EPIGENETICS
an emerging target class for drug screening

By Dr John Comley

Heightened awareness of the potential importance of epigenetic targets in many disease areas, and growing vendor interest in developing new assays to screen DNA methylation and histone modifications encouraged HTStec to undertake market research in this area in June 2010. The study concluded epigenetic screening was still very much in its infancy and there was a need for new and improved screening tools and assays. Assay specificity, producing active protein and the availability of good antibodies were all cited as key obstacles. Much attention is coming from oncology areas and interest is particularly high in the assaying histone deacetylases (HDAC & Sirtuins), histone methyltransferases (HKMT & HRMT), histone demethylases (HDM), DNA methyltransferases (DNMT) and histone acetyl transferases (HAT). Epigenetic target assays are increasingly being attempted utilising a wide range of different screening technologies. Some of the more generic approaches currently being developed and validated are suitable for all types of histone substrates, and have application across a diverse range of histone modifications. The breadth of assays becoming available will soon extend well beyond the HDAC & Sirtuins. These approaches now need to be translated into a set of robust ready-to-use assay kits or tool-box reagents to open up the epigenetic field to HTS. An increasing number of vendors also now offer fee-for-service screening and profiling against epigenetic targets, such that outsourced compound testing against a panel of epigenetic assays is becoming a real possibility. In conclusion, the tools required to support epigenetic screening are fast emerging as our knowledge and experience with these targets increases, such that we can expect to see greater adoption or external use of these assays by Pharma and Biotech lead discovery programmes over the coming years.

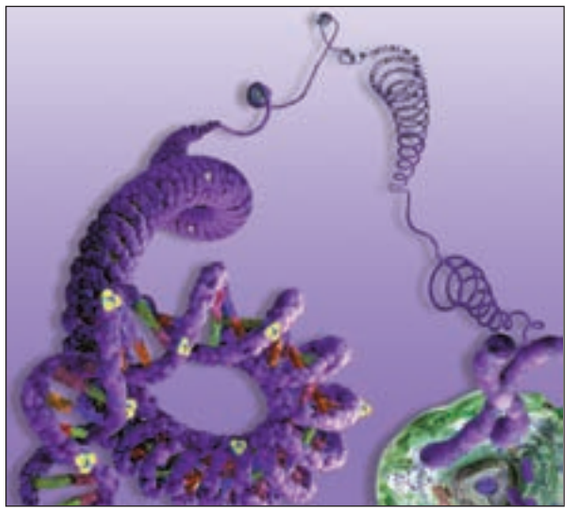


Image supplied by Active Motif

Figure 1: This image depicts the wrapping of DNA around histone octamers (greenish/blue spheres) to form nucleosomes, the organisation of nucleosomes into higher order chromatin structure and the compaction of chromatin into chromosomes (a metaphase chromosome in this case). The blue and yellow balls represent DNA methylation, ie methyl groups added to cytosine residues which serves as an epigenetic mark regulating many genome-dependant processes

Epigenetic mechanisms are inheritable factors that regulate genetic expression without changing the DNA sequence[1]. These factors are observable as DNA methylation, histone modification and small regulatory RNAs. In recent years the field of epigenetics has grown into one of the most dynamic areas of biological research, and is increasingly the focus of drug screening activities. Histones, the protein components of chromatin around which DNA can wind for compaction and gene regulation, play a key role in epigenetics. Histone modification occurs when the binding of epigenetic factors to histone 'tails' alters the extent to which DNA is wrapped around histones and the availability of genes in the DNA to be activated. Some of the epigenetic factors that bind to histones include methyl, acetyl, phosphate groups and ubiquitin and sumo proteins. All of these factors and processes can have an effect on health, possibly resulting in cancer, autoimmune disease, mental disorders, or diabetes among other illnesses.

The epigenetics research field has been mainly dominated by DNA methylation studies after bisulfite treatment (this provides complete cytosine to uracil conversion in DNA for rapid and precise methylation detection by a host of downstream procedures) and chromatin immunoprecipitation (so-called ChIP, a method used to determine the location of DNA binding sites on the genome for a particular protein of interest). However, realisation that the key epigenetic enzymes classes/proteins themselves represent important therapeutic targets has prompted the development of a range of biochemical enzyme assays that are directly amenable to screening and the investigation of cellular epigenetic modification assays.

Alerted by a recent SBS session devoted to epigenetics[2] and in response to growing vendor interest in this area, HTStec undertook a survey in June 2010 to document current practices and preferences in epigenetic enzyme screening assays, and to understand future user requirements[3]. In this article we discuss some of the survey findings and review details of how vendors are starting to address the needs of the screening community.

Current perception of epigenetic screening

The current perception of epigenetic screening by the majority (52%) of survey respondents was 'one of today's most exciting biological target areas, somewhat comparable to kinase situation, just time is needed to develop', this was followed by 'it

Figure 2: Current perception of epigenetic screening

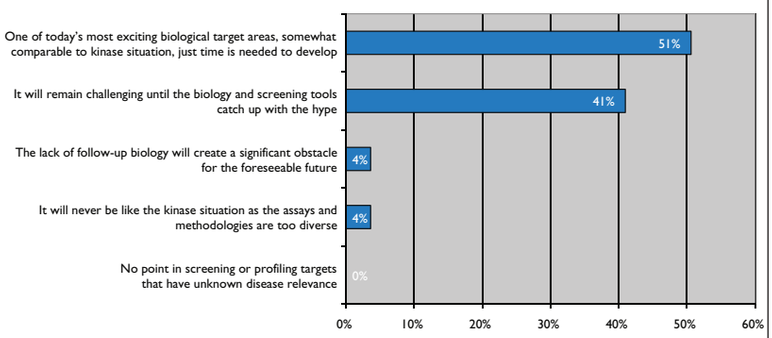


Figure 3: Therapeutic/disease areas targeting/using epigenetic enzyme assays

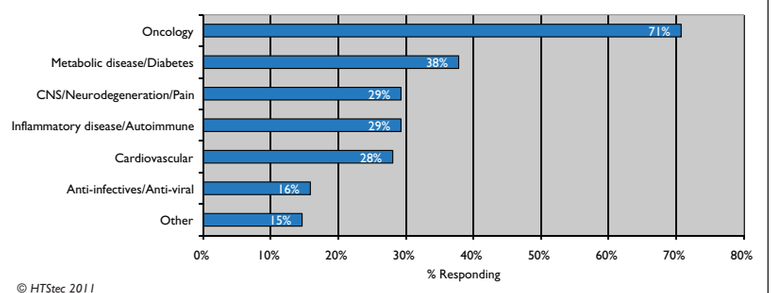


Figure 4: Key epigenetic enzyme classes/proteins of greatest interest

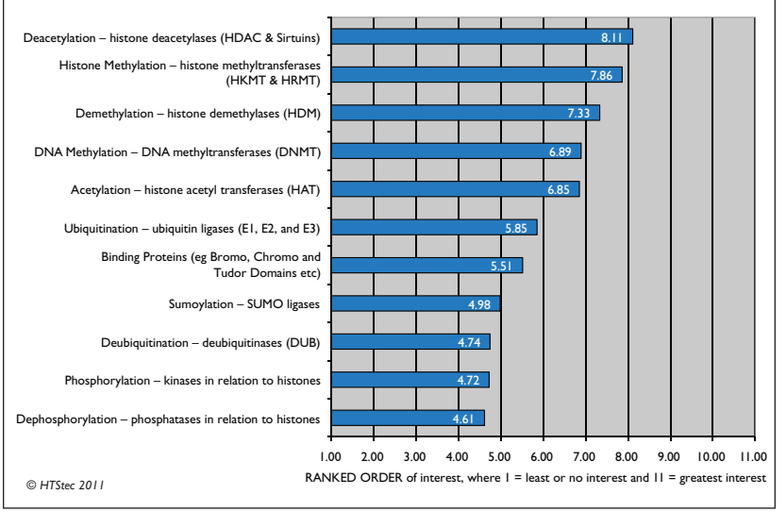


Figure 5: Assay technologies that have proven useful in epigenetic enzyme assays

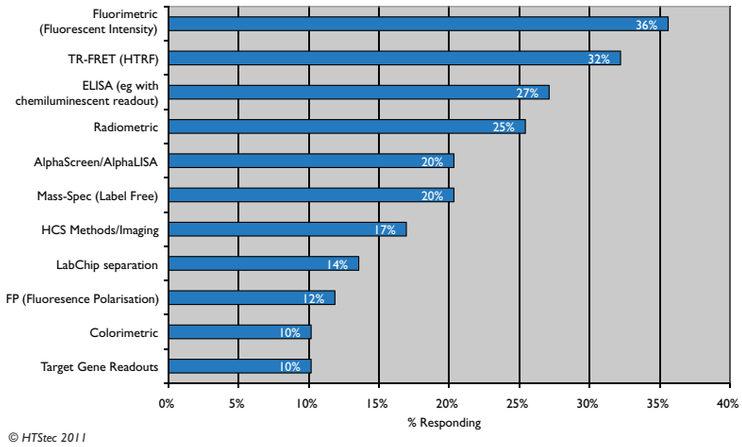


Figure 6: Substrates most commonly used for epigenetic enzyme assays

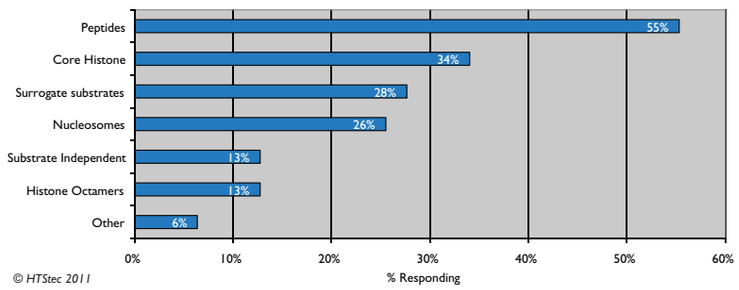
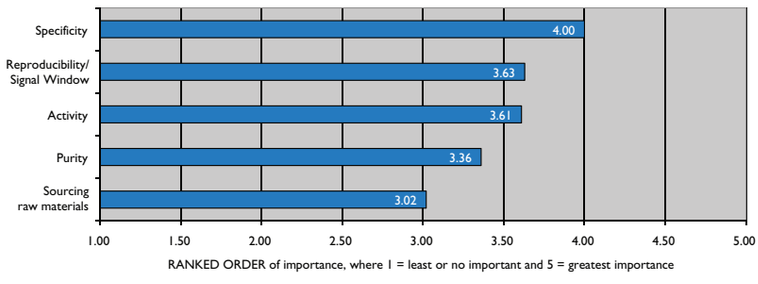


Figure 7: Most important challenges in epigenetic enzyme assay development for HTS



will remain challenging until the biology and screening tools catch up with the hype' with 41% responding. Only 8% of respondents had more negative perceptions about epigenetic screening (**Figure 2**).

Therapeutic areas targeting epigenetic enzyme assays

The majority (72%) of respondents were targeting, using or planning to use epigenetic assays within the oncology therapeutic area. Second greatest use/planned use of epigenetic assays was within the metabolic disease/diabetes therapeutic area (38% targeting). This was followed by CNS/neurodegeneration/pain therapeutic area (30% targeting), inflammatory disease/autoimmune therapeutic area (29% targeting) and then cardiovascular therapeutic area (28% targeting) (**Figure 3**).

Epigenetic enzyme classes/proteins of greatest interest

The epigenetic enzyme classes/proteins ranked of greatest interest to respondents were deacetylation – histone deacetylases (HDAC & Sirtuins). This was followed closely by histone methylation – histone methyltransferases (HKMT & HRMT), and then demethylation – histone demethylases (HDM). Ranked of least interest was dephosphorylation – phosphatases in relation to histones (**Figure 4**).

Assay formats proven most useful for epigenetic screening

The assay formats/detection technologies that have proven most useful to date in epigenetic enzyme assays by respondents were fluorimetric (fluorescent intensity), followed by TR-FRET (HTRF), ELISA and then radiometric. Least useful to date were target gene readouts and colorimetric assays (**Figure 5**).

Epigenetic enzymes assay substrates

The identification and selection of appropriate substrates for epigenetic enzyme assays is subject to varying opinion and investigation. The survey showed respondents most commonly used peptides as substrates for their epigenetic enzyme assays (55% using). This was followed by core histone (34% using); surrogate substrates (28% using) and then nucleosomes (26% using) (**Figure 6**).

Main challenges to epigenetic enzyme assay development

A lot of effort is currently going into development of new epigenetic assays. Specificity was ranked as

the most important challenge in assay development of epigenetic enzyme targets suitable for HTS applications. This was followed by reproducibility/signal window and activity, then purity. Sourcing raw materials was ranked the least important challenge (**Figure 7**).

Main obstacles in exploiting epigenetic targets

The availability of good antibodies was ranked as the most limiting (major obstacle) in the exploitation of epigenetic enzyme targets today. It was closely followed by lack of known specific inhibitors; production of active protein is difficult; and then physiological substrates not yet identified. Least limiting was no specific compound libraries are available (**Figure 8**).

Epigenetic cellular modification assays

It would however be wrong to assume that all screening efforts are focused on biochemical enzyme epigenetic assays today, when in fact respondents indicated that they are spending around 50% of their effort investigating epigenetic cellular modification assays. Of these the most investigated today were methylation (68% investigating), followed by acetylation (39% investigating), and then phosphorylation (24% investigating). Least investigated were sumoylation (only 11% investigating) (**Figure 9**).

Latest developments in epigenetic drug screening

The following vendor snapshots provide additional details and describe some of the latest developments in assays, kits, associated reagents and tools used for the screening and investigation of epigenetic target enzymes and proteins:

Active Motif (www.activemotif.com) develops innovative tools and reagents that help researchers investigate nuclear function with particular emphasis on chromatin dynamics and elucidating the mechanism and regulation of epigenetic events. In addition to offering validated antibodies and assays to enable the discovery and characterisation of key epigenetic processes, such as histone modification and DNA methylation Active Motif now also provides complete service solutions for the genome-wide analysis of epigenetic events, including DNA methylation, histone modification and transcriptional regulation. Active Motif offers antibody-based tools for a wide range of histone modifications as well as assays against histone modifying enzymes such as

Figure 8: Major limitations of screening epigenetic enzyme targets

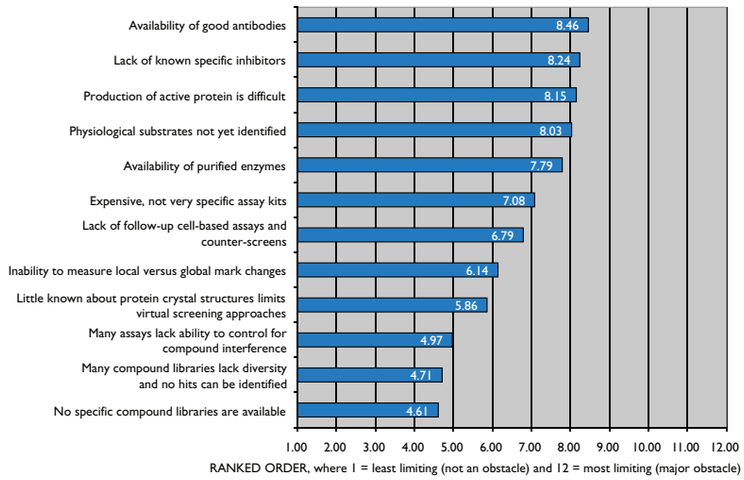
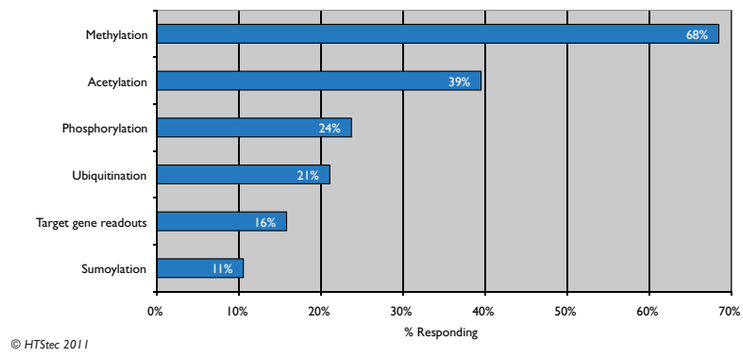


Figure 9: Most investigated epigenetic cellular modification assays



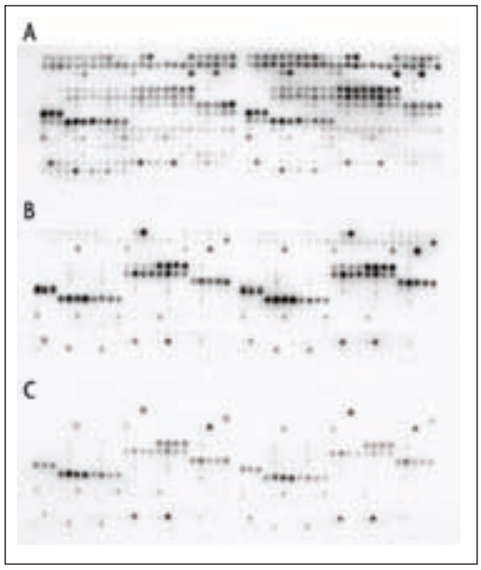


Figure 10
ECL detection of Active Motif's MODified Histone Peptide Arrays treated with HMT G9a. MODified histone peptide Arrays were treated with: A) 25 M G9a methyltransferase; B) 25 M G9a mutant H904K; C) no enzyme control, overnight in the presence of 1 mM AdoMet. The arrays were detected using a Histone H3 dimethyl Lys9 antibody. Novel methylation sites were observed on array A, which was treated with wild-type G9a histone methyltransferase, showing the activity of the histone modifying enzyme on the peptide substrate

HAT, HDAC and Histone demethylase. It provides a wide selection of antibodies and recombinant proteins to allow researchers to develop their own screening assays. Using a proprietary technique, Active Motif has generated full length recombinant histones to study site- and degree-specific methylation as well as acetylation modifications. Its offering of recombinant proteins also includes a variety of histone modifying enzymes. Since it is known that some histone modifying enzymes prefer nucleosomes as a substrate over purified histones Active Motif now offers hela mononucleosomes for use in screening assays. Many important regulators of genome function contain protein motifs that interact with specific histone modifications. To facilitate the study of these proteins, it offers its MODified™ histone peptide array for analysis of up to four different modification combinations within the same peptide sequence. The peptide arrays are also useful tools for validating the specificity of Active Motif's expanding line-up of antibodies to histone modifications (**Figure 10**).

The **Biocius** (www.biocius.com) RapidFire label-free mass spectrometry platform enables high throughput analysis of a wide range of epigenetic target enzymes. The system is uniquely capable of detecting multiple modification events across a single, native substrate; a challenging endeavour with other technologies using optically or radioactively labelled surrogate substrates. For example, lysine can accommodate up to three methyl groups, thus a variety of methylated states are possible depending on the activity of methylases and demethylases acting upon the protein. RapidFire mass spectrometry can detect and distinguish any of the methylation patterns: unmethylated, single, di- or tri-methylated. The RapidFire platform has been used to assess the activity of a variety of epigenetic enzymes, including protein acetylases/deacetylases, protein methylases/demethylases and DNA methylases/demethylases. Recent RapidFire data even demonstrates the simultaneous monitoring of SIRT-1 mediated deacetylation at several sites in the p53 gene sequence. Additionally, RapidFire technology demonstrates much faster analysis

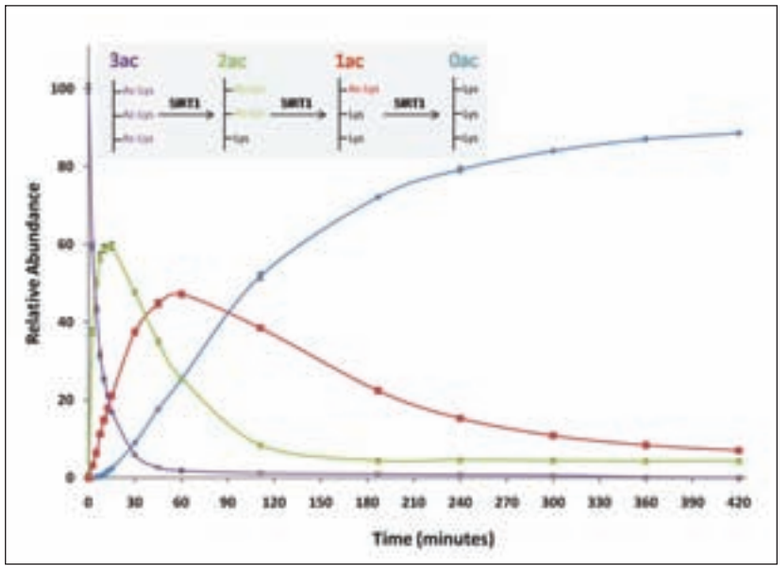


Figure 11: Sequential deacetylation of a triply-acetylated p53 peptide by SIRT1 using Biocius RapidFire

Figure 12: The Biocius RapidFire® RF300 Mass Spectrometry System processes epigenetic samples in 6-8 seconds each

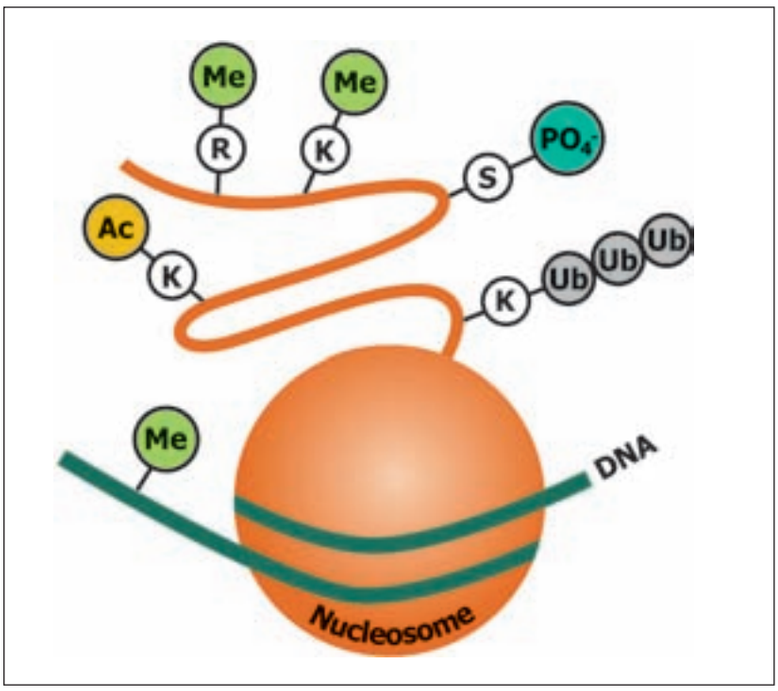


Figure 13: Post-translational modifications of histone protein that can influence gene transcription. BioFocus supports drug discovery for various epigenetic target families including histone methyltransferases, histone deacetylases, histone demethylases, ubiquitin E3 ligases and deubiquitnases. Abbreviations: Me, methyl; Ac, acetyl; Ub, ubiquitin; PO4-, phosphate

times than other traditional label-free technologies (6-10 second cycles), greatly increasing the efficiency of screening programmes for epigenetic and other enzyme targets (**Figures 11** and **12**).

A range of target assays are available at **BioFocus** (www.biofocus.com) with a focus on histone methyltransferases (HMTs) and HDACs, with histone demethylases, ubiquitin E3 ligases and deubiquitnases also available. For HMT hit finding campaigns, a subset of its 900,000 diverse compound library is screened typically with a radiometric assay format. Using this approach BioFocus has recently identified a number of novel chemotypes which were progressed to optimisation. For further characterisation of hits, orthogonal assay technologies, such as Caliper lab-on-a-chip, fluorescence lifetime technology and binding assays have successfully been used. BioFocus has successfully employed protein family structural superposition followed by sequence alignment to generate chemogenomic models that have been developed into protein family 'roadmaps'. Key residues are identified, aiding the understanding of pocket-based family homology and selectivity. Using the information of such models enables the understanding of protein targets for which structural information is not available. BioFocus uses this ensemble of information to design new target-family focused libraries, to prioritise compounds for specific assays (eg hit expansion) and/or to predict potential selectivity targets. It has used this approach to analyse the histone methyl transferase (HMT) sub-family, protein lysine methyltransferases (PKMTs). Structural superposition reveals high conservation in the primary and secondary structure around the S-adenosyl methionine (SAM) pocket. This observation can help prioritise compounds for screening; for example, adenosine is present in both ATP and SAM, therefore screening minimally functionalised compounds designed for the ATP site in kinases may provide a useful component of a screening deck for hit identification against this novel protein class (**Figure 13**).

BPS Bioscience (www.bpsbioscience.com) manufactures a broad portfolio of sensitive, specific epigenetic assay kits designed to determine enzyme activity or for inhibitor screening. Its portfolio includes both chemiluminescent 96- or 384-well plate assays and homogeneous AlphELISA/AlphaScreen assays. Many epigenetic enzymes are present in cells as complexes of multiple regulatory subunits, so they can be difficult to express as functional enzymes. For example, its EZH2 complex is

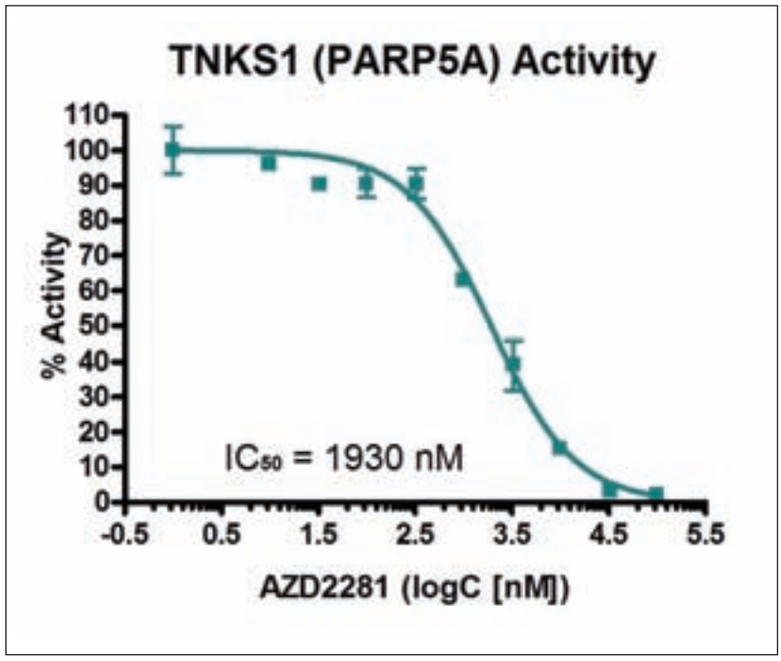


Figure 14: IC50 assay of PARP5A (TNKS1) enzyme activity, using PARP inhibitor AZD2281, measured using BPS Bioscience's Tankyrase-1 Chemiluminescent Assay Kit, #80565. Luminescence was detected using a Bio-Tek fluorescent microplate reader. IC50 = 1.93μM

co-expressed as a complex of five different proteins (EZH2, EED, SUZ12, RbAp48 and AEBP2) to maximise activity. Similarly, its DNA methyltransferase (DNMT) 3A and 3B enzymes are co-expressed with the regulatory factor DNMT3L, resulting in a functional complex similar to the native conformation of these proteins. These and other enzymes are the basis of a sequential panel of unique assays that allow researchers to assess the total epigenomic state of a cell, as well as study individual pathways. These kits estimate levels of cellular DNA methylation and histone methylation/demethylation by measuring DNA methyltransferase, histone methyltransferase (HMT) and

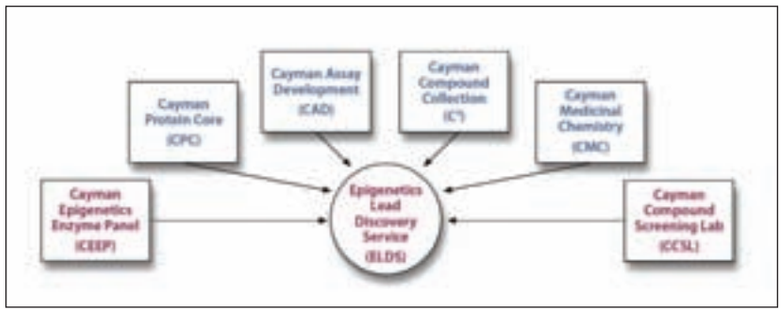
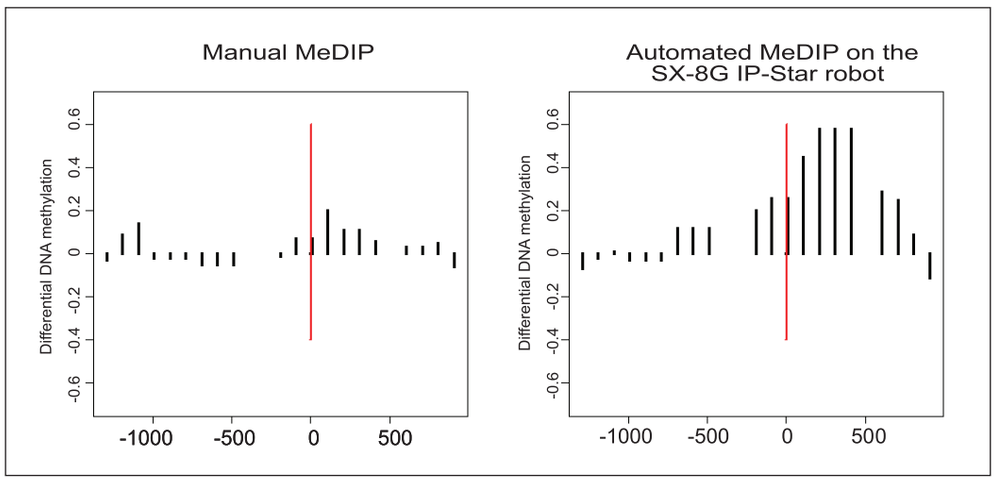


Figure 15: Cayman Epigenetics Lead Discovery (CELD) services will continually expand capabilities to other core areas of Cayman expertise

histone demethylase (HDM) activity. The kits focus on several key epigenetic targets and include four different DNMT kits, 16 HMT kits, six HDM kits, three HDAC kits, six poly [ADP Ribose] polymerase (PARP) kits (including Tankyrase 1 and 2), as well as a HAT (histone acetyltransferase) assay. PARP is involved in chromatin remodelling and control of DNA methylation, and PARP and its isozymes are products unique to BPS. In addition to providing assay kits and enzymes, BPS also offers screening and profiling services to determine the IC50 of test compounds or to screen an inhibitor against a panel of active enzymes. BPS's enzyme profiling panel currently includes all 11 HDACs, four SIRTs, six PARPs, 15 HMTs, three DNMTs and four HDMs (**Figure 14**).

Cayman Chemical (www.caymanchem.com) now offers an Epigenetics Lead Discovery Service, a collaborative programme for identifying novel epigenetic therapeutics by screening against a panel of targets. This Service places three Cayman strengths to your advantage. First, a Protein Core Team produces pure and functional recombinant proteins. Over 25 different proteins, including human HDACs, SIRTs, KMTs, KDMs and histones, are currently available. Second, an Assay Development Group creates versatile, dependable and affordable assay kits for each enzyme. To date, they have developed assay kits containing SIRT1, SIRT2, SIRT3, SIRT6, HDAC8, LSD1, SET7/9, SET8, JMJD2A and JMJD2D; these are designed to screen compounds that might alter activity. Other available assays measure overall DNA methylation, SAM-dependent methyltransferase activity, and JmjC- or LSD-type KDM activity. Finally, Cayman's outstanding chemists synthesise the latest inhibitors and modulators for epigenetics research. These products can be found on Cayman's website and purchased individually. However, independent researchers and companies alike have successfully worked with Cayman chemists for assistance in route design, hit-to-lead chemistry and scale-up synthesis for preclinical studies. Now, Cayman has established a dedicated screening lab to help meet epigenetics research and development needs (**Figure 15**).

Chromatin modulations play a central role in shaping the epigenome and many of the enzymes that mediate the covalent modification of the DNA and the protein components of the chromatin are deregulated in human diseases. Antibody or small molecule-based interventions modulating the activity of the DNA/chromatin-modifying enzymes



(Data courtesy of Dr Jorg Tost, Laboratory for Epigenetics. CEA Institut de Genomique, Evry, France)

Figure 16
Genome-wide differential DNA methylation between wildtype MEFs and MEFs mutated for a protein associated with the MLL complex. The Methylated DNA immunoprecipitation (MeDIP) was performed manually (left) or with the automated MeDIP protocol performed on the Diagenode IP-Star system and analysed on NimbleGEN promoter and CpG island tiling arrays. Data is centred around the transcription start site (x-axis, red line). Positive values on the y axis indicate hypermethylation in the mutated cell lines. Data shows an improved signal-to-noise ratio in the automated MeDIP preparation

have been shown to be able to revert malignant cells to a more normal epigenetic state and have now entered the clinics following their FDA approval. Despite the field being still in its infancy, epigenetic modulators are expected to be an attractive new class of chemotherapeutic agents. Consequently, there is currently great interest in the genome-wide identification and thorough analysis of the effects of novel modulators of the epigenetic machinery. To test their effects in cell line and in *in vivo* systems, reliable high-throughput technologies are required both for read-out as well as sample preparation. The advances in second generation sequencing technologies have enabled the analysis of large variety of epigenetic alterations at unprecedented resolution, sensitivity and speed. The use of dedicated robotics for sample preparation (eg SX-8G IP-Star Robot), **Diagenode** (www.diagenode.com) performing the liquid handling steps of the immunoprecipitation process and processing up to 16 samples in parallel, leads to a cost- and time-effective sample processing and a significant reduction of the inter-experimental variation inherent to experiments with multiple washing and incubation steps. The automated sample preparation of the IP-Star can be directly integrated into standard Next Generation Sequencing library preparation workflow using the enriched and/or immunoprecipitation fraction of the genome obtained from the robot without the need for additional experimental steps. The IP-Star can automate ChIP assays for both abundant histone modifications as well as site specific transcription factor ensuring high quality and more reproducible ChIP-seq results. For MeDIP-seq or MBD-seq, ligation of the respective sequencing adaptors prior to the immunoprecipitation on the IP-Star assesses the efficiency of the enrichment using standard qPCR analysis with sample independent control oligonucleotides added to the automated sample preparation process. The automation of the immunoprecipitation and/or affinity enrichment increases significantly the signal-to-noise ratio permitting a more rapid and reliable genome-wide identification of enrichment as assessed by high-resolution quantitative technologies, tiling microarrays and second generation sequencing (**Figure 16**).

EMD Millipore (www.millipore.com/epigenetics) offers a comprehensive range of products for the analysis of epigenetic regulation. EMD Millipore's offering of kits, assays, proteins, peptides, antibodies and inhibitors enables multiple approaches for laboratories, validating potential hits, identifying potential targets, or evaluating the effects of candidate compounds. For this purpose, EMD Millipore offers a variety of recombinant proteins, antibodies and inhibitors for laboratories creating customised assays. This is complemented by a variety of off-the-shelf kits for analysis of histone modifications, DNA methylation status and protein interactions utilising approaches such as ELISA, xMAP® and flow cytometry. Examples include HDAC Assay Kits that provide a simple, two-step procedure for colorimetric, fluorometric or radiometric detection of histone deacetylase activity in 96- or 384-well plates. For the measurement of acetylation, the HAT Assay Kit uses biotinylated histone peptides to measure histone acetyltransferase activity. For multiplex analysis utilising multiplex bead technology, the H2A.X Phosphorylation Multiplex Assay Kits enables bead-based multiplex measurement of phosphorylated histone H2A.X (Ser139). In labs performing cell

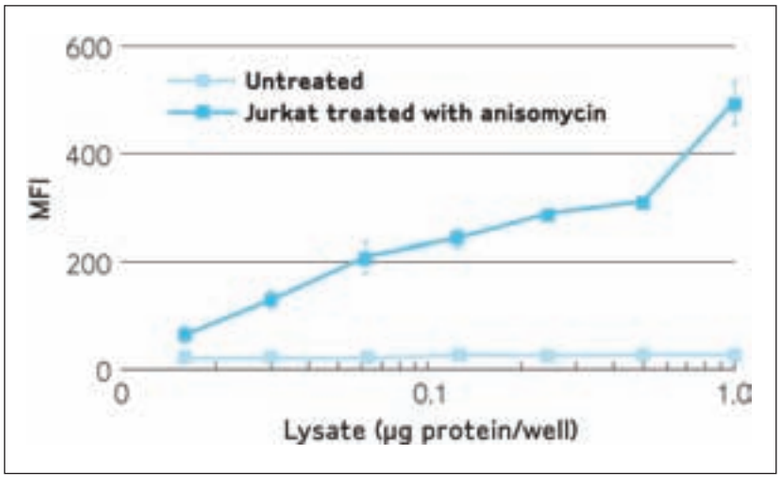


Figure 17: EMD Millipore's MILLIPLEX MAP detection of changes in phosphorylation of histone H2A.X (Ser139) in Jurkat cells stimulated with or without 25mM anisomycin. The Median Fluorescent Intensity (MFI) was measured using the Luminex instrument

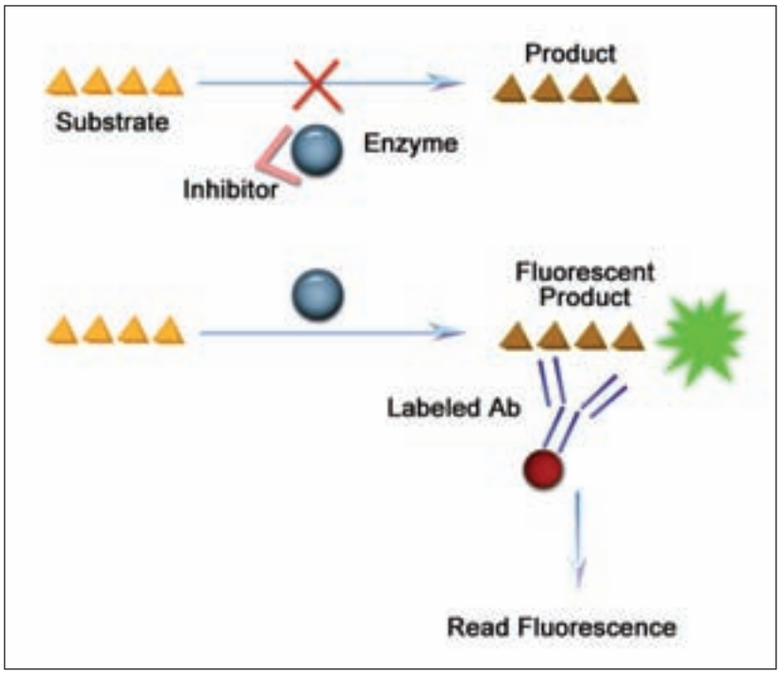


Figure 18: The principle of Epigentek's Epigenase™ for screening of inhibitors targeting epigenetic enzymes. In the absence of inhibitors, the enzyme and substrate coated on microplate wells interact with each other to form an enzyme-converted end product, which is then bound with a fluorescence antibody or ligand. The fluorescence intensity of the bound antibody/ligand can be detected with a fluorescence microplate reader. In the presence of an inhibitor, the compound may bind the enzyme to block the formation of the enzyme-converted end product. Therefore, if there is no fluorescence antibody/ligand binding to the wells, this would result in a much lower fluorescence intensity than the uninhibited control. In this mechanism, the inhibitors can be identified accordingly

cycle inhibitor profiling studies, the Phospho-Histone H3 Ser10 and Cyclin B1 Assay facilitates distinction among G2, M and G0/G1/S phases of the cell cycle. For laboratories wanting to analyse specific DNA-protein interactions without performing electrophoretic mobility shift assays, they offer EZ-TFA transcription factor assays. These 96-well plate-based DNA binding activity assays are available as universal kits for virtually any target of interest or as a pre-configured target-specific assay. As epigenetics solution provider, EMD Millipore also offers products for chromatin immunoprecipitation (ChIP), RNA binding protein immunoprecipitation (RIP), and DNA methylation analysis (**Figure 17**).

Epigentek (www.epigentek.com) was the first to pioneer a drug discovery service programme in epigenetics. Utilising its proprietary Epigenase™ screening platform, a fluorescence-coupled enzyme amplification technology, both DNA/histone modifying enzyme inhibitors and histone modification pattern modulators can be screened in a high throughput format (using 96, 384, or 1536-well microplates) with both single dose and EC50 profiling. The DNA/histone modifying enzyme panels include all 11 HDACs and five SIRTs, five HATs, 18 histone methyltransferases, 12 histone demethylases, four DNA methyltransferases, and 12 histone kinases. Histone modification pattern panels include DNA methylation, histone acetylation, histone methylation, histone phosphorylation and histone sumoylation (**Figure 18**).

For assessing global epigenetic modifications **Invitrogen** (www.invitrogen.com) is expanding its tools to investigate not only phosphorylation and ubiquitination, but also acetylation and methylation. Its new biochemical assay for identifying HDAC inhibitors uses a LanthaScreen® Eu-anti tag antibody with a fluorescently labelled tracer to measure the binding affinity of compounds rather than enzyme activity. This approach does not require a substrate, therefore avoiding issues related to the specificity of existing substrates. In addition, the signal is unaffected by the presence of residual endogenous HDACs from the host expression system because it is dependent on the presence of an epitope tag on the HDAC of interest. On the cellular side, Invitrogen has increased its LanthaScreen® cellular assay offering to include the analysis of histone H3 site-specific acetylation, methylation and phosphorylation in a wide range of cell backgrounds, including primary cells. These assays utilise cells expressing GFP-Histone H3 transiently delivered via BacMam and terbium

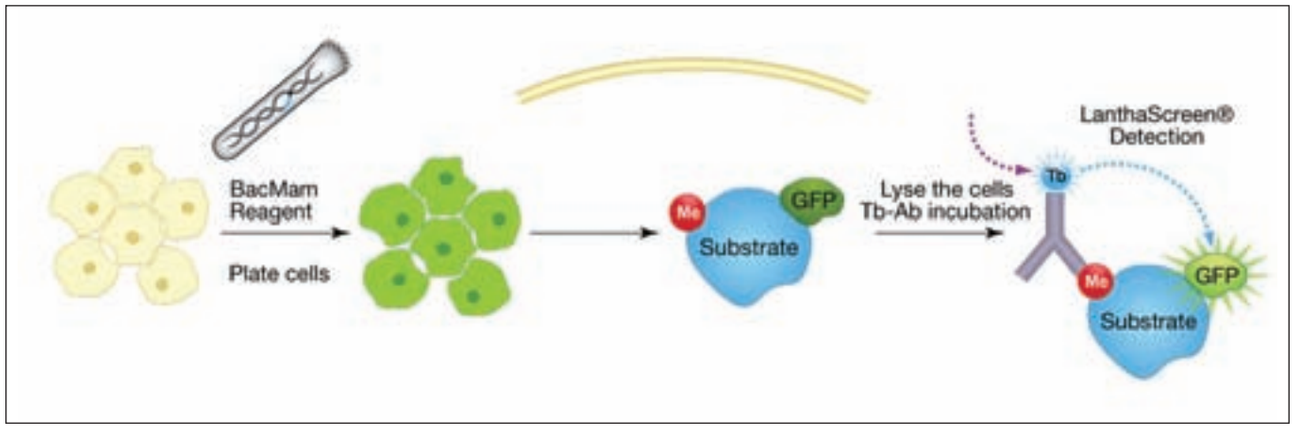


Figure 19: Invitrogen's BacMam-enabled cellular assay work flow. Cells are incubated with BacMam reagent encoding a GFP-fusion protein. 24 to 48 hours later, the cells are lysed in the presence of a terbium labelled anti-modification (such as methylation as shown) specific antibody. The level of modification on the GFP-fusion protein is determined via the LanthaScreen® TR-FRET readout

labelled Histone H3 modification specific antibodies. To identify local regions of the genome associated with specific protein modifications, Invitrogen has developed the MAGnify™ ChIP System. Based upon magnetic beads, it provides a faster and more reliable method than traditional ChIP protocols and is highly amenable to analysis by Next Gen Sequencing (ChIP-Seq) (**Figure 19**).

Rapid and sensitive assays to probe the activity of enzymes involved in histone modification are essential to perform high-throughput screenings aimed at finding hits that could provide a starting point to new drug discovery. PerkinElmer's (www.perkinelmer.com) LANCE® Ultra and AlphaLISA® assay platforms are non-radioactive, homogeneous, anti-body-based technologies commonly employed for such applications. Using these platforms, PerkinElmer recently launched a series of reagents to monitor epigenetic modifications elicited on four highly studied histone H3 marks, H3K4, H3K9, H3K27 and H3K36, by writer and erasers of the histone code. Technical notes are currently available that demonstrate the use of these reagents to measure the *in vitro* catalytic activities of histone acetyltransferases and deacetylases, and also methyltransferases (HMT) and demethylases (**Figure 1**). The assays are performed in two simple steps: the enzymatic reaction, followed by product detection with either an anti-mark europium-labelled antibody (LANCE Ultra), or anti-mark AlphaLISA Acceptor Beads. Anti-mark antibodies were selected on the basis of their specificity against the modification of interest. Of note is that both AlphaLISA and LANCE Ultra assays require low peptide substrate and enzyme concentrations (in the nanomolar range), which is sufficient to generate robust and highly reproducible assay signals. This represents a significant advantage over alternative, less sensitive technologies that generally require micromolar substrate and enzyme concentrations, and greatly simplifies working near cofactor Km concentrations when applicable. PerkinElmer's AlphaLISA reagents have also been shown to allow measurement of HMT activity using full length purified histone H3 protein, giving added flexibility to choose the most appropriate substrate. With the all-in-one-well format, absence of washes, and small number of assay steps, the LANCE Ultra and AlphaLISA platforms allow straightforward automation of epigenetic assays for screening (hit finding) and orthogonal testing (hit-to-lead) of histone modifying enzymes (**Figure 20**).

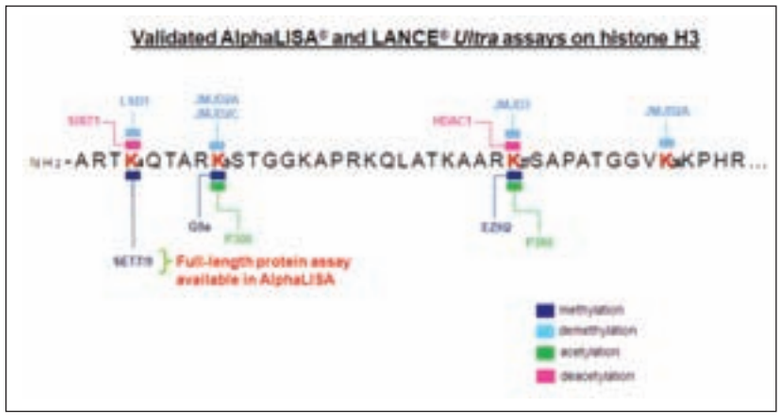


Figure 20: Summary of available PerkinElmer AlphaLISA and LANCE Ultra reagents used to measure modifications on histone 3. All detection reagents have been well characterised for their specificity at detecting the modification of interest and validated using different enzyme classes depicted on the figure. More information on the specificity of the mark detected is available online and reagents for other histone modifications should be available shortly

The histone deacetylase enzyme family contains many attractive targets for drug discovery because these enzymes are among the most critical post-translational regulators of transcriptional processes and gene expression. To date, methods developed for interrogating HDAC class I and II and Sirtuin activities have been complicated by insufficient or labour-intensive throughput, detection platform interferences, and/or poor sensitivity owing to low catalytic activity. **Promega** (www.promega.com) has developed two highly sensitive, single addition, 'add-mix-measure' assays (HDAC-Glo™ I/II Assay and Screening System and SIRT-Glo™ Assay and Screening System) which obviate these traditional limitations in screening environments. These pan-selective assays utilise novel luminogenic substrates that contain optimised peptide sequences culminating in an acetyllysine conjugated to aminoluciferin. When combined in a homogeneous reagent with recombinant Ultra-Glo™ luciferase and a lysine-specific developer enzyme, this near simultaneous, coupled reaction chemistry produces a 'glow-type' luminescent signal that is stable, proportional and quantifiable using standard luminometry in plate-based formats. The assays produce large signal windows with low variation (Z' > 0.8) making them suitable for miniaturisation into high density plate formats. Although both assay systems can be used with recombinant enzyme sources, the HDAC-Glo™ I/II Assay offers additional utility in lytic or non-lytic cell-based formats with primary and cancer cell types. Furthermore, the HDAC-Glo™ I/II Assay can be multiplexed in same-well formats with spectrally distinct viability or cytotoxicity assays to assess the cellular consequences of prolonged HDAC inhibition; useful for evaluating both on-target efficacy and off-target safety. Lastly, convenient counter-screen chemistries are available for both assays which are useful for confirming deacetylase specific effects (Figure 21).

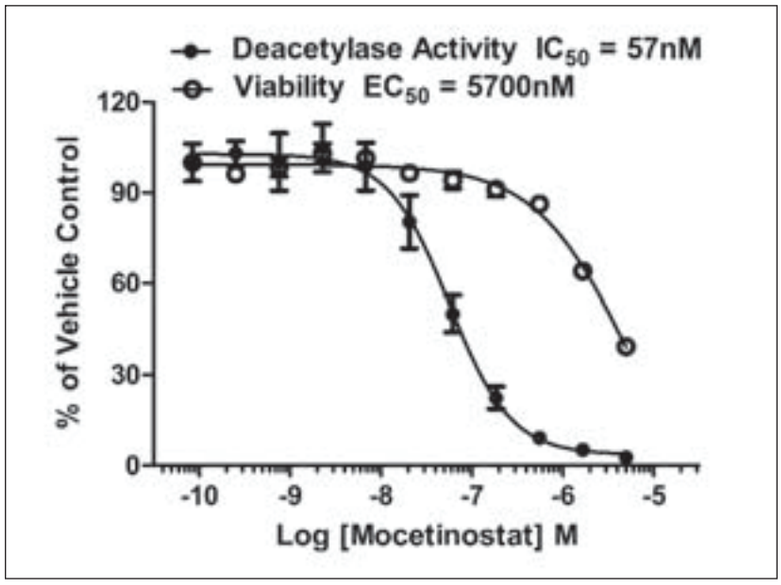


Figure 21: Serial dilutions of the HDAC inhibitor Mocetinostat were applied to K562 cells for 48hrs. Cell viability was determined using Promega's CellTiter-Fluor™ in a same-well, multiplexed format prior to the addition of Promega's HDAC-Glo™ I/II Assay Reagent to detect remaining HDAC activity

Reaction Biology (RB) (www.reactionbiology.com) is a premier service provider for drug profiling, screening and early drug discovery collaborations. Its proprietary HotSpot™ technology is an innovative, high-throughput platform for screening and profiling small molecules against any biological target which may be assayed by radioisotope detection. For transferase enzymes in particular, such assays are recognised as the 'gold standard'. RB has become the first and only service provider to provide screening and profiling against all major classes of epigenetic transferases via the radioisotope approach: histone lysine and arginine methyltransferases (HMTs), DNA methyltransferases (DNMTs), Histone acetylases (HATs), and histone-modifying kinases (and all other kinase types). All HotSpot™ assays are performed in a miniaturised reaction format to reduce cost and waste, while

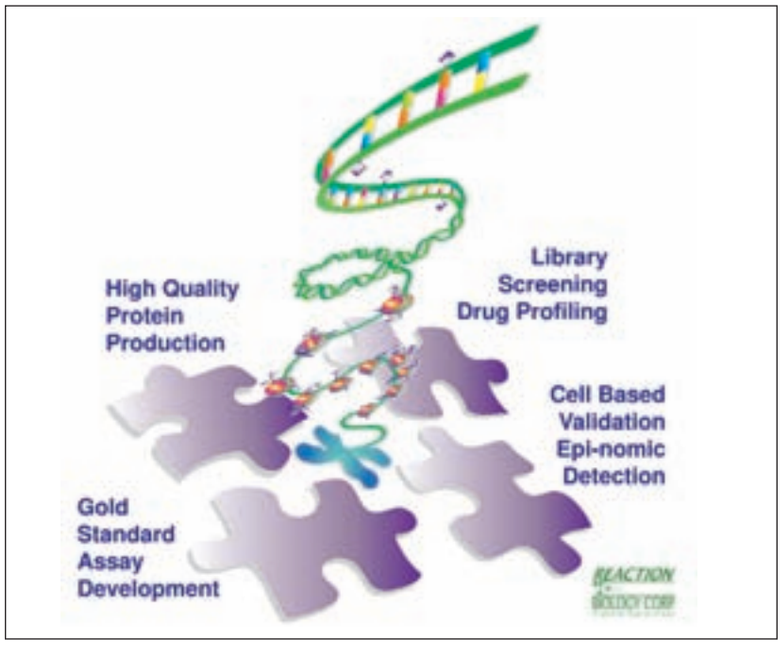


Figure 22: Reaction Biology takes a comprehensive approach to serving the epigenetic drug discovery industry, providing high quality proteins, robust and low cost assay development, library screening, drug profiling and validation of drug activity in cellular assays

Table 1: Epigenetic enzyme classes/proteins for which vendors have developed screening assays, kits, reagents or tools

Epigenetic Enzyme Classes/Proteins	Active Motif	Biocius	BioFocus	BPS Bioscienc
Acetylation – histone acetyl transferases (HAT)	✔	✔	✔	✔
Deacetylation – histone deacetylases (HDAC & Sirtuins)	✔	✔	✔	✔
Histone Methylation – histone methyltransferases (HKMT & HRMT)	✔	✔	✔	✔
DNA Methylation – DNA methyltransferases (DNMT)	✔	✔	✔	✔
Demethylation – histone demethylases (HDM)	✔	✔	✔	✔
Ubiquitination – ubiquitin ligases (E1, E2, and E3)	✔	✔	✔	✔
Deubiquitination – deubiquitinases (DUB)	✔	✔	✔	✔
Sumoylation – SUMO ligases	✔	✔	✔	
Phosphorylation – kinases in relation to histones	✔	✔	✔	
Dephosphorylation – phosphatases in relation to histones		✔	✔	
Binding Proteins (eg Bromo, Chromo & Tudor Domains etc)	✔		✔	
Offers Fee-For-Service Testing Against Epigenetic Targets		✔	✔	✔

maintaining excellent data quality. Substrates require no special modifications (eg biotin, fluorophors or other tags). This means various biologically relevant substrates may be used, including full nucleosomes, histone proteins or peptides. Since detection does not rely on antibodies, coupling enzymes or fluorescence, major causes of false positives, false negatives and compound interference are eliminated. RB also offers fluorescence-based detection for all HDAC and Sirtuin isoforms with a variety of substrates. Histone Demethylase and deubiquitinase (DUBs) assays are also under development. Solving the puzzle of early stage discovery/development for emerging targets within the epigenetic field requires a comprehensive approach. One critical piece of the puzzle is the investment in a deep understanding of the biology of the target, and subsequent production of high quality recombinant proteins for these challenging targets. Next, the development of relevant and powerful assay platforms allows for the sensitive detection of target activity and chemical inhibition. Access to high quality chemical compound libraries and screening expertise then allows for rapid hit identification and hit-to-lead development. The puzzle can then be further solved by the use of relevant cell-based assays for hit/lead validation. By embracing this multifaceted approach, RB has come to the forefront of research service providers within the epigenetic drug discovery industry (**Figure 22**).

Discussion

At the time of the SBS session devoted to epigenetics[2] it was easy to draw the conclusion that epigenetic screening was very much in its infancy, as only a few vendors appeared to be offering suitable screening tools and assays, and most of these were for HDAC & Sirtuins. This was also the feedback from HTStec's survey last summer. However, putting together this review it is apparent that quite a lot has changed in the interim. In **Table 1** the product offerings of the 12 companies discussed in this article are compared with respect to their support of screening assays, kits, reagents and tool provision for the epigenetic enzymes and proteins targets listed. What this table reveals it that the breadth of assays now covered extends well beyond the HDAC & Sirtuins, with some vendors supporting the entire list of targets. This would not have been achieved without the availability of more specific antibodies

BPS Bioscience	Cayman Chemical	Diagenode	EMD Millipore	Epigentek	Invitrogen	PerkinElmer	Promega	Reaction Biology
✔	✔	✔	✔	✔	✔	✔		✔
✔	✔	✔	✔	✔	✔	✔	✔	✔
✔	✔	✔	✔	✔	✔	✔		✔
✔	✔	✔	✔	✔	✔	✔		✔
✔	✔	✔	✔	✔	✔	✔		✔
✔		✔	✔		✔	✔		✔
✔		✔			✔			✔
		✔	✔		✔	✔		
		✔	✔	✔	✔	✔		✔
		✔	✔		✔	✔		✔
				✔		✔		
✔	✔		✔	✔	✔			✔

or label-free methods permitting the detection of multiple modification events across a single native substrate. Although some of the first reported assays for HDACs were mainly fluorimetric (based on fluorescent intensity), epigenetic targets are increasingly being assayed by a broad range of different screening technologies. While many perceive radiometric approaches as the 'gold standard', the jury is out as to which technology will prove most predictive and suited to routine primary screening. As the survey pointed out, there are important challenges, such as assay specificity and the retention of activity in purified proteins, which must be addressed. However, there does appear to be advantages associated with some of the more generic approaches currently being validated, for example suitability to all types of histone substrates and application across a diverse range of histone modifications. These approaches now need to be translated in a set of robust ready-to-use assay kits or tool-box reagents to really open up the field to HTS. **Table 1** also highlights the increasing number of vendors that now offer fee-for-service screening and profiling against epigenetic targets. It is premature to describe this activity as comparable to kinase outsourcing, but clearly the industry is gearing up in the expectation that many companies will choose to outsource compound testing against a panel of epigenetic assays. The survey showed that one of the main drivers for this was to access an assay/screening technology respondents do not have or use in-house. In conclusion, the tools required to support epigenetic screening are fast emerging as our knowledge of these targets increases, such that we can expect to see greater adoption or external use of these assays by Pharma and Biotech lead discovery programmes over the coming years. DDW

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