TITLE: Development of a high-throughput fluorescence polarization assay to detect inhibitors of the FAK-paxillin interaction

AUTHORS

Timothy Marlowe^{abcd*}, Carlos Alvarado^a, Andrew Rivera^{ad}, Felicia Lenzo^c, Rohini Nott^a, Dena Bondugji^{ac}, Justin Montoya^f, Alana Hurley^a, Matt Kaplan^g, Andrew Capaldi^g, and William Cance^{abc*}

^aUniversity of Arizona Cancer Center, 625 N 6th Street, Phoenix, AZ 85004, USA

^bInterdisciplinary Oncology, University of Arizona College of Medicine – Phoenix, 475 N 5th Street, Phoenix, AZ, 85004, USA

^cPharmacology and Toxicology, University of Arizona College of Pharmacy, P.O. Box 210207, Tucson, AZ, 85721, USA

^dMolecular Analysis Core, University of Arizona College of Medicine – Phoenix, 475 N 5th Street, Phoenix, AZ 85004, USA

^eCell Stress Biology, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263, USA

^fChild Health, University of Arizona College of Medicine – Phoenix, 475 N 5th Street, Phoenix, AZ 85004, USA

^gFunctional Genomics Core, University of Arizona College of Pharmacy, 1657 E. Helen Street, Tucson, AZ 85719, USA

***Correspondence email:** tmarlowe@email.arizona.edu; wcance@email.arizona.edu

Keywords: Focal Adhesion Kinase; FAT Domain; Drug Discovery

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

ABSTRACT

Focal adhesion kinase (FAK) is a promising cancer drug target due to its massive overexpression in multiple solid tumors and critical role in the integration of signals that control proliferation, invasion, apoptosis, and metastasis. Previous FAK drug discovery and high-throughput screening has exclusively focused on the identification of inhibitors that target the kinase domain of FAK. Because FAK is both a kinase and scaffolding protein, the development of novel screening assays that detect inhibitors of FAK protein-protein interactions remains a critical need. In this report, we describe the development of a high-throughput fluorescence polarization (FP) screening assay that measures the interactions between FAK and paxillin, a focal adhesion-associated protein. We designed a TAMRA-tagged paxillin peptide based on the paxillin LD2 motif that binds to the focal adhesion targeting (FAT) domain with significant dynamic range, specificity, variability, stability, and a Z'-factor suitable for high-throughput screening. In addition, we performed a pilot screen of 1,593 compounds using this FP assay, showing its feasibility for high-throughput drug screening. Finally, we identified 3 compounds that show dose-dependent competition of FAT-paxillin binding. This assay represents the first described high-throughput screening assay for FAK scaffold inhibitors and can accelerate drug discovery efforts for this promising drug target.

INTRODUCTION

Focal adhesion kinase (FAK) is a 125 kDa non-receptor tyrosine kinase that is overexpressed in a variety of human malignancies, including melanoma, breast, colon, ovarian, pancreatic, and glioblastoma.¹ We were the first to show overexpression of FAK in human tumor samples² and years of experimental validation have led to the conclusion that FAK is a critical component for human cancer progression. From a biological standpoint, FAK is involved in cell motility, invasion, angiocrine signaling, lymphangiogenesis, metastasis, and epithelial-mesenchymal transition (EMT).^{3,4} FAK also

sequesters and inactivates pro-apoptotic proteins, such as p53 and RIP, to enhance the survival signals necessary for a cancer to invade and metastasize.^{5, 6} FAK's role in cancer has been confirmed by multiple methodologies including clinical prognostic studies, genetically-engineered mouse models, gene knockout studies, and site-directed mutagenesis.⁷⁻⁹

FAK functions as both a kinase and scaffolding protein, and has three major domains: the N-terminal 4.1, ezrin, radixin, moesin (FERM) domain, the central kinase domain, and the C-terminal focal adhesion targeting (FAT) domain. FAK knockdown results in robust activation of apoptosis and growth arrest in cancer cells with minimal effects in normal cells.^{10, 11} Conversely, FAK-kinase inhibitors have a partial effect on apoptosis/tumor growth^{12, 13} and it is hypothesized that the FAK scaffold is the major modulator of FAK-dependent anti-apoptosis.¹⁴ FAK directly binds p53 to suppress p53-mediated apoptosis⁵ and disruption of the FAT domain by adenoviral FAK-CD was shown to induce apoptosis in cancer cells.¹⁵ We showed that FAK-kinase inhibitors do not drastically inhibit FAK phosphorylation at autophosphorylation residue Y397 and Receptor Tyrosine Kinases (RTKs) transphosphorylate FAK as a drug resistance mechanism.¹⁶ Furthermore, FAK-kinase inhibitors have shown limited efficacy in Phase I/II clinical trials.^{17, 18}

Recent attention has been focused on the FAT domain of FAK as an alternative approach to target FAK in cancer.¹⁹ The FAT domain is a four-helical bundle at the C-terminus of FAK that contains key residue Y925 and is involved in multiple protein-protein interactions at the focal adhesion site. Numerous data have emerged showing the importance of the FAT domain as the initiator of FAK activation through its multiple interactions with Paxillin, Leupaxin, CD4, and DCC.²⁰⁻²² The integrity of the FAT domain is essential for localization of FAK to focal adhesions, association with integrins/RTKs, and downstream FAK signaling.²³ More specifically, FAK localization to the focal adhesion is mediated by the FAT-Paxillin interaction and mutation of the binding site was shown have drastic effects on FAK phosphorylation, paxillin phosphorylation, focal adhesion turnover, cell adhesion, migration, and invasion.^{24, 25} Paxillin contains alpha helical binding motifs termed LD motifs, after the first two amino

1
2
3 acids of the consensus sequence (LDXLLXXL). Two such LD motifs (LD2 and LD4) are required for
4
5 binding to the FAT domain of FAK.²⁶ The FAT-Paxillin LD2/LD4 interaction has been well-
6
7 characterized (Figure 1A), has a K_D of 10-50 μ M, and has been validated by multiple orthogonal assays
8
9 (X-ray, SPR, ITC, NMR, and mutagenesis).^{20, 27, 28} LD2 and LD4 interact at two separate hydrophobic
10
11 patches on the FAT domain (Helix 1-4, Helix 2-3) and disruption of both sites is required for maximal
12
13 biological effect.^{20, 27, 29} In all, these data validate the FAT-paxillin binding site as alternative cancer drug
14
15 target.
16
17

18
19 Here, we report the development of a high-throughput FP assay to measure FAK-paxillin
20
21 interactions and utilize in small molecule drug screening efforts. We show the structure-based
22
23 optimization of a TAMRA-paxillin LD2 probe that binds to both the Helix 1-4 and Helix 2-3 binding
24
25 sites on the FAK FAT domain. We use surface plasmon resonance as well as site-directed mutagenesis to
26
27 validate the specificity of the signal. We evaluate the high-throughput characteristics of the assay and
28
29 perform a pilot screen using the NCI Diversity Set V 1,593 compound library and automated liquid
30
31 handling systems. Finally, we identify 3 compounds that display dose-dependent inhibition of FAT-
32
33 paxillin binding. These results demonstrate a novel drug screening approach for the identification of
34
35 potential FAK scaffold inhibitors.
36
37
38
39
40

41 **METHODS**

42
43 **Reagents, chemicals, and peptides**

44
45
46 TAMRA-tagged and unlabeled peptides were acquired as lyophilized powder through Biomatik
47
48 Corporation. HPLC and mass spectrometry data confirmed proper identity and purity >95% for each
49
50 peptide. Peptides were dissolved at 1-10 mM in 40 mM Tris (pH 8.1). 384-well plates for assay
51
52 optimization studies were acquired from ThermoFisher (Nunc cat#264576, 242764, 262260, 267461),
53
54
55
56
57
58
59
60

Greiner (cat#781209, 784900) and Corning (cat# 3575, 4514). The NCI diversity set V compound library of 1,593 was graciously provided Dr. David Azorsa (University of Arizona).

Expression and Purification of His-tagged FAT and Avitag-FAT

The FAT domain of FAK (residues 892-1052) were cloned into the pET15b vector to produce an N-terminally His(6)-tagged FAT domain. The peT15b-FAT vector was transformed into *E. coli* BL21(DE3) chemically competent cells (ThermoFisher). Bacteria was grown in LB broth at 37 °C to an absorbance of 0.8 at wavelength 600 nm. 0.2 mM of IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to induce protein expression. Bacteria were further incubated for 4 hours at 37 °C after which cells were collected through centrifugation at 5000 x g for 10 minutes. Cell pellets were resuspended using lysis buffer (20 mM Tris [pH 8.0], 200 mM NaCl, 5 mM β-mercaptoethanol (β-me), 10 mM imidazole, and 1X Halt protease inhibitor cocktail (ThermoFisher) and frozen overnight at -80°C.

Upon thawing, cells were further lysed through sonication (mild sonication for 2 minutes, 50% cycle, 5 seconds per cycle) and insoluble components were then removed through centrifugation at 12,000 rpm for 15 minutes. The cell lysate was incubated with HisPur Ni-NTA resin (ThermoFisher) overnight while gently shaking at 4°C. The resin was previously equilibrated with lysis buffer. For purification, the nickel resin with bound His-tagged FAT was transferred to a spin column and washed with (20 mM Tris [pH 8.0], 200 mM NaCl, 5 mM β-me, and 25 mM imidazole). The protein was eluted using 20 mM Tris (pH 8.0), 200 mM NaCl, 5 mM β-me, and 250 mM Imidazole. The eluted protein was buffer exchanged using Zeba spin desalting columns (ThermoFisher) into 20 mM Tris pH 8.0, 200 mM NaCl, and 5 mM β-me. Coomassie-stained SDS PAGE analysis of the purified protein demonstrated around 90% purity.

Fluorescence Polarization Assay

The FP assay buffer used was 20 mM Tris, 200 mM NaCl, 0.05% β-me, 0.1% Triton X-100, 5% glycerol, and 1X Halt protease inhibitor cocktail. All final FP reactions are placed into a 384-well plate

(NUNC 267461) at 30 μ L and shaken for 3 hours at room temperature to reach equilibrium. The plates are read on a PerkinElmer plate reader with software Envision Manager 1.13. Bodipy TMR FP optical module (2100-4100) was used as the mirror. The excitation filter (2100-5830) utilized wavelength at 531 nm and both emission filters (2100-5800 and 2100-5810) were at wavelengths of 579 nm. The baseline mP of TAMRA-LD2-L10D only was set to 15 mP through the gain optimization wizard on the Envision Manager software. The optimization set the G-Factor to 1.01 and the number of flashes per well at 10.

Saturation binding assays

For equilibrium dissociation constant (K_D) determination for TAMRA-LD2 and TAMRA-LD2-L10D, FAT was titrated from 0.2 μ M to 200 μ M into FP buffer containing 0.1 μ M TAMRA-LD2 or TAMRA-LD2-L10D for a final volume of 30 μ L. Wells with no FAT were used as a baseline value which was subtracted from the raw values to produce Δ mP values. The saturation binding data was processed through GraphPad Prism using the One site – Total model to produce a saturation curve and a calculated K_D with standard error (SE).

Competition Assay

For IC_{50} determination of unlabeled-LD2 as an inhibitor, LD2 was titrated from 1 μ M to 250 μ M into FP Buffer containing 20 μ M FAT and 0.1 μ M TAMRA-LD2 or TAMRA-LD2-L10D. Wells with no FAT were used as a baseline value which was subtracted from the raw values to produce Δ mP values. The plate was read at every hour for four hours to test for time differences in IC_{50} . The titration data was processed through GraphPad Prism to produce a dose response curve and a calculated IC_{50} with standard error (SE). A four-parameter dose-response inhibition model was utilized and the bottom fit was constrained to the lower plateau of the curve. K_i was determined from the following calculation³⁰:

$$K_i = \frac{I_{50}}{\frac{L_{50}}{K_D} + \frac{P_0}{K_D} + 1} \quad (\text{Eq. 1})$$

Where I_{50} is the concentration of free inhibitor at 50% inhibition. L_{50} is the concentration of free ligand at 50% inhibition. P_0 is the concentration of free protein and K_D is calculated from the saturation curve in Figure 1. I_{50} is calculated by the following equation:

$$I_{50} = IC_{50} - P_T + PL_{50} \left(\frac{K_D}{L_{50}} + 1 \right) \quad (\text{Eq. 2})$$

Where P_T is the total protein concentration, L_T is the total labeled-ligand concentration, P_0 is the positive root of $P_0^2 + (K_D + L_T) \cdot P_0 - P_T$, $PL_0 = P - P_0$, $PL_{50} = PL_0/2$, $L_0 = L_T - PL_0$, and $L_{50} = L_T - PL_{50}$. The compounds for the dose response curves were serially diluted one to one from a concentration of 5 mM to 5 μ M in DMSO. 2 μ L of compound at each respective concentration were then added to 30 μ L of a master mix containing 20 μ M FAT and 0.1 μ M TAMRA-LD2-L10D for a final DMSO concentration of 6.25% in each well, and for a final compound concentration range of 312.5 μ M to 0.31 μ M. The dose response curves were fitted on GraphPad Prism and the K_i was calculated using equations 1 and 2. Standard error (SE) of the K_i was calculated similarly using the above equation. Commercially available compounds were purchased for follow up studies at higher concentrations.

Surface Plasmon Resonance (SPR)

SPR binding studies were performed on a ForteBio Pioneer FE SPR system. In brief, a SADH Streptavidin in Dextran Hydrogel biosensor (ForteBio) was docked onto the flow cell and preconditioned with two injections of 10 mM NaOH, 1 M NaCl for 1 min at 50 μ L/min. Subsequently, biotinylated

Avitag-FAT protein was diluted in running buffer (20 mM Tris-HCl, 200 mM NaCl, 0.05% Tween-20) and injected at 10 μ l/min to achieve approximately 1,000 RU of immobilized protein on channel 1. Empty channel 2 served as the reference control. After achieving a stable baseline, a concentration series (200 μ M-0.01 μ M) of paxillin LD2 WT and L10D peptide was prepared in final running buffer (20 mM Tris-HCl, 200 mM NaCl, 0.05% Tween-20, 5% DMSO) and injected using the OneStep gradient injection method at a flow rate of 75 μ l/min. 3% sucrose was utilized as a bulk standard control for OneStep injection and a DMSO calibration curve was performed using a concentration range of 3.5% to 6.5% DMSO. Raw SPR data were appropriately processed in Qdat software (ForteBio) by normalizing the baseline prior to injection, aligning the channels, subtraction of the reference channel, and blank subtraction. Kinetic data were fitted to a pseudo-first order 1:1 interaction binding model using to calculate K_D . In addition, a steady-state model and R_{eq} data points were used to validate the binding affinity. Visual inspection of the SPR sensograms was performed to verify appropriate model fitting, lack of mass transport effects, return to baseline, and lack of irregular kinetics.

High Throughput Screening

The small molecule diversity set library, NCI Diversity Set V, was obtained through the NCI. 2 μ L of each small molecule in DMSO was aliquoted into 384-well NUNC plates using the Acoustic Transfer System Gen 4 from Biosero. A master mix of 20 μ M His-tagged FAT and 0.1 μ M TAMRA-LD2-L10D in FP buffer was added into each well by a Tecan Liquid Handling machine utilizing the MCA96 pipetting head, for a final drug concentration of 312.5 μ M. A negative control of 20 μ M FAT, 0.1 μ M TAMRA-LD2-L10D and 6.25% DMSO, a positive control of 0.1 μ M TAMRA-LD2-L10D and 6.25% DMSO, and a positive control of 20 μ M FAT, 0.1 μ M TAMRA-LD2-L10D, 250 μ M unlabeled LD2 and 6.25% DMSO were added to each plate for baseline referencing and Z' -factor calculation. Data was processed through GraphPad Prism to produce a hit plot. Z' -factor calculations for HTS efficiency was calculated by³¹:

$$Z' - factor = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \quad (\text{Eq. 3})$$

Where σ_p and σ_n are the standard deviations of the positive and negative controls respectively. μ_p and μ_n are the average values for the positive and negative controls respectively. A Z' -factor between 0.5-1.0 represents an excellent assay with 1.0 being an ideal assay. For each repetition of the assay, 16-32 control wells per control were ran for the purpose of Z' -factor calculations.

Molecular Modeling

The PyMOL molecular graphics program (Schrödinger, Inc.) was utilized for general structural representation and examination of the FAK FAT: paxillin LD2 complex (PDB 1OW8). Residues that were proximal to the interaction interface ($< 5\text{\AA}$) and showed key intermolecular contacts were selected for mutagenesis. The PyMOL mutagenesis wizard was utilized to predict effect of mutations on FAT-LD2 interaction, protein folding, and steric clashes. Publication-quality images were generated using the ray-trace command.

Site Directed Mutagenesis of the FAT Domain

Mutation primers were designed through the Agilent QuickChange Primer Design website and ordered through Invitrogen. Primer sequences are listed in Supplemental Table S1. Site-directed mutagenesis was performed using the QuickChange Lightning mutagenesis kit. Mutations were confirmed through sequencing at the University of Arizona Genetics Core.

RESULTS

Development of initial TAMRA-LD2 probe and establishment of fluorescence polarization assay.

To develop a high-throughput FP assay capable of detecting inhibitors of the FAK-paxillin interaction, we first started with the design of a fluorescently-labeled paxillin probe to be used in the assay. We utilized the X-ray crystal structure of the FAK FAT-paxillin LD2 complex (PDB 1OW8) to guide design efforts. As shown in Figure 1A, the paxillin LD2 peptide is a 13-amino acid (NLSELDRLLELN) amphipathic alpha helix that binds to two hydrophobic patches on the FAT domain (Helix 1-4 and Helix 2-3 binding sites). Using this structure, we determined that the N-terminus of paxillin LD2 is orientated towards solvent and is a logical site for peptide modification. To fluorescently modify LD2 peptide without perturbing binding, we added a 6-carbon linker on the N-terminus (6-aminohexanoic acid) for linking to 5-TAMRA. Next, we performed FP saturation binding studies using a constant concentration of TAMRA-LD2 probe (0.1 μ M) and increasing concentrations of purified FAT domain protein (0.1-200 μ M). We identified a clean binding isotherm with a calculated K_D of 48.6 μ M (Figure 1B). To validate the specificity of the binding interaction, we performed competition binding studies with constant concentrations of TAMRA-LD2 probe (0.1 μ M) and FAT protein (20 μ M), while titrating increasing concentrations of unlabeled LD2 peptide (0.1-500 μ M). We observed dose-dependent competition with a calculated K_i of 45.9 μ M (Figure 1C), which was comparable to the K_D obtained in saturation binding experiments. A time course demonstrated no change in IC_{50} over time. Finally, to validate that the FP-derived K_D was comparable to independent methodology, we performed surface plasmon resonance (SPR) studies with biotinylated FAT domain protein and unlabeled paxillin LD2 peptide. We found a K_D of 51.5 μ M in SPR binding studies (Figure 1D), which was similar to the FP-derived K_D of 48.6 μ M, thus validating the accuracy of the FP assay.

Optimization of TAMRA-LD2 probe and FP assay.

Upon further inspection of FP saturation and competition binding curves, we noticed that the maximum binding (B_{max}) level was relatively lower compared to other published similar FP assays.^{32, 33} Given the high hydrophobicity of our TAMRA-LD2 probe, we hypothesized that the peptide could be

self-associating or non-specifically binding to the plate surface, therefore preventing maximum binding to FAK FAT protein. To test this, we examined the X-ray crystal structure of the FAT-paxillin LD2 complex (PDB 1OW8) and identified Leucine 10 as a potential residue for hydrophobic-to-hydrophilic substitution (Figure 2A). Leucine 10 is orientated towards the solvent in the complex and is not part of the hydrophobic interaction surface. We synthesized a TAMRA-LD2-L10D (leucine to aspartic acid) probe and performed FP saturation studies in comparison with TAMRA-LD2 WT (Figure 2B). Interestingly, TAMRA-LD2-L10D gave a slightly higher $B_{\max}$ compared to WT (81 vs. 75) while showing a similar K_D . Also, at any given concentration of FAT, the measured assay window is larger for TAMRA-LD2-L10D than TAMRA-LD2. Furthermore, the intrinsic polarization (measured in mP) of TAMRA-LD2-L10D was lower compared to WT (19.5 vs. 35.8 mP) despite its similar molecular weight, thus suggesting a reduced amount of self-association/non-specific binding by the L10D peptide (Supplemental Figure S1). Next, we compared the ability of unlabeled LD2 to competitively inhibit TAMRA-LD2 WT:FAT binding versus TAMRA-LD2-L10D:FAT binding (Figure 1C). Unlabeled LD2 effectively inhibited both interactions with similar inhibition constants (K_i of WT = 45.9 μ M, K_i of L10D = 52.3 μ M). These results led us to use TAMRA-LD2-L10D over TAMRA-LD2 for all future studies. We also tested whether the 6X his-tag from recombinant FAT purification would interfere with binding compared to the cleaved version and found minimal difference in binding affinities (K_D of His-tagged = 49.7 μ M, K_D of Untagged = 47.3 μ M, Figure 1D).

Validation of binding specificity by site-directed mutagenesis.

To validate that the FP binding signal was in fact due to specific binding at the FAT-paxillin interface, we designed a series of mutant FAT proteins at both the Helix 1-4 and Helix 2-3 binding sites. In addition, mutant proteins could be utilized to characterize Helix 1-4 or Helix 2-3 selective binding of future small molecules. We used the X-ray crystal structure of the FAT-paxillin LD2 complex (PDB 1OW8) as well as a previously described report²⁷ to select a series of surface residues at each binding site

suitable for mutation (Figure 3A). Using site-directed mutagenesis and mutant-containing primers, we made 18 total mutant constructs with mutations engineered at both paxillin binding sites. Subsequently, we performed small-scale 6X his-tag protein purification and examined elutions for protein yield and purity (Figure 3B). Single-site mutants L1035D, L994E, I936E, I936A, and KER (K955A, E948A, R962A) had high levels of initial yield and purity however mutants I936D, I998A, I998D, V932A, and V932D had limited yield/purity. Two-site mutants KER+I936D, KER+V932A, and I936E+L994E had high levels of yield/purity while I936E+I998A, I936E+I998D, KER+V932D, KER+H1025A, KER+L1035D, and I936E+L965D had limited yield/purity. We moved forward with protein constructs that gave high yield/purity and tested them in FP saturation studies in comparison to FAT WT (Figure 3C). Single site mutants L994E ($K_D = 82.3 \mu\text{M}$) and I936A ($K_D = 70.8 \mu\text{M}$) had slightly lower binding affinities compared to FAT WT ($K_D = 48.6 \mu\text{M}$), suggesting reduction to a single-site binding mode. Interestingly, single site mutant L1035D displayed a linear-shaped binding curve and a weak K_D of 4.5mM, suggesting substantial disruption of protein folding by mutation and therefore TAMRA-LD2-L10D binding. We also performed similar FP saturation studies with two-site mutants and demonstrated that KER+V932A and I936E+L994E mutants had no binding (K_D could not be calculated) while KER+I936D mutant had a weak binding affinity ($K_D = 266 \mu\text{M}$). In all, these data showed the specificity of FAT-paxillin interactions measured by FP and identified single-site mutants that could be utilized for site-specific binding.

Optimization and validation of high-throughput characteristics.

Next, we sought to optimize 384-well plate material and vendor in order to achieve the best possible assay window and signal variability. We tested 8 different 384-well plates that varied in plate material (polypropylene, polystyrene, non-binding surface), well volume, well shape (round, square, flat bottom, round bottom), and vendor (Figure 4A). We analyzed positive controls (0.1 μM TAMRA-LD2-L10D only) and negative controls (50 μM FAT + 0.1 μM TAMRA-LD2-L10D) in all plates; and

calculated assay window (ΔmP) and Z' -Factor (equation 3). Plate NUNC 267461, which is a shallow well, square shaped, rounded bottom, black polypropylene 384-well plate, had the overall best combination of Z' -Factor (0.72) and assay window (52.2 mP). This plate was selected for all subsequent assays. Because this assay would be utilized in screening of compounds dissolved in DMSO, we next tested the tolerability of the FP assay to increasing concentrations of DMSO. We performed assay window studies using 20 μM of FAT protein, as this lower concentration would have lower % occupancy of probe and therefore would be more suitable for competition studies. As shown in Figure 4B, FP binding signal was tolerated up to 13.33% DMSO, indicating suitability for compound screening applications. Next, we performed time-course studies on an entire 384-well plate with both positive and negative controls to monitor signal stability and variation over time (Figure 4C). After 2 hours of assay incubation time, the Z' -factor was > 0.60 , indicating good signal separation and an excellent assay. This signal variability was stable up to 4 hours. 3 hours was selected as an appropriate incubation time for producing a good Z' -factor. Finally, to test for potential edge and drift-effects over a complete 384-well plate, we plotted well number versus mP for both positive and negative controls. As shown in Figure 4D, positive and negative control signal was uniformly distributed over all areas of the plate and no trends were observed at the plate edge. The positive control has a standard deviation of 2.07 and the negative control a standard deviation of 1.9, with the overall Z' -factor for the plate being 0.63. In all, these data validated the use of our FP assay for high-throughput screening.

Pilot Screen

To further validate our FP assay for high-throughput drug screening, we proceeded with a pilot drug screen using our Tecan Freedom Evo 100 liquid handling robot. Tecan scripts were programmed using aqueous buffer and DMSO as test solutions. We selected the Diversity Set V library of 1,593 compounds available from the National Cancer Institute to use for our pilot screen. Compounds were screened at a final concentration of 312.5 μM (6.25% DMSO) in 384-well format (Figure 5A). Both

1
2
3 fluorescence polarization and fluorescent intensity values were measured for each plate. Because
4 naturally fluorescent or quenching compounds can perturb fluorescence polarization measurements and
5 thus can serve as false positives,^{34, 35} fluorescence intensity was utilized as an internal counter-screen.
6
7 Compounds that caused a > 25% increase or decrease in fluorescence intensity were excluded as hits.
8
9 Manual inspection of these wells confirmed that the majority of fluorescent or quenching compounds had
10 a red/orange tint. In addition, we noticed that all compounds that precipitated upon dilution in the assay
11 mixture had an associated decrease in mP, sometimes lower than TAMRA-LD2-L10D control. As such,
12 precipitating compounds were also excluded as hits. In total, after removal of 13 interfering compounds,
13 we identified 16 single-point hits that had a decreased mP value with > 40% inhibition compared to the
14 negative control average (FAT + TAMRA-LD2-L10D + DMSO). This corresponded to a hit rate of
15 1.0%. To validate that identified single-point hits had dose-dependent pharmacological activity, we
16 tested all 16 hits in FP dose-response curve studies from 312.5 μ M to 0.3 μ M (Figure 5B and Table 1).
17
18 We found 3 validated hits that showed dose-dependent inhibition of FAT-paxillin binding. After
19 purchasing commercially available compounds and repeating the dose response at higher concentrations,
20 the most potent compound (compound #1223) showed a calculated K_i of 111.2 μ M. 13 of 16 original
21 hits did not show sufficient dose-dependent inhibition to be considered validated hits. In all, this pilot
22 screen confirmed the use of our FP assay for high-throughput screening and identified 3 potential FAT-
23 paxillin inhibitors for follow-up studies.
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

47 **DISCUSSION**
48
49

50 FAK is widely expressed in multiple different tumor types and is a central node to multiple
51 oncogenic signaling pathways.^{1, 36} Despite FAK's biological importance in cancer, drug discovery
52 efforts have solely focused on targeting the kinase domain and have not addressed the critical scaffolding
53
54
55
56
57
58
59
60

functions of the protein.^{37, 38} This report represents a significant advancement to the FAK drug discovery field as we disclose the first described high-throughput screening assay for non-catalytic FAK inhibitor discovery. The FAK FAT-paxillin interaction is gaining traction as an alternative site for FAK inhibitor discovery due to its importance in regulating invasion, metastasis, apoptosis, as well as the breadth of x-ray crystallographic information available.^{19, 20} Furthermore, traditional ATP-competitive FAK-kinase inhibitors have had limited success in clinical trials and resistance mechanisms have emerged.^{17, 18} These studies highlight the need for FAK-paxillin inhibitors in order to alternatively target FAK in cancer.

In order to develop a FAK-paxillin screening assay, we performed significant assay validation and optimization including validation by SPR, site-directed mutagenesis, competition studies, plate optimization, and high-throughput metrics. In addition, we performed structure-guided optimization of the TAMRA-LD2 probe to promote lower background mP signal and a greater assay window. Intriguingly, when we tested small scale protein purification of 18 FAT mutants, only a small amount had sufficient yield and purity for FP studies. We speculate that specific FAT mutations may confer low bacterial expression, impact protein folding, decrease protein stability, or increase proteolytic cleavage. Our results further emphasize the need to empirically test multiple mutant constructs when attempting a site-directed mutagenesis approach. Another observation was that our FP assay was very stable at high concentrations of DMSO (> 5%). This DMSO tolerability may be due to the high thermostability of four-helical bundle proteins like the FAK FAT domain that have a high degree of hydrophobic packing and electrostatic interactions to stabilize 3D structure.³⁹ It is interestingly to note that in plate optimization studies, polypropylene plates seemed to correlate best with assay performance, perhaps due to the amphipathic nature of the LD2 peptide. Future studies may include further optimization of the TAMRA-LD2 probe to reduce potential aggregation or modification of the FAK protein construct (e.g., length) to allow greater mP change upon binding.

Upon processing the data from our FP pilot screen, we applied a computational PAINS (Pan-Assay Interfering Substances) filter on the entire NCI Div5 library to identify compounds that had

1
2
3 potentially reactive or promiscuous moieties.⁴⁰ This process is standard in our laboratory as well as in
4 many high-throughput screening labs. Unexpectedly, we found that 163 of 1,593 total compounds
5 (10.2%) were positive for PAINS moieties after substructure filtering. These included known
6 reactive/promiscuous structures such Michael acceptors, azo compounds, quinones, and others. These
7 findings caution the use of publically available compound libraries such as the NCI Div5 library for drug
8 discovery applications and highlight the importance of flagging compounds that are positive for PAINS
9 moieties. Furthermore, we emphasize the careful consideration of rigorous follow-up assays to rule out
10 non-specific inhibition, compound aggregation, and pan-assay interference.
11
12
13
14
15
16
17
18
19

20
21 The major purpose of the pilot screen with the NCI Div5 library was to validate signal variability
22 from plate to plate, establish a method for counter screening/hit triage, and to establish a reasonable hit
23 rate. The use of our FAT-paxillin FP assay in a pilot drug screen has established the feasibility of this
24 assay for a potential larger drug screen using a larger library such as a 3D diversity set or a protein-
25 protein interaction-focused library. We obtained 8 hits from the pilot screen that showed valid dose-
26 dependent inhibition and intend to follow-up on these compounds using secondary assays such as SPR,
27 STD NMR, HSQC NMR, X-ray crystallography, aggregation studies, and various cell-based assays.
28 Future screening studies may include development of a SPR or NMR-based FAT domain assay for
29 fragment-based drug discovery.
30
31
32
33
34
35
36
37
38
39

40
41 In conclusion, we have presented a high-throughput fluorescence polarization assay that
42 measures FAK-paxillin interactions in 384-well format for drug discovery applications. To the best of our
43 knowledge, this represents the first described high-throughput assay focused on the detection on FAK-
44 scaffold inhibitors as opposed to FAK-kinase inhibitors. Through structure-based design of the TAMRA-
45 Paxillin LD2 probe, we were able to design an assay that had a sufficient assay window, low variability, a
46 Z'-factor of 0.63, stable 384-well signal, and straight-forward robotic automation. Future goals include
47 the screening of additional small molecule libraries to identify potent FAK-paxillin inhibitors that will be
48 tested as an alternative approach to target FAK in cancer.
49
50
51
52
53
54
55
56
57
58
59
60

LIST OF ABBREVIATIONS

Focal Adhesion Kinase (FAK)

Focal Adhesion Targeting (FAT)

Protein Data Bank (PDB)

DECLARATIONS**Ethics Approval**

Not applicable

Consent for publication

Not applicable

Competing Interests

All authors have approved the manuscript and declare no conflict of interest.

Funding information

National Cancer Institute (grant No. R01 CA065910 to William Cance, Timothy Marlowe).

Acknowledgements

We would like to thank Dr. David Azorsa for use of the NCI Diversity Set V for pilot screening studies.

FIGURE LEGENDS

Figure 1. Characterization of the FAT-Paxillin interaction through modeling, SPR, and FP. A. 3D model of the LD2 motif of Paxillin binding to both binding sites on FAT. The model was edited from a previously determined crystal structure to contain demonstrate both LD2 binding sites on the same FAT molecule. B. Binding curve between TAMRA-LD2 and FAT as determined through FP. TAMRA-LD2 was kept constant at a 0.1 μM while FAT was titrated from 200 μM down to 0.2 μM . The calculated K_D of the interaction was $48.6 \pm 11.4 \mu\text{M}$. C. Dose response curve of 0.1 μM TAMRA-LD2 and 20 μM FAT inhibited by unlabeled LD2 titrated from 250 μM down to 0.5 μM . The calculated K_i was $45.9 \pm 4.2 \mu\text{M}$. D. SPR binding curve of the FAT-Paxillin interaction. The determined K_D was $51.5 \pm 0.2 \mu\text{M}$, a similar value to that determined by FP. FP experiments were performed in triplicate and SPR in duplicate.

Figure 2. Peptide and protein optimization for the FP assay. A. 3D model of the mutated L10D LD2 motif of Paxillin binding to both binding sites on FAT with a close up view of the mutation. The mutation is on the non-binding side of the peptide to prevent any loss in binding while reducing hydrophobic aggregation of the peptide. B. Binding curve between 0.1 μM TAMRA-LD2-L10D and FAT titrated from 200 μM down to 0.2 μM , determined through FP. The calculated K_D of the interaction was $49.7 \pm 12.0 \mu\text{M}$. C. Dose response curve of 0.1 μM TAMRA-LD2-L10D and 20 μM FAT inhibited by unlabeled LD2 titrated from 250 μM down to 0.3 μM . The calculated K_i is $52.3 \mu\text{M} \pm 9.3 \mu\text{M}$. D. Binding curve comparison of His-tagged FAT and untagged FAT. The curves and calculated K_D indicate marginal difference between cleaved and tagged protein. Experiments were performed in triplicate.

Figure 3. Assay validation through mutagenesis of FAT. A. 3D model highlighting the key amino acid interactions in both Paxillin binding sites that were mutated as an assay control. B. SDS-PAGE of the purification of various FAT single and double site mutations. Certain mutations were unable to either express or purify. C. On the left, binding curve comparisons of WT FAT and various single site mutations. L994E and L1035D are mutations of the 2-3 helix binding site. I936A is a mutation is a

1
2
3 mutation of the 1-4 helix binding site. The mutations demonstrate a decrease in binding affinity from WT
4 FAT but not complete binding loss indicating the dual binding nature of the FAT-Paxillin interaction. On
5 the right, binding curve comparison of WT FAT and various double site mutations. All double site
6 mutations demonstrate a severe loss in binding affinity compared to WT FAT. The K_D indicated as ND
7 (non-determinable) could not be calculated due to lack of binding character in the curves. Experiments
8 were performed in triplicate.

9
10
11 **Figure 4. High throughput screening metric optimization.** A. Plate optimization using various 384
12 well plates. Assay window (difference between signal at 20 μ M FAT and 0.1 μ M TAMRA-LD2-L10D
13 (top) and 0.1 μ M TAMRA-LD2-L10D only (bottom)) was plotted for each plate as well as calculated Z' -
14 factor. NUNC 267461 was selected as the plate to move forward with, having the best combination of a
15 high assay window and low Z' -factor. B. DMSO effects on assay window at various concentrations of
16 DMSO. Assay window variability was not statistically significant up to 13.33% DMSO. C. Time
17 optimization by incubating a plate of controls while shaking at room temperature. Assay window and Z' -
18 factor were calculated at each time and while assay window remains consistent, Z' -factor increases over
19 time. D. Drift effect analysis by plotting two entire 384-well plates, one with FAT + TAMRA-LD2-L10D
20 and the other with only TAMRA-LD2-L10D. The plot demonstrates no drift effect with the current assay
21 conditions in the NUNC 267461 plates.

22
23
24 **Figure 5. Pilot screen utilizing NCI Diversity Set V.** A. Plot of the results from a single point (312.5
25 μ M) screen of all compounds in the set at standard FP conditions (20 μ M FAT + 0.1 μ M TAMRA-LD2-
26 L10D). Hit threshold was set at >40% inhibition. Compound under the hit threshold that exhibit
27 fluorescence 25% higher or lower than the average of the controls were labeled as fluorescent/quenchers
28 and excluded in the final list of hits. Other compounds under the threshold were observed to have formed
29 a precipitate at the highest concentration and also removed from the final list. Labeled on the plot are also
30 potential aggregators which increased FP signal relative to the positive control. The final list contained 16
31 hits to be validated through dose response. B. Six representative plots of the dose response curves for the

hits are presented. The three on the left represent good dose response inhibition while the plots on the right show false positives. The false positives demonstrate minimal decreases and potential increases in FP signal up to the final concentration which experiences a sharp drop. Dose-response curves were performed in triplicate.

Table 1. Dose response inhibition data for the 16 single-point hits. I_{max} indicates the maximum inhibitory effect relative to the TAMRA-LD2-L10D and TAMRA-LD2-L10D + FAT controls. Calculated $K_i \pm$ Standard Error (SE) is shown. Results show only 3/16 compounds are true validated hits, the rest had ND (non-determinable) K_i . Experiments were performed in triplicate.

REFERENCES

1. Golubovskaya, V. M.; Kweh, F. A.; Cance, W. G. Focal adhesion kinase and cancer. *Histol Histopathol* **2009**, *24*, 503-10.
2. Weiner, T. M.; Liu, E. T.; Craven, R. J.; et al. Expression of focal adhesion kinase gene and invasive cancer. *Lancet* **1993**, *342*, 1024-5.
3. McLean, G. W.; Carragher, N. O.; Avizienyte, E.; et al. The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. *Nat Rev Cancer* **2005**, *5*, 505-15.
4. Schaller, M. D.; Borgman, C. A.; Cobb, B. S.; et al. pp125fak a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proceedings of the National Academy of Sciences of the United States of America* **1992**, *89*, 5192-6.
5. Golubovskaya, V. M.; Finch, R.; Cance, W. G. Direct Interaction of the N-terminal domain of Focal Adhesion Kinase with the N-terminal transactivation domain of p53. *J. Biol. Chem.* **2005**, *280*, 25008-25021.
6. Kurenova, E.; Xu, L.-H.; Yang, X.; et al. Focal Adhesion Kinase Suppresses Apoptosis by Binding to the Death Domain of Receptor-Interacting Protein. *Mol. Cell. Biol.* **2004**, *24*, 4361-4371.
7. Lahlou, H.; Sanguin-Gendreau, V.; Zuo, D.; et al. Mammary epithelial-specific disruption of the focal adhesion kinase blocks mammary tumor progression. *Proc Natl Acad Sci U S A* **2007**, *104*, 20302-7.
8. Nishimura, M.; Machida, K.; Imaizumi, M.; et al. Tyrosine phosphorylation of 100-130 kDa proteins in lung cancer correlates with poor prognosis. *British Journal of Cancer* **1996**, *74*, 780-7.
9. Ilic, D.; Furuta, Y.; Kanazawa, S.; et al. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **1995**, *377*, 539-44.
10. Xu, L.-h.; Yang, X.-h.; Bradham, C. A.; et al. The focal adhesion kinase suppresses transformation-associated, anchorage-Independent apoptosis in human breast cancer cells. *J. Biol. Chem.* **2000**, *275*, 30597-30604.
11. Sonoda, Y.; Matsumoto, Y.; Funakoshi, M.; et al. Anti-apoptotic Role of Focal Adhesion Kinase (FAK). Induction of Inhibitor-Of-Apoptosis Proteins and Apoptosis Suppression by the

- Overexpression of Fak in a Human Leukemic Cell Line, HL-60. *J Biol Chem* **2000**, 275, 16309-16315.
12. Kessler, B. E.; Sharma, V.; Zhou, Q.; et al. FAK Expression, Not Kinase Activity, Is a Key Mediator of Thyroid Tumorigenesis and Protumorigenic Processes. *Mol Cancer Res* **2016**, 14, 869-82.
 13. Stokes, J. B.; Adair, S. J.; Slack-Davis, J.; et al. Inhibition of Focal Adhesion Kinase by PF-562,271 Inhibits the Growth and Metastasis of Pancreatic Cancer Concomitant with Altering the Tumor Microenvironment. *Molecular Cancer Therapeutics* **2011**, 10, 2135-45.
 14. Cance, W. G.; Golubovskaya, V. M. Focal adhesion kinase versus p53: apoptosis or survival? *Sci Signal* **2008**, 1, pe22.
 15. Xu, L. H.; Yang, X.; Craven, R. J.; et al. The COOH-terminal domain of the focal adhesion kinase induces loss of adhesion and cell death in human tumor cells. *Cell Growth Differ* **1998**, 9, 999-1005.
 16. Marlowe, T. A.; Lenzo, F. L.; Figel, S. A.; et al. Oncogenic Receptor Tyrosine Kinases Directly Phosphorylate Focal Adhesion Kinase (FAK) as a Resistance Mechanism to FAK-Kinase Inhibitors. *Mol Cancer Ther* **2016**, 15, 3028-3039.
 17. Infante, J. R.; Camidge, D. R.; Mileskin, L. R.; et al. Safety, pharmacokinetic, and pharmacodynamic phase I dose-escalation trial of PF-00562271, an inhibitor of focal adhesion kinase, in advanced solid tumors. *J Clin Oncol* **2012**, 30, 1527-33.
 18. Jones, S. F.; Siu, L. L.; Bendell, J. C.; et al. A phase I study of VS-6063, a second-generation focal adhesion kinase inhibitor, in patients with advanced solid tumors. *Invest New Drugs* **2015**, 33, 1100-7.
 19. Kanteti, R.; Batra, S. K.; Lennon, F. E.; et al. FAK and paxillin, two potential targets in pancreatic cancer. *Oncotarget* **2016**, 7, 31586-601.
 20. Hoellerer, M. K.; Noble, M. E.; Labesse, G.; et al. Molecular recognition of paxillin LD motifs by the focal adhesion targeting domain. *Structure* **2003**, 11, 1207-17.
 21. Garron, M. L.; Arthos, J.; Guichou, J. F.; et al. Structural basis for the interaction between focal adhesion kinase and CD4. *J Mol Biol* **2008**, 375, 1320-8.
 22. Xu, S.; Liu, Y.; Li, X.; et al. The binding of DCC-P3 motif and FAK-FAT domain mediates the initial step of netrin-1/DCC signaling for axon attraction. *Cell Discov* **2018**, 4, 8.
 23. Sieg, D. J.; Hauck, C. R.; Ilic, D.; et al. FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol* **2000**, 2, 249-256.
 24. Kaneda, T.; Sonoda, Y.; Ando, K.; et al. Mutation of Y925F in focal adhesion kinase (FAK) suppresses melanoma cell proliferation and metastasis. *Cancer Lett* **2008**, 270, 354-61.
 25. Deramaut, T. B.; Dujardin, D.; Noulet, F.; et al. Altering FAK-paxillin interactions reduces adhesion, migration and invasion processes. *PLoS One* **2014**, 9, e92059.
 26. Scheswohl, D. M.; Harrell, J. R.; Rajfur, Z.; et al. Multiple paxillin binding sites regulate FAK function. *J Mol Signal* **2008**, 3, 1.
 27. Gao, G.; Prutzman, K. C.; King, M. L.; et al. NMR solution structure of the focal adhesion targeting domain of focal adhesion kinase in complex with a paxillin LD peptide: evidence for a two-site binding model. *J Biol Chem* **2004**, 279, 8441-51.
 28. Hayashi, I.; Vuori, K.; Liddington, R. C. The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. *Nat Struct Biol* **2002**, 9, 101-6.
 29. Bertolucci, C. M.; Guibao, C. D.; Zheng, J. Structural features of the focal adhesion kinase-paxillin complex give insight into the dynamics of focal adhesion assembly. *Protein Sci* **2005**, 14, 644-52.
 30. Nikolovska-Coleska, Z.; Wang, R.; Fang, X.; et al. Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. *Anal Biochem* **2004**, 332, 261-73.

31. Zhang, J. H.; Chung, T. D.; Oldenburg, K. R. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* **1999**, *4*, 67-73.

32. Parker, G. J.; Law, T. L.; Lenocho, F. J.; et al. Development of high throughput screening assays using fluorescence polarization: nuclear receptor-ligand-binding and kinase/phosphatase assays. *J Biomol Screen* **2000**, *5*, 77-88.

33. Du, Y.; Moulick, K.; Rodina, A.; et al. High-throughput screening fluorescence polarization assay for tumor-specific Hsp90. *J Biomol Screen* **2007**, *12*, 915-24.

34. Kusba, J.; Bogdanov, V.; Gryczynski, I.; et al. Theory of light quenching: effects of fluorescence polarization, intensity, and anisotropy decays. *Biophys J* **1994**, *67*, 2024-40.

35. Turek-Etienne, T. C.; Small, E. C.; Soh, S. C.; et al. Evaluation of fluorescent compound interference in 4 fluorescence polarization assays: 2 kinases, 1 protease, and 1 phosphatase. *J Biomol Screen* **2003**, *8*, 176-84.

36. Cance, W. G.; Kurenova, E.; Marlowe, T.; et al. Disrupting the scaffold to improve focal adhesion kinase-targeted cancer therapeutics. *Science Signaling* **2013**, *6*, pe10.

37. Roberts, W. G.; Ung, E.; Whalen, P.; et al. Antitumor activity and pharmacology of a selective focal adhesion kinase inhibitor, PF-562,271. *Cancer Res* **2008**, *68*, 1935-44.

38. Tanjoni, I.; Walsh, C.; Uryu, S.; et al. PND-1186 FAK inhibitor selectively promotes tumor cell apoptosis in three-dimensional environments. *Cancer Biol Ther* **2010**, *9*, 764-77.

39. Bellesia, G.; Jewett, A. I.; Shea, J. E. Relative stability of de novo four-helix bundle proteins: insights from coarse grained molecular simulations. *Protein Sci* **2011**, *20*, 818-26.

40. Baell, J. B.; Holloway, G. A. New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J Med Chem* **2010**, *53*, 2719-40.

TABLE 1

Table 1
Sixteen compounds from Diversity Set V evaluated in FP for dose response inhibition with
calculated K_i and I_{max}

Compound	I_{max}	$K_i \pm SE$ (μM)
168	100	329.8 ± 24.1
316	ND	ND
447	ND	ND
459	ND	ND
518	ND	ND
650	ND	ND
743	ND	ND
906	ND	ND
994	ND	ND
1037	ND	ND
1122	ND	ND
1223	65.0	111.2 ± 14.5
1236	ND	ND
1324	ND	ND
1429	100	330.1 ± 31.1
1542	ND	ND

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

SUPPLEMENTAL METHODS & DATA

TITLE: Development of a high-throughput fluorescence polarization assay to detect inhibitors of the FAK-paxillin interaction

Correspondence email: tmarlowe@email.arizona.edu; wcance@email.arizona.edu

Table S1. Primer design for site-directed mutagenesis

Mutation	Primer Sequences
I936D	5'-GATTTTACTGGACATCTCGTCGACAGCTTTCACCAGGCCC-3'
	5'-GGGCCTGGTGAAAGCTGTCGACGAGATGTCCAGTAAATC-3'
I936A	5'-GATTTTACTGGACATCTCGGCGACAGCTTTCACCAGGCCC-3'
	5'-GGGCCTGGTGAAAGCTGTCGCCGAGATGTCCAGTAAATC-3'
I998A	5'-GCCAGTTTCATCTTGTGGCGAGCTCACCCAGGTCAGA-3'
	5'-TCTGACCTGGGTGAGCTCGCCAACAAGATGAACTGGC-3'
I998D	5'-GCCAGTTTCATCTTGTGGCGAGCTCACCCAGGTCAGA-3'
	5'-TCTGACCTGGGTGAGCTCGACAACAAGATGAACTGGC-3'
V932D	5'-ATCTCGATGACAGCTTTGTCCAGGCCCCGTCACATTC-3'
	5'-GAATGTGACGGGCCTGGACAAAGCTGTCATCGAGAT-3'
V932A	5'-CGATGACAGCTTTCGCCAGGCCCCGTCACAT-3'
	5'-ATGTGACGGGCCTGGCGAAAGCTGTCATCG-3'
L1035D	5'-GTCAATGACATCGTCTAAGTTTTGGCATCCACAGCCAGG-3'
	5'-CCTGGCTGTGGATGCCAAAACTTAGACGATGTCATTGAC-3'
L994E	5'-CTTGTTGATGAGCTCACCCCTCGTCAGAGTTCAATAGCTTC-3'
	5'-GAAGCTATTGAAGTCTGACGAGGGTGAGTCATCAACAAG-3'
H1025A	5'-AGCCAGGGCGGCAGCAGCAGTCAGCATTTGC-3'
	5'-GCAAATGCTGACTGCTGCTGCCGCCCTGGCT-3'
I936E	5'-GGATTTTACTGGACATCTCCTCGACAGCTTTCACCAGGCCC-3'
	5'-GGGCCTGGTGAAAGCTGTCGAGGAGATGTCCAGTAAATCC-3'
L965D	5'-TCGGCTTGGCCCTGAGGACATTAGACGCCACTGTGGAT-3'
	5'-ATCCACAGTGGCGTCTAATGTCCTCAGGGCCAAGCCGA-3'
K955A	5'-GGCCAAGCCGACTTCCGCCACCATAGGGACATAC-3'
	5'-GTATGTCCCTATGGTGGCGGAAGTCGGCTTGGCC-3'
E948A	5'-AGGGACATACTCCGCTGGTGGGGCTGG-3'
	5'-CCAGCCCCACCAGCGGAGTATGTCCCT-3'
R962A	5'-AGTGGCCAATAATGTCGCCAGGGCCAAGCCGAC-3'
	5'-GTCGGCTTGGCCCTGGCGACATTATTGGCCACT-3'

Figure S1. Intrinsic FP properties of TAMRA-LD2 and TAMRA-LD2-L10D fluorescent probes. mP measurement was obtained for TAMRA probes diluted into FP assay buffer at the concentration indicated. Multiple replicates over multiple plates were recorded (n = 16). Statistical analysis was done using the Student's t-test. Results indicate a lower intrinsic FP signal for TAMRA-LD2-L10D.