Optimization of Enzyme Reactions for Screening and Profiling Wild Type EZH2 with the Transcreener EPIGEN Methyltransferase Assay

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Abstract

Post translational modification of histone proteins by Histone Methyltransferases play an important role in gene regulation. Misregulation of these enzymes have been implicated in initiation and progression of several diseases, including cancer. EZH2 is the catalytic component of the polycomb group repressive Complex (PRC2), that selectively methylates histone H3 lysine 27 (H3K27). Numerous studies have shown that EZH2 is overexpressed in prostate, breast, bladder, brain, and other tumor types and is recognized as a molecular marker for cancer progression and aggressiveness. This poster describes the development, optimization and validation of Transcreener EPIGEN Methyltransferase assay for EZH2. The study was conducted using wild type EZH2 and the assay was developed using peptide substrates as well as the full length histone protein. This poster describes each step that was performed to develop an assay: Antibody optimization, Substrate optimization, Determining Optimal Enzyme concentration leading up to a HTS assay for lead identification. Finally a dose response curve was performed for two key EZH2 inhibitors- GSK 343 and UNC 1999 using the PRC2 complex with Transcreener EPIGEN Methyltransferase assay. To aid in drug discovery Bellbrook Labs developed an EZH2 Transzyme Methyltransferase Assay kit which contains enzyme, substrate and the Epigen Methyltransferase kit.

Universal, HTS-proven Methyltransferase Detection in a Mix-and Read Format

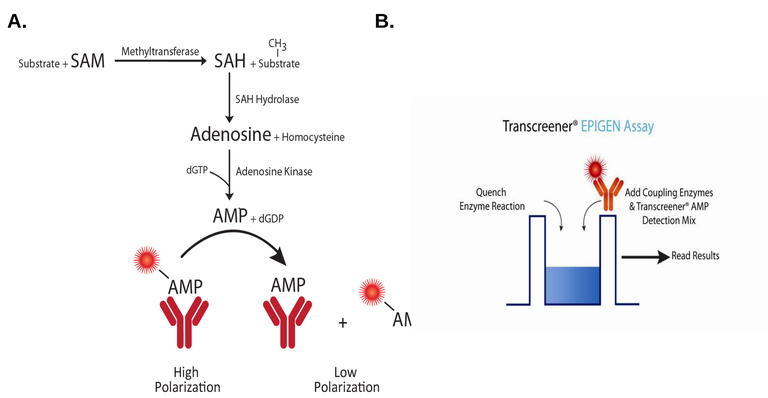


Figure 1. The Transcreener EPIGEN Methyltransferase Assay. A. The SAH produced in a methyltransferase reaction is converted to AMP in two enzymatic steps. The AMP is detected using the extensively validated Transcreener® AMP/GMP Assay, a competitive immunoassay, with a far red fluorescence polarization readout. B. The assays are a mix-and-read, homogenous format and can be run in stop time or continuous mode in either 384 or 1536 well plates.

Precalibration of Detection Reagents for HTS Quality Assay Windows

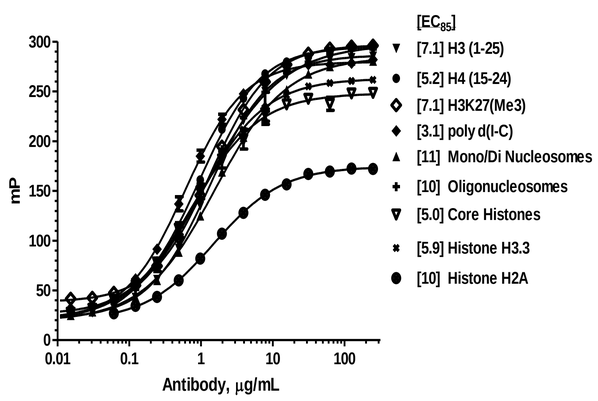
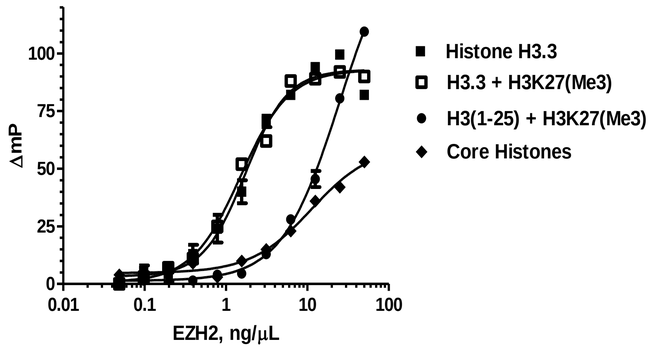


Figure 2. Optimization of AMP^2 antibody concentration for use with different methyltransferase acceptor substrates. Tracer (4 nM) was titrated with AMP antibody in the presence of 2 µM SAM and the indicated substrate at its optimal concentration. The antibody concentration resulting in 85% saturation [EC_{85}] will yield a good assay window (> 100mP) for detection of initial velocity levels of SAH production. This information is provided with each of the Transzyme kits.

Selection of Optimal Acceptor Substrate



	Histone H3.3	Histone H3.3 + H3K27(Me3)	H3(1-25) + H3K27(Me3)	Core Histones
EC_{80}	3.9	3.9	75.9	43.9

Figure 3. Substrate Selection for EZH2: Enzymes were titrated in 15 µL reactions in the presence of 2 µM SAM and substrate at appropriate concentrations. Reactions were performed in a kinetic format to monitor reaction progress over time (3 hour time point shown). Full length Histone H3.3 gave the most robust assay window compared to H3 peptides or core histones. Addition of tri-methylated H3K37(Me3) peptide to Histone H3.3 did not enhance the assay window. The EC_{80}, which reflects 10-15% SAM conversion, gives an estimation of how much EZH2 enzyme is required for a robust assay.

Determination of Enzyme Concentration for End Point Assay

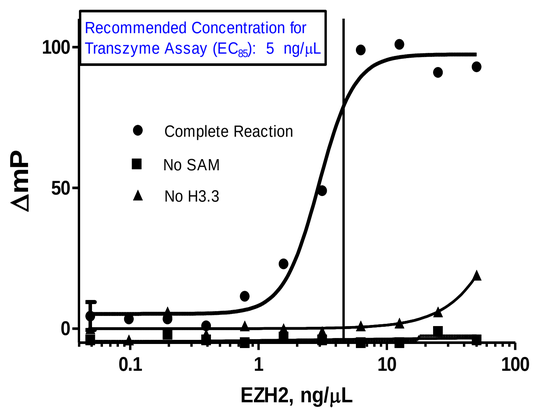


Figure 4. Determination of EZH2 concentration for End Point Assay: EZH2 was titrated in 15 µL reactions in the presence of 2 µM SAM and 3 ng/µL Histone H3.3 for 3 hours at 30°C and then quenched with stop reagent followed by addition of detection reagents. The EC_{85} concentration was determined to be ~ 5 ng/µL EZH2.

Linearity and Assay Robustness

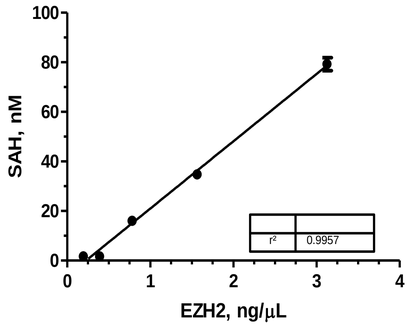


Figure 5. Tranzyme assay kits follow Michaelis Menten parameters.

The linear correlation between the enzyme concentration and product formation demonstrates initial velocity conditions and adherence to Michaelis-Menten parameters.

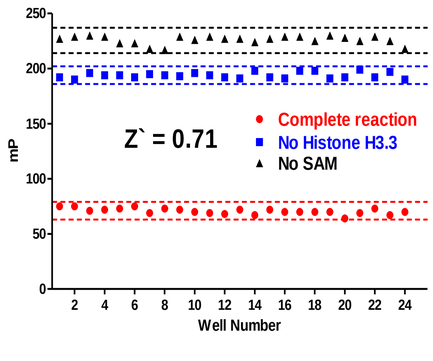
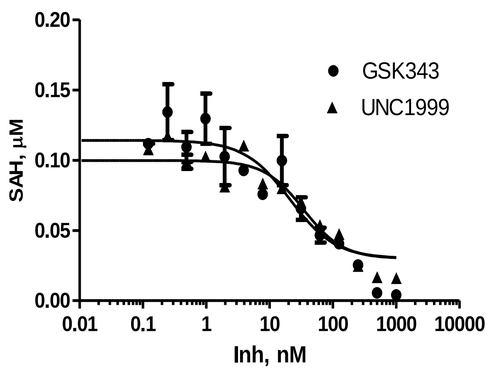


Figure 6. Z`-factor Determination for EZH2:

The EZH2 Transzyme kit was used to determine Z' (n = 24) in a 384 well format. 5 ng/µL of EZH2 and 3 ng/µL of histone H3.3 were stopped after 3 hours at 30ºC. A Z' of 0.71 was achieved.

Dose Response Curves



	GSK343	UNC1999
$IC_{50\ (nM)}$	21.04	34.53

Figure 7. Dose Response Curves with EZH2 Specific Probes. Inhibitors GSK343 and UNC1999 were serially diluted and pre-incubated with 5 ng/µL f EZH2 followed by addition of 3 ng/µL Histone H3.3. After 3 hours stop mix, and detection reagents were added. Signal was then converted to product formation and IC_{50} were determined.

TRANSZYME METHYLTRANSFERASE ASSAY KITS

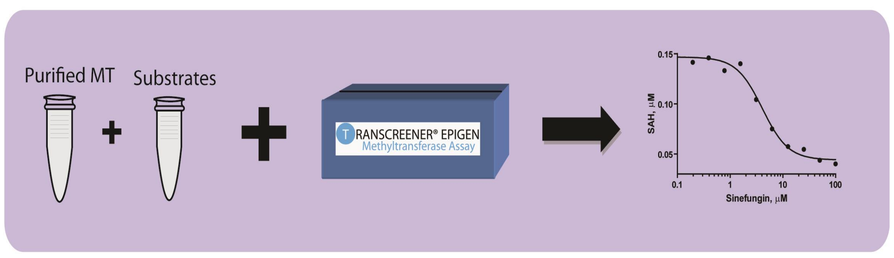


Figure 8. The EZH2 Transzyme Methyltransferase Assay kit combines Transcreener® EPIGEN Methyltransferase Assay detection reagents with purified and validated EZH2 from Reaction Biology, and the Histone H3-3 substrate. These kits are designed to allow investigators to begin screening or running dose response experiments without any assay development or pilot experiments. Enzyme and detection reagents are pre-calibrated to produce outstanding assay windows under initial velocity conditions, insuring accurate kinetics and inhibitor potency measurements.

Conclusions

- **A Transzyme assay kit for EZH2 was developed combining purified enzyme from Reaction Biology, substrate and Transcreener EPIGEN Methyltrasnferase detection reagents.**
- **Optimal enzyme and substrate concentrations were determined to be: 5 ng/µL EZH2 and 3 ng/µL Histone H3.3 in a 20 µL reaction for 3 hours at 30˚C**
- **These pre-calibrated conditions gave an outstanding *Z' of 0.71* under initial velocity conditions.**
- **The Transzyme EZH2 kit enables determination of inhibitors potency in a turn key format.**
- **HTS-Ready: Transcreener® HTS assay technology has been validated in over 50 million wells of screening.**

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