Fast molecular interaction screening of epigenetic gene regulator G9a with fragments from a large chemical space

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Abstract

One of the latest trends in drug discovery screening shows a rise in the number of explorative targets. In order to evaluate these targets, the industry needs screening technologies that offer flexibility in characterizing binding events between more heterologous targets and ligands from large or new chemical spaces. Dianthus NT.23PicoDuo swiftly detects and quantifies molecular interactions in solution, independent of the molecular mass of the two interacting molecules, making it a perfect match for fragment-based screening with compounds representing a large chemical space. In this application note, we show data from single-dose screening of a fragment library with a broad molecular mass and complexity range against the target molecule histone methyltransferase (HMT) G9a, followed by affinity constant determination (K_d) for a selection of hit molecules. At the end of the study, we screened 5,784 data points including controls in less than 8 hours of instrument time, using less than 20 µg of target protein. Affinities were in the high micromolar range as expected from fragments, and in good agreement with published values for the positive control.

Introduction

The inefficiency of the drug discovery process is a widely accepted problem by everyone in the pharmaceutical industry. Investment in pharmaceutical research and development continues to increase, while the number of new drugs approved by the US Food and Drug Administration remains low[1]. One area that is crucial to making drug discovery more efficient is the screening process. Current trends demand technologies that can characterize binding events involving more heterogeneous targets and compound libraries that are not larger, but rather made up of more diverse molecules from large and new chemical spaces. There is also a need for faster tools, as computer-aided iterative screening is becoming more popular as an alternative to high throughput screening.

Dianthus is the screening tool that makes drug discovery more cost-effective — it detects and quantifies molecular interactions in solution, independent of the mass and size of the two interacting molecules, and does it at an unmatched speed with flexible throughput.

To quantify molecular interactions, Dianthus uses Temperature Related Intensity Change (TRIC), a technology with 10 years of proven success in molecular interaction research[2]. TRIC measures the strength of the interaction by detecting a variation in the fluorescence signal of a fluorescently labeled or intrinsically fluorescent target as a result of a very rapid and precise IR-laser induced temperature change. The binding of a ligand in close proximity to the fluorophore or a ligand-induced conformational change in the target strongly affects the extent of the change in fluorescence between the unbound (no ligand) and bound (with ligand) state of the target. Changes in fluorescence upon activation of the IR laser are monitored to characterize the interaction and derive affinity constants[2].

The fragment-based lead discovery study presented here, utilizes a library with 2,490 fragments from a large chemical space in two steps: single-dose screening and affinity screening. The target molecule, G9a (also known as EHMT2), has been the focus of oncological drug discovery research over the last decade and Irregularities in its activity have been associated with a variety of oncogenic phenotypes, such as breast or lung cancer[3]. G9a is a histone methyltransferase (HMT) that acts in methylation of H3K9 (lysine 9 in histone 3). HMTs are enzymes involved in epigenetic gene regulation by methylation of histone proteins in either Lysine or Arginine residues[4]. Finding inhibitors with different modes

of action for G9a could be an important step to treat cancer patients with such phenotypes[5]. To detect and then quantify the interactions between G9s and the fragments, G9a was labeled with a red fluorescent probe and assays were performed in 384-well microplates using the Dianthus NT.23PicoDuo system.

Results

Screening a chemical library of such a broad variety of molecular masses, reaching from ions to more complex organic molecules, requires a method capable of detecting binding events independent of molecular mass and binding site. Dianthus, in comparison to other biophysical technologies, does this, making it an ideal tool for fragment based lead discovery.

For the single-dose assay, a library of 2,490 fragment containing molecules ranging in size from ~40 Da to ~1 kDa, was screened. Each fragment (ligand) was tested at a final concentration of 1 mM, while the target, G9a, was used at a final concentration of 5 nM. Each ligand dilution was prepared and tested in duplicate. S-adenosyl-L-methionine (SAM), a G9a natural ligand, was used as a positive control at regular intervals during the screen at a final concentration of 100 µM. Data was analyzed with NanoTemper's DI.Screening Analysis software.

Figure 1 shows the signal amplitude obtained for each ligand and each replicate of the positive control SAM (y-axis) plotted against the chronological order in which they were measured (x-axis). The area of insignificant ΔFnorm (pink shaded area) corresponds to three standard deviations from the signal recorded from all DMSO references. Ligands with signals that fall within this area are categorized as non-binders.

All replicates of the positive control SAM (yellow dots) show a significant signal amplitude and very little variability throughout the considerably large single-dose screen, spanning sixteen 384-microwell plates. Some data points were categorized as autofluorescence (purple dots) or aggregation (green dots), both categories part of the quality checks performed automatically by the software. Molecules interacting with the target were identified as hits (light blue dots).

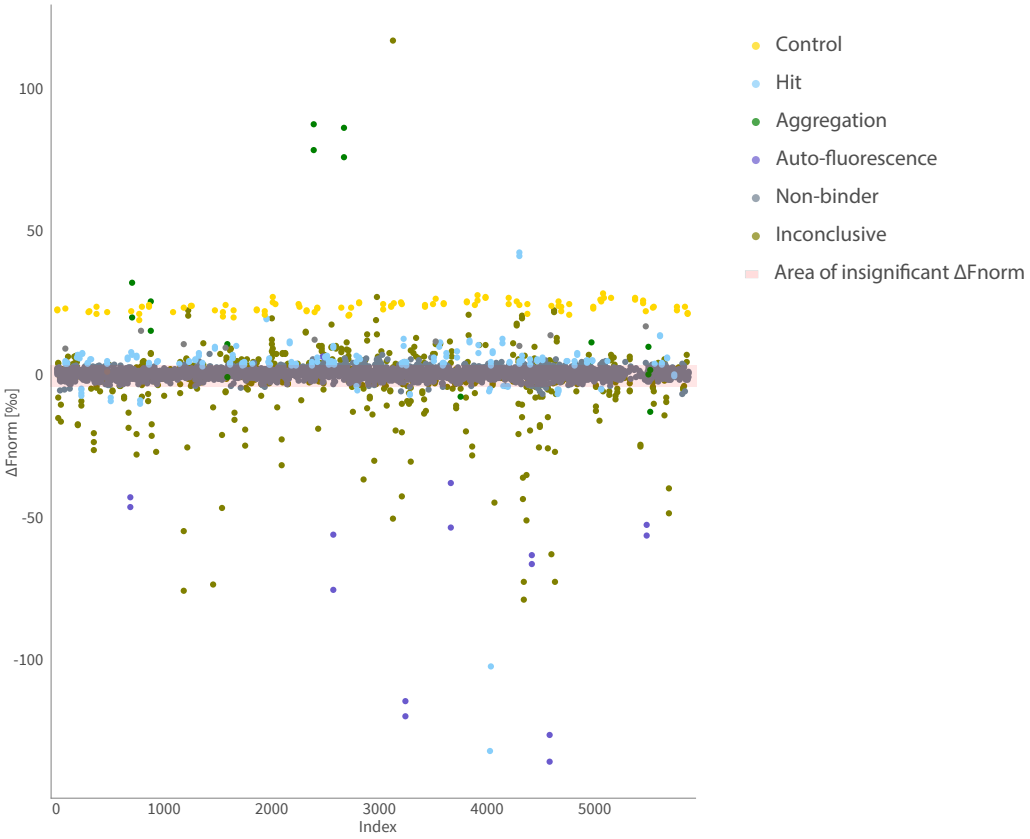


Figure 1: *Scatter plot showing the ΔFnorm response of all tested fragments in duplicate. The points are colored corresponding to the category they were sorted into by the software algorithm.*

Figure 2 shows the summary of the results seen in the scatter plot. The majority of the fragments (77.1%) were automatically identified by the software as non-binders since their signals fell within the area of insignificant ΔFnorm,

an expected outcome from using a non-specific library. Eighty-eight fragments (3.5%) were identified as hits. They ranged from ~100 Da to ~350 Da in molecular weight and from ~90 to ~400 in molecule complexity according to the Bertz/Hendrickson/Ihlenfeldt formula[6,7]. From the list of fragment hits, a few were selected for the second step, affinity screening, involving titration experiments to determine specificity and affinity (K_d).

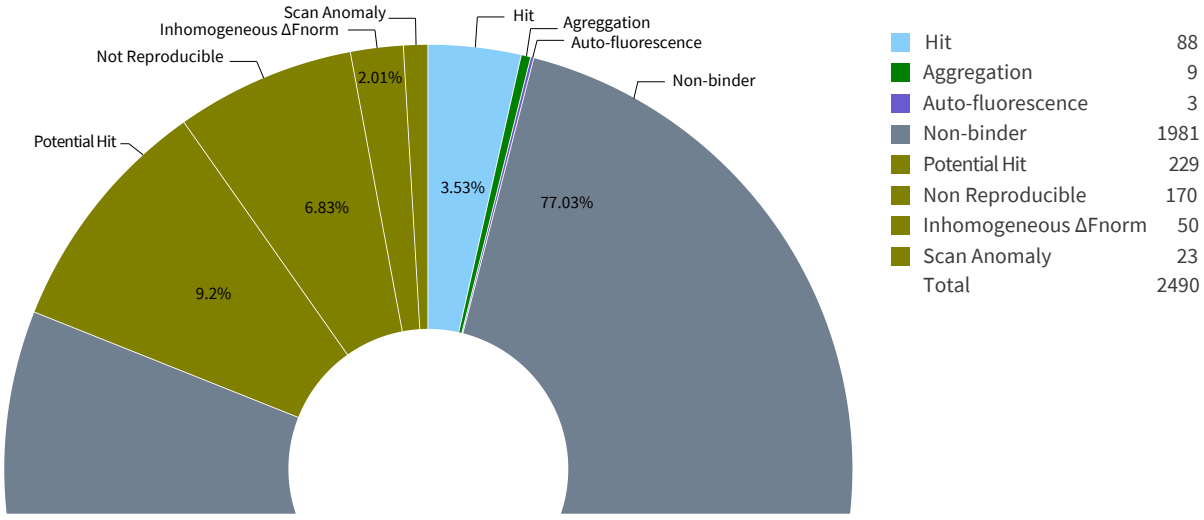
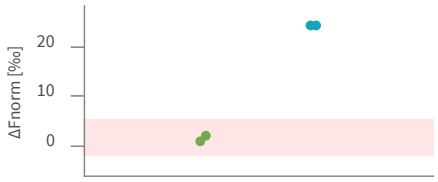
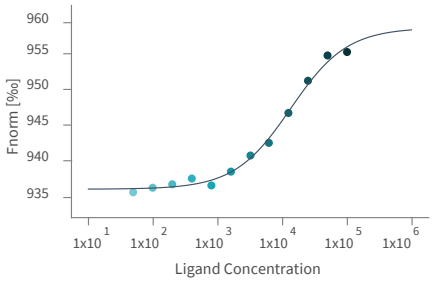
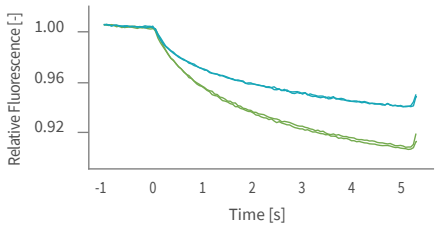
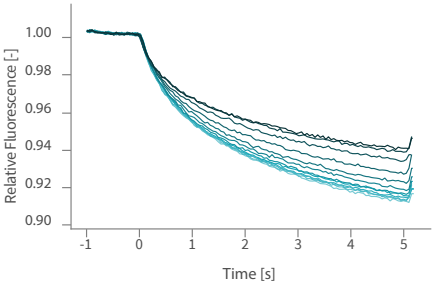
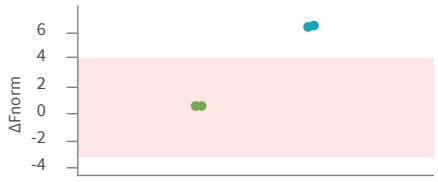
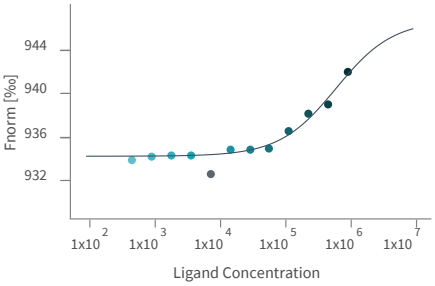
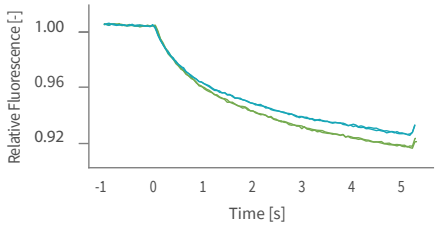
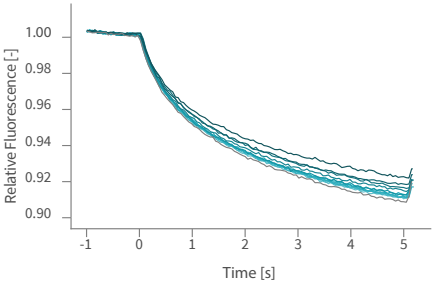


Figure 2: *Single-dose screen summary. Fragments are sorted into categories based on sorting algorithm in DI.Screening Analysis software.*

For the affinity screening, a 12-point, 0.5-fold dilution series of the selected fragments were prepared, starting at 1 mM. Then, labeled G9a was added at 5 nM final concentration to each of the 12 ligand dilutions. The dose-dependent response curves, resulting from Fnorm analysis after a 2.5 second IR-laser on-time, were approximated with a fit model that describes the law of mass action. Fragments were characterized by different binding affinities and signal amplitudes.

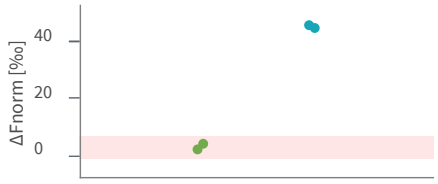
Table 1 shows four fragments and the positive control SAM. The figures demonstrate good correlation between the single-dose and affinity screens.

Fragment	Single-dose screen (A) ΔFnorm (B) TRIC trace	Affinity screen (C) Fnorm dose response curve (D) TRIC trace
Positive control SAM	(A) 	(C) 
	(B) 	(D) 
Fragment A12935	(A) 	(C) 
	(B) 	(D) 

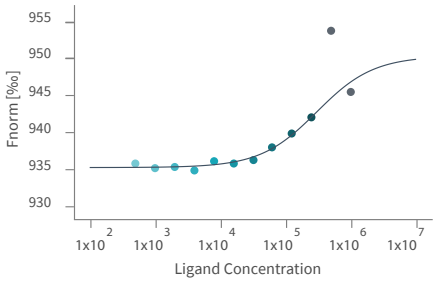
Fragment	Single-dose screen (A) ΔFnorm (B) TRIC trace	Affinity screen (C) Fnorm dose response curve (D) TRIC trace

Fragment C18598

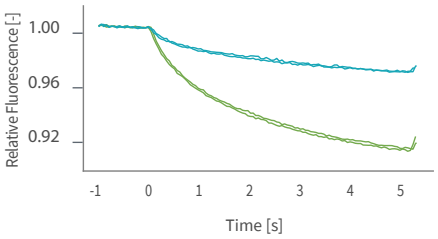
(A)



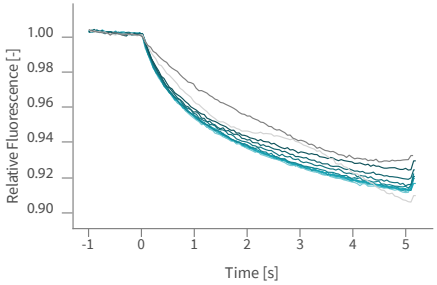
(C)



(B)

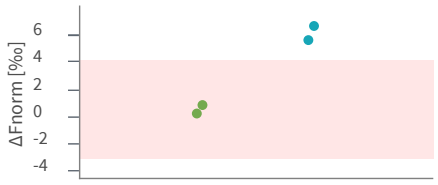


(D)

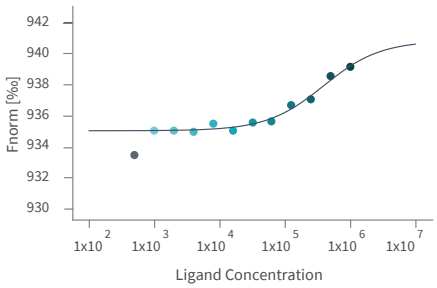


Fragment A25749

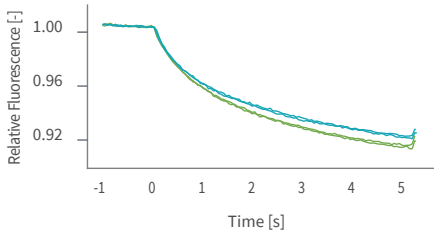
(A)



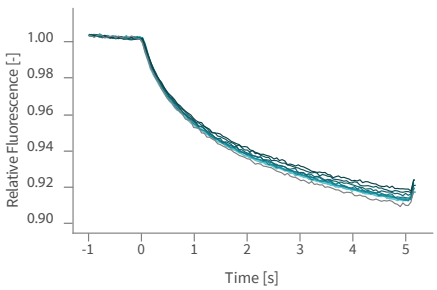
(C)



(B)



(D)



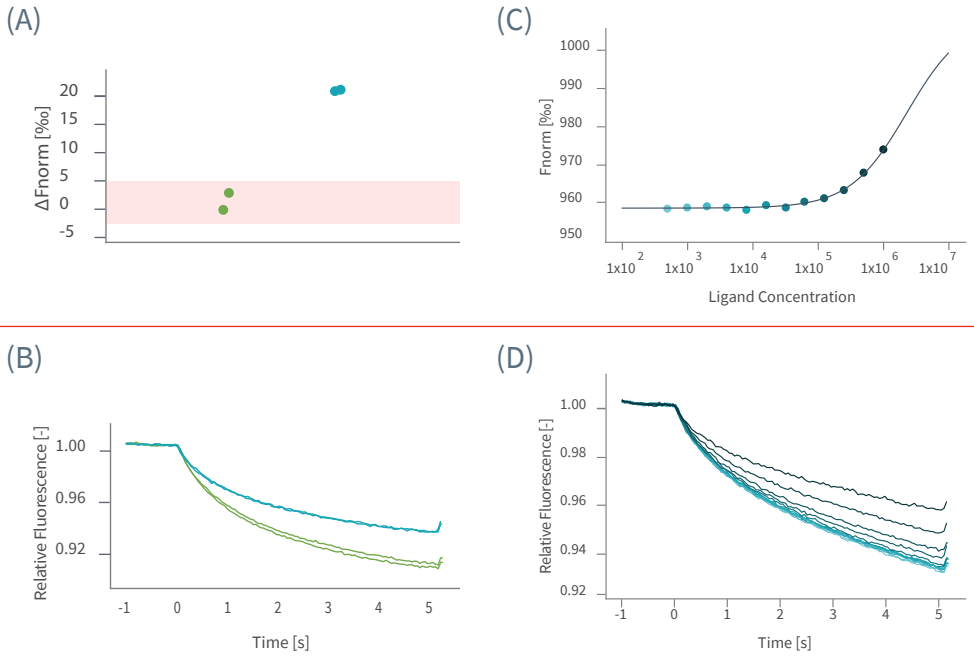
Fragment	Single-dose screen (A) ΔFnorm (B) TRIC trace	Affinity screen (C) Fnorm dose response curve (D) TRIC trace
Fragment D85021		

Table 1: *Column on the left shows the scatter plot with the ΔFnorm response (A) and TRIC signal plotted as the relative fluorescence vs. time (B) from the single-dose screen. Dots and traces in green correspond to the signal measured from the reference (DMSO), dots and traces in blue represent signal measured from the positive control (SAM) and fragments. Column on the right represents the TRIC signal (C) and dose response curves resulting from the titrations of selected fragment molecules and SAM against G9a (D). Dots and traces in grey correspond to data that was identified by the software to be biased by aggregation. Concentrations in the dose response plots are indicated in nM.*

Overall, affinities were in the higher micromolar range, as expected from fragment molecules (Table 2). Importantly, the measured dissociation constant for SAM, K_d=13.3 µM, was in good agreement with published values derived from ITC data[8].

Ligand	K_d (mM)	Amplitude, ΔFnorm (‰)	S/N ratio
SAM	0.013	22.5	38.0
A12935	0.656	12.5	37.7
C18598*	>0.299	15.1	41.7
A25749	0.386	5.8	28.2
D85021*	>2.270	50.2	136.8

Table 2: *K_d values, amplitudes and S/N ratios for positive control SAM and the four fragments selected for affinity screening. *Saturation of binding curve is insufficient to determine a more precise estimation of the K_d value.*

Discussion

Dianthus NT.23PicoDuo was used to test the binding of a 2,490-fragment library to the cancer target G9a. By using TRIC as the biophysical technology to first detect and then quantify interactions, novel candidates were identified with high micromolar affinities for G9a from a library of fragments with a broad range of molecular mass and complexity. The entire fragment library was screened in duplicates (5,784 data points including controls) in less than 8 hours of instrument time, using less than 20 µg of target protein. Not only were novel G9a ligands identified in the single-dose experiments, but straightforward additional testing in the affinity screen step was used to further expand the hit list and investigate interesting compound-induced effects, such as aggregation. However, further testing and especially optimization of the identified interactors would be necessary in order to increase compound affinity, which can be performed with the same system and the same advantages shown for lead ID. Using Dianthus NT.23PicoDuo saved many days of screening time compared to other high throughput screening systems by at least a factor of 6 in some cases. Combining its speed, flexible throughput and ability to screen fragments from a large chemical space, Dianthus is the perfect fit and primary choice for fragment-based lead discovery.

Materials and Methods

G9a was labeled following the labeling protocol as specified in NanoTemper Technologies RED-NHS 2nd Generation labeling kit. The assay buffer for all experiments was 50 mM HEPES, pH 8.0, 150 mM NaCl, 2 mM GSH, 0.005% TWEEN® 20 and a final volume of 20 µL per datapoint was used for both the single-dose and the affinity screen in Dianthus 384-well microwell plates. The fragment library was dissolved in 100% DMSO at a concentration of 100 mM and SAM was dissolved in water to a concentration of 10 mM. In all experiments with SAM as a ligand, a final amount of 1% DMSO was added to mimic the conditions for fragment dilutions. We used a HAMILTON STARlet system for liquid handling. First, we diluted the fragments from the 100 mM stock solution into assay buffer to 4 mM and 4% DMSO. In a second step, the fragments were further diluted into a G9a-containing solution to a final concentration of 1 mM and 1%DMSO. Labeled G9a was used at a final concentration of 5 nM. The first dilution was into a conventional microwell plate, while the second dilution step occurred in the Dianthus 384-well microplate, where the measurements took place.

For affinity measurements, fragments were pre-diluted to 2 mM stocks in assay buffer, and 0.5-fold dilution were prepared in assay buffer containing

2% DMSO. Each fragment dilution was then mixed with equal volumes of labeled G9a containing assay buffer, resulting in final concentrations of 1% DMSO and 5 nM labeled G9a. In all experiments with SAM as a ligand, a final amount of 1% DMSO was added to mimic the conditions for fragment dilutions.

After the 384-well microplates were loaded with the fragment + G9a mix, they were equilibrated for 2.5 hr and centrifuged for 30 sec at 1000 x *g* before loading onto Dianthus NT.23PicoDuo. The system was set to 20°C as set temperature. The samples were first measured for 1 sec without heating and for 5 sec with the IR-laser turned on. The two optical systems in Dianthus were used in parallel, resulting in an overall measurement time of ~30 min per plate. Measured fluorescence values collected are displayed as relative fluorescence, where the fluorescence obtained at ambient temperature is normalized to one, and as normalized fluorescence (Fnorm) which describes the ratio between fluorescence values (F1) after and the fluorescence values (F0) prior to IR laser activation and is typically given in ‰. The dissociation constant or K_d, is obtained by fitting a dose-response curve to a plot of Fnorm vs. ligand concentration.

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