Exact Mass Acid Cleaved [M + linker]	 Photocleaved
1		1534.66 1532.49	544.99 543.06
2		1437.99 1436.53	448.34 447.11
3		1288.49 1287.56	299.08 298.13
4		1389.53 1389.61 [M - boc]	N/D 500.24
5		1440.61 1439.63 [M - boc]	550.54 550.25
6		1388.82 1387.63 [M - boc]	N/D 498.26
7		1423.41 1421.63	455.55 432.20 [M + Na]
8		1389.55 1388.56	398.11 399.14

9		1291.52 1290.58	302.09 301.15
10		1393.06 1391.61	N/D 402.19
11		N/D 1383.54	394.62 394.11
12		1425.71 1424.64	436.15 435.22
13		N/D 1373.58	407.16 384.15 [M + Na]
(+)		1569.70 1568.68	579.22 579.26
(-)		1480.83 1479.44	489.16 488.14