Assay development for epigenetic regulation of histone 3 lysine 36 methylation using AlphaLISA

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Abstract

In the last several years the field of histone methylation biology has grown significantly. However, the development of new cellular assays for methylating and demethylating enzymes has lagged behind, relying largely on reporter or high content assays. Here we describe the development of a homogeneous AlphaLISA cell based assay measuring modulation of the histone H3 Lys36 dimethyl mark by lysine-modifying enzymes. In order to detect global modulation of the methyl mark using a non-HCS assay, a nearly homogeneous population of enzyme expressing cells is needed. To circumvent the need for stable cell lines, we utilized the Maxcyte STX system for cellular electroporation, which enabled cDNA transfection efficiencies greater than 90%. In addition, this technology allowed us to control the expression level by adding different concentrations of the expression plasmid. We have confirmed the ability of the enzyme to modulate histone methyl mark levels on H3K36 in a concentration dependent manner, using mass spectrometry, western blot, and the H3K36me2 AlphaLISA assay. Finally, to account for cell viability effects caused by compound treatment, we investigated the use of a Total Histone AlphaLISA and Cell Titer Glow. In summary, a H3K36Me2 AlphaLISA was successfully developed and miniaturized to 1536-format, with multiplexed control of cell viability.

Objectives

Identify a high efficiency and scalable transfection method.

Develop a generic non-HCS HTS-compatible cell based assay for detection of methyl mark modulation.

Identify a normalization method to account for cell loss after compound treatment.

Materials and Methods

Maxcyte

Collect cells and resuspend in MaxCyte EP buffer & pellet.
Resuspend cells in EP buffer at 1x10^8 cells/mL and add DNA at 250ug/ml.
Combine cells and DNA in a OC-100 processing assembly (PA).
Process cells using Maxcyte U2OS cell protocol.
Transfer processed cells to a single well of 96 well plate.
Incubate plate @ 37°C +5%CO_2 for 20 min.
Transfer cells to warm medium.

AlphaLISA – 1536 Format

Add 1uL of Cell-Lysis buffer to the cell layer (3uL) for 15 minutes.
Add 2uL of Cell-Extraction buffer for 10 minutes.
Add 2uL of the AlphaLISA Acceptor beads (H3K36Me2; 20ug/ml final) and Biotin-anti H3 (C-term; 3nM final) Ab mixture to all wells and incubate for 60 minutes.
Add 2uL of the AlphaLISA Donor Beads (20ug/mL final) to all wells and incubate for 30 minutes.
Read on an Envision®.

Western Blot

Load 5ug per lane of total protein on 10% SDS PAGE.
Transfer samples to PVDF membrane and block with 5% dry-milk.
Incubate overnight with primary antibody at 4°C.
-Cell Signaling, Inc. - Rabbit Di-Methyl-Histone H3 (Lys36) #2901.
Incubate for 60 minutes with secondary antibody.
-Sigma Aldrich - Mouse monoclonal Anti-β-Actin antibody - clone AC-15 #A5441.
Develop on film using SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific #34087.

Assay Principle

Maxcyte STX ™

Figure 1: MaxCyte STX™ Transfection System technology is based on electroporation.

Applying an electrical field to cells causes an increase in the permeability of the cell plasma membrane. DNA, RNA, siRNA or other biomolecules can enter the cells.

The MaxCyte system employs a continuous flow design and process volumes from 50uL up to 100mL.

AlphaLISA

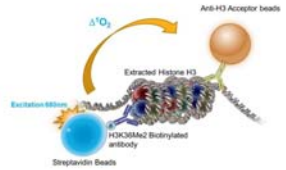

Figure 2: AlphaLISA: Assay principle of a cellular assay for the detection of dimethyl histone 3 lysine 36.
Following a homogeneous histone extraction protocol. AlphaLISA Acceptor beads (conjugated with H3K36Me2 antibody) and a biotinylated anti-Histone H3 (C-terminus) antibody are incubated with the extracted histone lysate. The biotinylated antibody is then captured by Streptavidin (SA) Donor beads.

Results

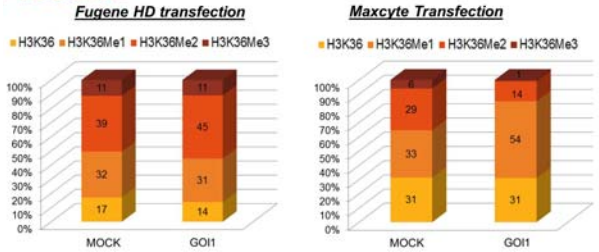

Figure 3. H3K36 Mass Spectrometry Analyses.
U2OS cells were transfected with either FugeneHD (6:2) or Maxcyte (250ug/ml). 5 x 10^6 cells were analyzed by LC-MS and H3K36 level were measured.

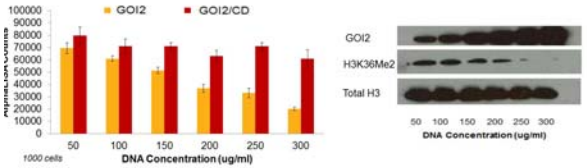

Figure 4. Maxcyte transfection optimization
U2OS cells were transfected in OC-100 processing assemblies with 6 different concentrations of plasmid encoding our GOI2. Transfected cells were incubated for 24 hours before plating. 48 hours post-transfection cells were either processed for AlphaLISA or Western Blot.

Results (cont.)

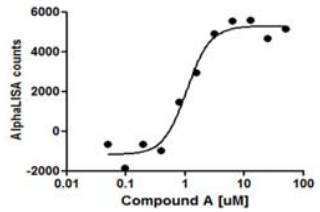

Figure 5. Pilot screen using AlphaLISA; compounds increasing H3K36Me2 AlphaLISA signal in concentration dependent manner were found. Compound A shown as an example.
U2OS cells were Maxcyte transfected with GOI2 for 16 hours before cell plating in 1536. The assay plate was then incubated at 37°C +5%CO_2 for 8 hours prior compound addition. The assay was then incubated for an additional 24 hours before proceeding with AlphaLISA detection.

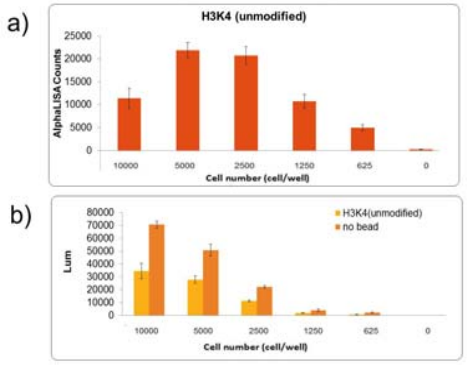

Figure 6. Normalization for cell number
a) Total H3 AlphaLISA was used on a duplicate plate to normalize the cell number.
b) Cell Titer Glow (CTG) was added to each well after the final AlphaLISA read. CTG was tested in presence and in absence of AlphaLISA beads.

Conclusion

The Maxcyte STX system allows for high efficiency transfection and can be scale from small scale to HTS scale.

We have developed a cell based AlphaLISA for the detection of dimethylation of histone H3 at lysine 36. The assay was successfully miniaturized to 1536 format.

We were able to use AlphaLISA Total H3 or Cell Titer Glow in order to normalize the assay and to account for cell number after compound treatment.