Kinome Profiling with Continuous Assays and Integration of Automated Linear Range Determination to Accelerate Generation of Improved Drug Treatments

AssayQuant® TECHNOLOGIES INC.

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ABSTRACT

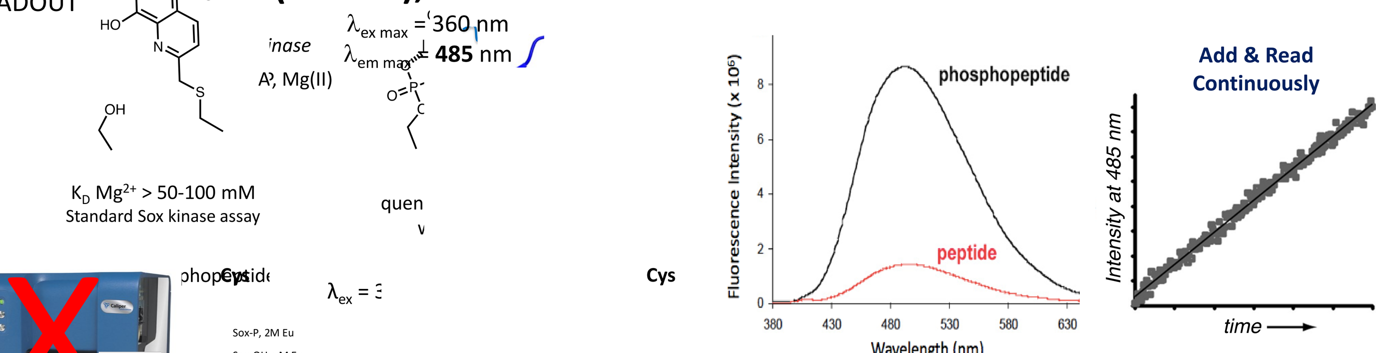
Together with biochemical and cellular potency information, selectivity data are critical to predict potential off-target activities that could result in toxicity during drug treatment. Profiling against a broad selection of kinases in the human kinome is traditionally conducted with a variety of endpoint assay formats, including indirect competitive displacement and enzymatic assays with radioactively labeled ATP or fluorophore-based peptide substrates, or by following ADP generation. When the progress curve is linear throughout the experiment, a single endpoint reading can approximate the initial reaction rate. However, if there is a delay in the onset of the reaction, or there are other changes in reaction rate over time, then an endpoint reading will be inaccurate. Continuous assays generate progress curves and provide critical time-dependent information. Although progress curves have been measured routinely for over a century, no automated method has yet been reported to identify the initial velocity of the reaction, essential for high-throughput assessment of enzyme activity and drug effectiveness. We have developed a novel automated protocol to streamline and optimize the process. Our heuristic algorithm analyzes the progress curve to identify the most relevant linear range and determine an initial rate, which is then used to characterize the modulation of enzyme activity by inhibitors or activators of the target. This continuous assay format, where the full progress curve of the reaction is captured, has been applied to characterize the modulation of protein kinase and phosphatase enzyme activity, which together represent over 30% of all drug development efforts. Our analysis highlights distinct advantages over endpoint determinations, including 1) the true reaction rate is captured directly from the slope of the progress curve with many time points, yielding much higher confidence in this critical parameter, 2) it takes into account both initial lag in enzyme activity (potentially indicating incomplete activation) and pre-mature saturation of the signal over time (consistent with, for example, substrate depletion or enzyme instability), 3) the occurrence of noise in an assay (often the consequence of compound insolubility) might be undetected or underappreciated in an endpoint assay, and 4) compound-specific patterns that provide important insights about drug mechanism and efficacy can be identified and characterized. If compound-induced non-linearity is present in progress curves, endpoint assays can greatly underestimate the true potency of compounds. However, with a continuous assay, such deviations from linearity may instead highlight time-dependent inhibition and enable structure-kinetic relationship optimization. The data shown emphasize the utility of this approach to identify highly valuable and exploitable inhibitory mechanisms that would be missed entirely with an endpoint assay. These kinome profiling data using a continuous kinase assay combined with an automated linear range finding algorithm will enable more effective treatments across all disease areas.

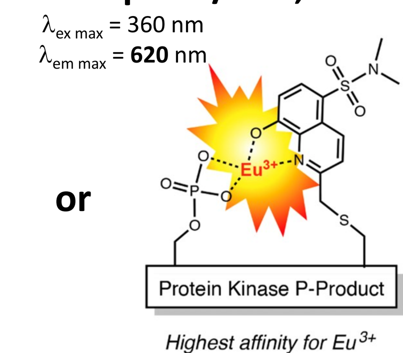
FIGURE 1. ENDPOINT VS. CONTINUOUS ASSAYS

	Endpoint Assay – one read at a single timepoint	Continuous read over full progress curve
Pros	• Quicker, easier, can stack plates for read	• Better assay development and optimization • Internal control for assay behavior • Higher confidence in reading • Detection of interesting and exploitable MOA
Cons	• Critical assumption that is often wrong: final signal reflects the initial reaction rate • Lower confidence in reading • Important mode-of-action details of inhibition can be missed	• Greater need for reader time

FIGURE 2. THE PHOSPHOSENS® ASSAY – CONTINUOUS & ENDPOINT/RED FORMATS

A. Continuous (Kinetic), FI





or

B. Endpoint/Red, TRF

$\lambda_{ex\ max}$ = 360 nm
$\lambda_{em\ max}$ = 620 nm

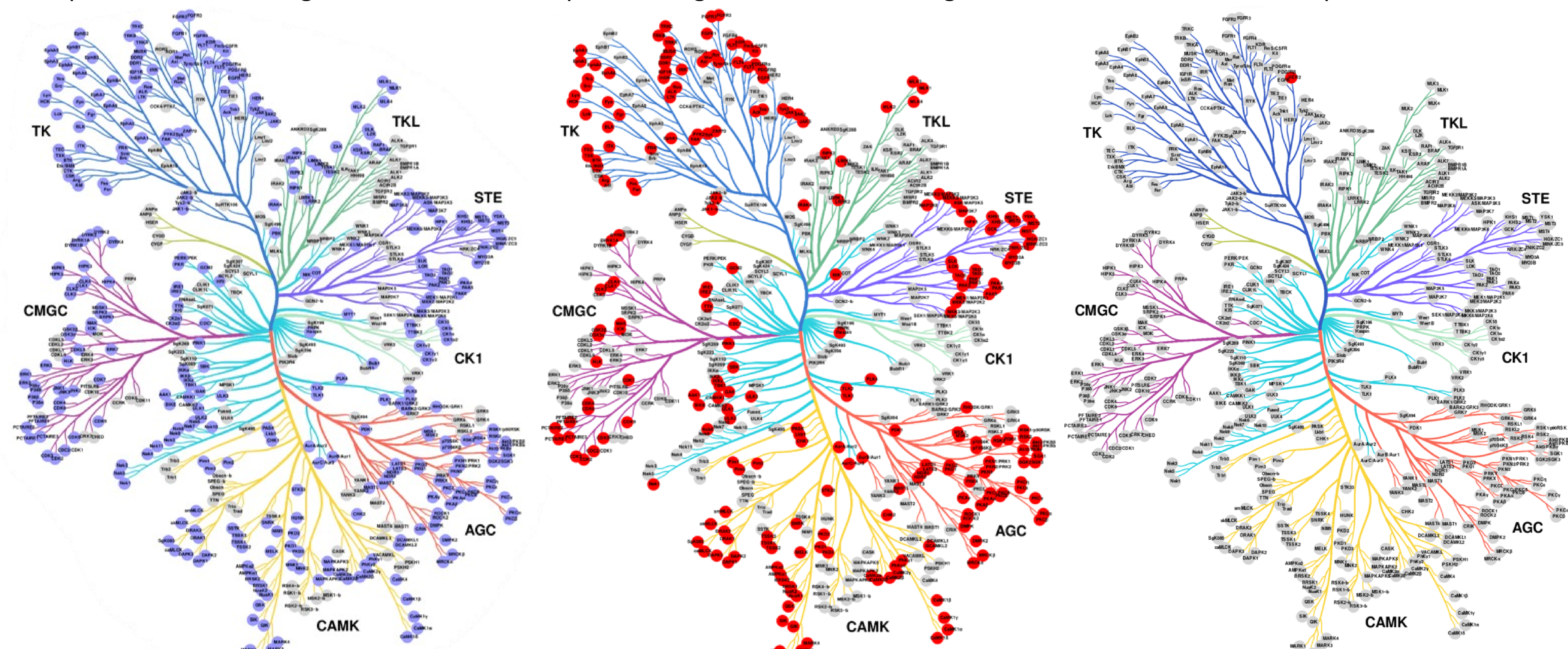
Protein Kinase P-Product

Highest affinity for Eu^{3+}

Peptide sequences are synthesized using solid-phase methods with the Sox fluorophore coupled through the sulfhydryl group of a cysteine residue proximal to a protein kinase phosphorylation site, such as a tyrosine, serine or threonine. Upon addition of a kinase, the peptide is phosphorylated. In the presence of magnesium ion, a chelation complex is formed with the phosphate group, resulting in fluorescence enhancement of the Sox fluorophore that can be **monitored continuously** as fluorescence intensity **(A)**. Kinase inhibitors prevent phosphorylation and thus fluorescence. At any point, Europium ion can be added, to displace the magnesium ion, resulting in a long wavelength, time-resolved fluorescence (TRF) **endpoint/Red format (B)** that is useful for high-throughput or structure activity relationship (SAR) applications.

FIGURE 3. KINOME PROFILING

Kinome profiling is a critical assay to determine the selectivity of a kinase inhibitor. It is not enough to know how strongly a compound inhibits a target kinase. It is also important to gain an understanding of what other kinases the compound inhibits.



>400 unique wild-type kinases in the AssayQuant Kinome Profiling Panel

Some non-selective inhibitors, such as Staurosporine, hit a wide variety of kinases

Whereas some inhibitors are exquisitely selective, such as this Her2 inhibitor

FIGURE 4. ALGORITHM FOR AUTOMATED LINEAR RANGE FINDING

- Manual methods are insufficient: they are subjective and lead to user-to-user and enzyme-to-enzyme variability and take too long for high throughput data analysis.
- A fully automated method avoids variability of rate measurements between users, enzymes, and experiments, and enables high-throughput data analysis.
- An automated method must identify and exclude slower lag and plateau phases (A) that introduce deviations from linearity in the progress curve.

1. Identify the region of 10 consecutive data points with the steepest slope (highest gradient), e.g., using the SLOPE function of Excel. This is the Maximum Slope.

2. Determine the standard error of the Maximum Slope, e.g., with the LINEST function of Excel.

3. Calculate the slope value that is 3 slope standard error values below the Maximum Slope. This is the Minimum Slope.

4. Starting with the first time point of the Maximum Slope range, calculate the slope to each subsequent time point. Then identify the last time point with slope above the Minimum Slope. This is the last time point of the linear range.

5. Starting with the last time point of the Maximum Slope range, calculate the slope to each previous time point. Then identify the first time point with slope above the Minimum Slope. This is the first time point of the linear range.

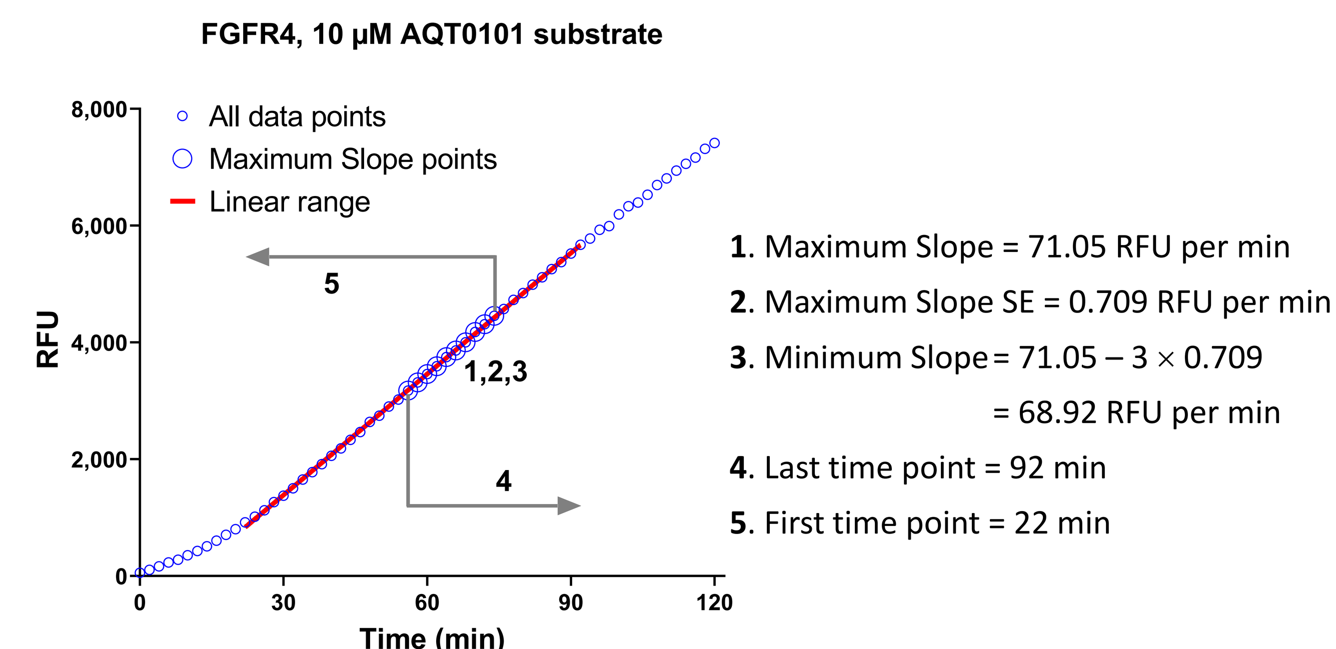


FIGURE 5. EXAMPLES OF AUTOMATED LINEAR RANGE FINDING APPLIED TO KINOME PROFILING

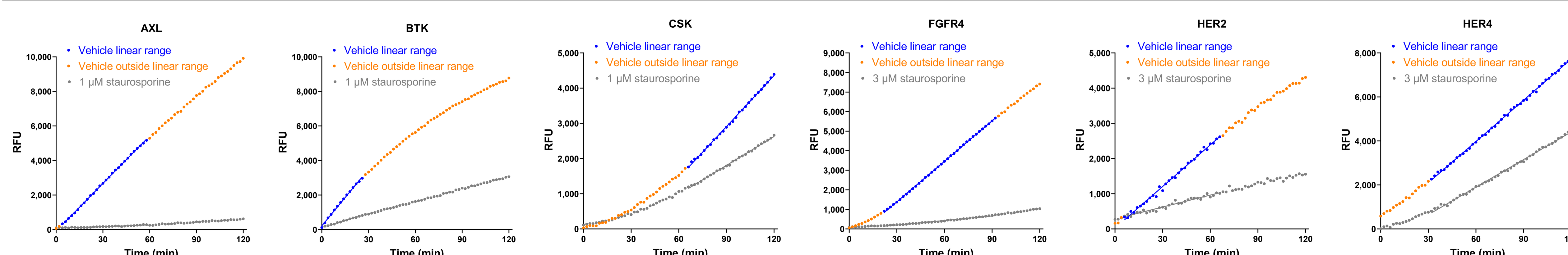
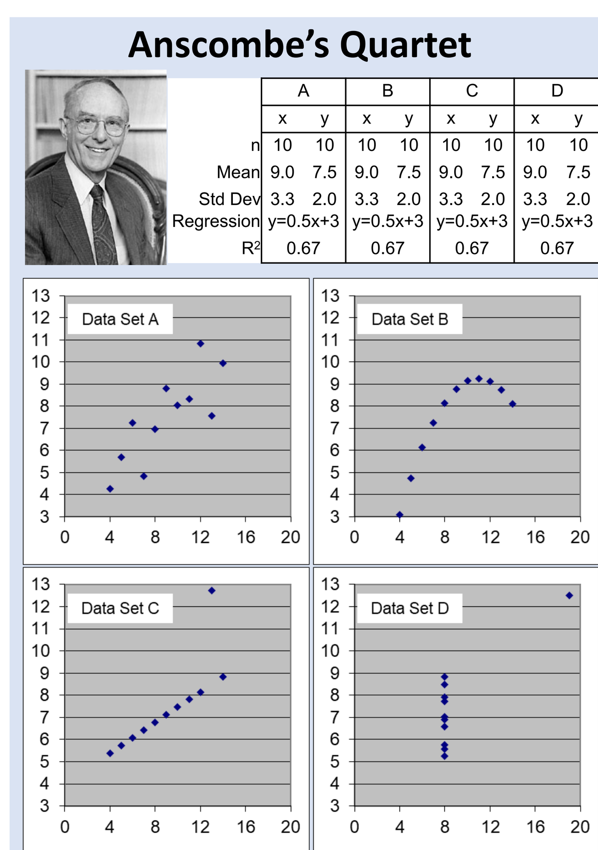


FIGURE 6. BEYOND ENDPOINTS: WHAT IS BEHIND THE RAW DATA?

Anscombe's Quartet

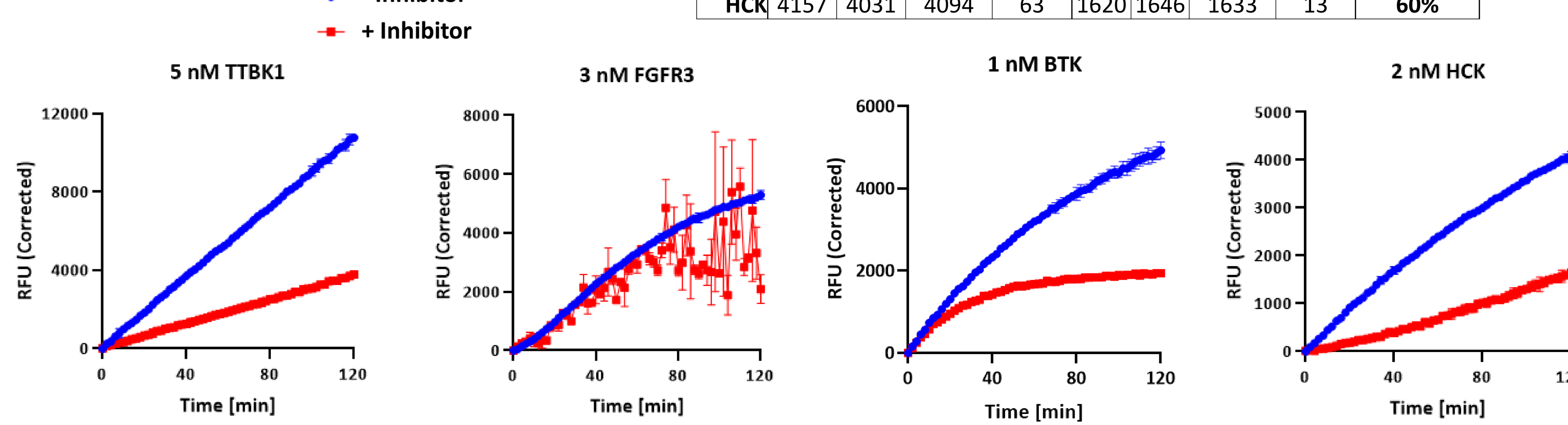


In 1973, Francis Anscombe developed a useful teaching tool to stress the importance of examining raw data before drawing experimental conclusions. Four data sets, with ten pairs of x,y data in each, have identical averages, linear regressions, and confidence measures. However, looking at the data itself it is clear there is something fundamentally different going on between these data sets.

%Inhibition data from single concentration endpoint readings. Like Anscombe's quartet, the effect of inhibitors for these four kinases appears to be the same in an endpoint assay: around 60-65% Inhibition. However, if we look at the progress curve that leads up to this endpoint, additional rich observations become available.

AssayQuant's Quartet

	-Inhibitor				+Inhibitor				
	RepA	RepB	average	avedev	RepA	RepB	average	avedev	%Inhibition
TTBK1	10683	10861	10772	89	3717	3850	3784	67	65%
FGFR3	5399	5183	5291	108	2434	1743	2089	346	61%
BTK	5071	4789	4930	141	1923	1956	1940	17	61%
HCK	4157	4031	4094	63	1620	1646	1633	13	60%



This inhibitor's effect on TTBK1 activity is an example of the underlying assumption for endpoint reads: the final read is a good approximation of the initial rate. Of course, with sixty data points for each replicate as compared to only one endpoint, there is much more confidence in the rates from the continuous format

Artifacts in the experiment are far more easily detected with a continuous assay. The noise in the assay (likely due to compound insolubility) might have been predicted from the slightly higher average deviation in the endpoint readings. But the noise is far clearer in the continuous format

Saturating progress curves might suggest substrate depletion, enzyme instability, product inhibition, but most importantly, it might also suggest the time-dependent inhibition (TDI) associated with irreversible or slow off-rate inhibitors. Detection of this MOA is not possible with a single endpoint assay

The introduction or extension of an activity lag before the establishment of an initial rate might be an indication of another interesting MOA, e.g., the ability of the inhibitor to bind the active vs. inactive conformation of a kinase, or it might be an indication of the ability to inhibit specific oligomeric forms of the kinase

SUMMARY: KINOME PROFILING WITH A CONTINUOUS ASSAY FORMAT

- **Fully automated linear range finding substantially increases the speed, reliability, and reproducibility of initial rate determination for enzyme kinetic studies, enabling high throughput screening in a kinetic enzyme assay format**
- **The algorithm utilizes basic arithmetic and linear regression statistics and so can be implemented easily and quickly in industrial data analysis packages, such as Scigilian.**
- **Initial rate determinations from continuous assay progress curves are based upon dozens of points, yielding much more confidence in the measure. In contrast, endpoint assays yield just that single read, and therefore much more uncertainty**
- **Visualization of the entire progress curve allows a continuous assay to identify aspects of an inhibitor's mode of action that are invisible in an endpoint assay, such as:**
  - **An activity lag before the initiation of reaction**
  - **Substrate depletion**
  - **Enzyme instability**
  - **Product inhibition**
  - **Time-dependent inhibition indicative of irreversible or slow off-rate inhibitors**
  - **Compound-dependent lag extension**