An Overview on Screening Methods for Lysine Specific Demethylase 1 (LSD1) Inhibitors

Yi-Chao Zheng, Jiao Chang, Ting Zhang, Feng-Zhi Suo, Xiao-Bing Chen, Ying Liu, Bing Zhao, Bin Yu, and Hong-Min Liu

Abstract: Background: In the past few years, great attention has been paid to the identification and characterization of selective and potent inhibitors of the first identified histone demethylase LSD1, which may erase mono- and di-methylated histone 3 lysine 4 and 9. As the aberrant overexpression of LSD1 is involved in various pathological processes, especially cancer, obtaining selective and potent LSD1 inhibitors has emerged as a crucial issue in medicinal chemistry research.

Method: Until now, several LSD1 inhibitor screening models have been established, including enzyme coupled assay, LC-MS based assay, and FRET based assay. Nevertheless, due to some special instrument requirement and additional costs of LC-MS and FRET, the enzyme coupled assay is the most widely applied method for LSD1 inhibitor screening.

Result: We summarized and compared several reported in vitro LSD1 inhibitor screening models. Each of them has distinct advantages and disadvantages, and none of these methods is perfect. In order to exclude the false positive results, at least one additional method should be applied to screen LSD1 inhibitors.

Keywords: LSD1, inhibitor, screening model, enzyme coupled assay, LC-MS, FRET.

1. INTRODUCTION

The histone code features reversible lysine modifications as a major contributor for the regulation of chromatin accessibility, gene expression, and cellular growth. Acetylation and methylation of the lysine side chain are considered the dominant and best-studied post-translational modifications in histones. Lysine acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), whereas lysine methylation is regulated by histone lysine methyltransferases (HMTs) and histone lysine demethylases (KDMs) [1]. Whereas acetylation of the lysine only occurs once per residue, lysine methylation can occur as mono-, di-, and trimethylation forms. LSD1 is the first identified KDM in 2004 [2]. Historically, it was uncertain as to whether protein lysine methylation was reversible. Since then, tens of KDMs were characterized, and divided into two groups, including flavin adenine dinucleotide (FAD) dependent KDMs and Jumonji C (JMJC) domain containing KDMs [1]. In the FAD dependent KDMs family, there are three members, lysine demethylase 1 (LSD1), LSD1+8a [3] and LSD2 [4-7], and all of them utilize the cofactor FAD during catalysis of demethylation. Meanwhile, they may erase mono-, di- methylated lysine residue. During...
the reaction, each catalytic cycle of methyl removal produces a molecule of formaldehyde and H$_2$O$_2$, respectively, while consuming O$_2$. Molecular oxygen is used as the electron acceptor and methyl group oxidation is then proceeded via hydride transfer from the N-methyl group onto FAD, forming an imine, which is unstable to hydrolysis [8]. To produce the resultant imine, the methyl group of lysine is oxidized and hydrolyzed to form formaldehyde (Scheme 1). In this reaction, the amount of H$_2$O$_2$ produced in the demethylation indicates the activity of the FAD dependent KDM. Hence, quantification of the byproduct, H$_2$O$_2$, is an option to evaluate the activity of the KDM.

![Scheme 1](image)

**Scheme 1.** The mechanism of LSD1 catalysis.

Until now, plenty of articles have indicated that LSD1 is overexpressed in several kinds of cancers and contributes to the cancer cell proliferation [1, 9, 10]. Meanwhile, three of LSD1 inhibitors have entered into clinic trials for the treatment of small cell lung cancer and acute myelogenous leukemia, including ORY1001 (also called RG6016) from Oyrzon, GSK2879552 from GlaxoSmithKline and INCB059872 from Incyte [9-12]. Hence, LSD1 is a promising target for cancer therapy, especially small cell lung cancer and leukemia, and many medicinal chemists try to pursue potent and selective LSD1 inhibitor. Until now, there are several methods for the screening of LSD1 inhibitors. All these methods can be divided into three groups, label free method, fluorescence resonance energy transfer (FRET) based method and byproduct quantification method. For the label free method, mass spectrometry platform is applied to monitor the amount of LSD1 substrate H3K4me2 [13, 14]; surface plasmon resonance (SPR), isothermal titration calorimetry (ITC) and bio-layer interferometry (BLI) are applied to monitor the protein- molecule interaction. Nevertheless, all of these methods required additional expensive equipment and professional skills for the operation, and for the compound screening, it will take long time for the operation as well complicated steps for sample preparation. For the FRET based method, time-resolved fluorescence resonance energy transfer (TR-FRET) has been reported for LSD1 inhibitor screening [15]. Besides, there are also some commercially available kits from PerkinElmer and CisBio for the FRET based LSD1 inhibitor screening. These methods are sensitive, robust and scalable. However, all these FRET based methods need additional tag for the screening, which may impact the conformation of the target protein. Sometimes, antibody required in these assays may also lead to additional cost and unexpected unspecificity. Hence, the byproduct quantification method is widely used primarily for enzyme inhibitor screening as this method is costs acceptable, scalable, sensitive, and easy to operate and do not need additional equipment, and so is LSD1 [16-25].

Until now, several LSD1 inhibitor screening methods have been reported. We have also applied some of these methods in our LSD1 inhibitor evaluation [1, 10, 16]. Here, several LSD1 inhibitor screening models will be stated.

### 2.1. Mass spectrometry Based LSD1 Inhibitor Screening Model

As a molecular balance, mass spectrometry was used to obtain the accurate mass of the specific molecule, and it has been applied for the LSD1 inhibitor screening [13, 14]. Products with LSD1 substrate were contained in an assay well, then the assay used Rapid-Fire chromatography in line with a triple stage quadrupole detection method to measure the assay well [14]. After that, self-assembled monolayer desorption/ionization (SAMDI) mass was applied in 384-well format and before injecting into a mass spectrometer, the sample can be rapidly purified prior to injection into a mass spectrometer, in this way, the assay will get rid of the complex chromatographic steps [13]. Both of these two methods utilize a short amino acid peptide as a substrate, which corresponds to the first 21 amino acid residues of histone 3, and they are all based on measurement of the amount of the direct formation of unmethylated and mono-methylated peptide, and their quantity can indicate the process of LSD1 demethylation.

### 2.2. Luminol Coupled Assay for LSD1

Luminol (5-amino-2,3-dihydro-1,4-phthalalidine) is a cyclic acyl-hydrazide. The luminol reaction is in line with a way of direct chemiluminescence [26]. The excited state of 3-aminophthalate generated during the luminol oxidation process can perfectly match the chemiluminescence spectrum of luminol, so excited 3-aminophthalate dianion can be considered as the light-emitting intermediate molecular [27, 28]. The chemi-
luminescence of luminol was demonstrated at 425 nm, \( \lambda_{\text{Max}} \) in the system of alkaline solution of luminol oxidized by oxidizing agents, such as hydrogen peroxide, ozone, halogens, etc. And hydrogen peroxide is the strongest oxidant that can increase the light intensity of luminol [29].

As one molecular hydrogen peroxide can be produced during the demethylation of LSD1 acting on H3K4me2 [2], the quantity of hydrogen peroxide can be obtained by the application of luminol. After the LSD1 recombinant was incubated with substrate peptide and different candidate compounds, the generated \( \text{H}_2\text{O}_2 \) can be quantified by adding luminol and horseradish peroxidase (HRP), and the quantity of \( \text{H}_2\text{O}_2 \) may illustrate the LSD1 activity (Fig. 1). The amount of \( \text{H}_2\text{O}_2 \) would be decreased when the process of demethylation by LSD1 was suppressed by LSD1 inhibitors [30-33]. Hence, luminescence-based method can be used for the detection of the enzymatic activity of LSD1 as well as the activity of the inhibitors (Fig. 1) [30].

![Fig. (1). Work flow of luminol coupled assay for LSD1.](image)

Chemiluminescence-based methods are widely used in various assays because of its easy availability, simple operation and low cost. Nevertheless, the chemiluminescence should be monitored immediately after the adding of luminol and HRP as the light cannot last for a long time unless the luminol was replaced by some other new chemiluminescence reagent, such as Lumigen. Hence, when luminol was applied for LSD1 inhibitor screening, additional injector is required for the multi-detection microplate reader, so that each well can be monitored as soon as luminol and HRP were added into the mixture. Besides, compound that may have inherent luminescence or interact with HRP or \( \text{H}_2\text{O}_2 \) may also lead to the false positive result.

2.3. Amplex Red Coupled Assay for LSD1

Amplex red reagent, in combination with HRP, has been used to detect \( \text{H}_2\text{O}_2 \) released from various biological samples, including cells, or generated in enzyme-coupled reactions [34]. Furthermore, amplex red reagent can be used in an ultrasensitive assay for peroxidase activity when \( \text{H}_2\text{O}_2 \) is excess. In the presence of peroxidase, the amplex red reagent reacts with \( \text{H}_2\text{O}_2 \) in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. Resorufin has excitation and emission maxima of approximately 563 nm and 587 nm, respectively, and because the extinction coefficient is high (58000 ± 5000 cm\(^{-1}\)M\(^{-1}\)), this assay can be monitored fluorometrically or spectrophotometrically. This reaction has been used to detect as little as 10 picomoles of \( \text{H}_2\text{O}_2 \) in a 100 \( \mu \text{L} \) volume or 1 \( \times 10^{-5} \) U/mL of HRP [34]. With this method, amplex red coupled assay for LSD1 was established as reported [22]. Until now, plenty of articles have applied this method for primary screening as well as LSD1 activity evaluation (Fig. 1) [16, 17, 22, 35-37].

Until now, some commercialized kits also use this method for LSD1 inhibitor screening as this experiment can be performed within 45 min with strong sensitivity and low costs. Nevertheless, there are still some limitations on this method. When the candidate compound shows similar spectrum with excitation wavelength at 563 nm and emission wavelength at 587 nm, self-fluorescence of the candidate compound may interfere with the result indicated by resorufin, which may lead to false positive results of the candidate compound. Meanwhile, compounds with strong reducibility may also interact with HRP or \( \text{H}_2\text{O}_2 \), which leads to the false positive results. Hence, for high throughput screening, amplex red coupled assay for LSD1 can be applied for the first round screening, but additional experiments will be needed for further confirmation.

![Fig. (2). Work flow of amplex red coupled assay for LSD1.](image)

2.4. 4-aminoantipyrine Coupled Assay for LSD1

Furthermore, activity of LSD1 can also be monitored by absorbance. As reported, 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) and 4-aminoantipyrine (4-AA) can be oxidized by HRP to form quinone dye in the presence of \( \text{H}_2\text{O}_2 \) [38]. The amount of reacted quinone dye can be monitored at 545-556 nm. The color of quinone dye was directly correlated with the amount of \( \text{H}_2\text{O}_2 \). By measuring the absorbance of quinone dye with similar work flow as Fig. (2), the amount of hydrogen peroxide was indirectly determined, then the activity of LSD1 can be elucidated (Fig. 1) [39]. If LSD1 inhibitors are added in the demethylation process of LSD1, the amount of \( \text{H}_2\text{O}_2 \) will reduce. In that way, inhibitory effect of LSD1 inhibitor against the recombinant can be evaluated.

So far, several articles have applied this method for detecting LSD1 activity or the activity of the candidate compound against LSD1 [13, 40]. Nevertheless, this
assay is not so sensitive. In our laboratory, we failed to apply this method to detect LSD1 activity due to its poor sensitivity, and amplex red coupled assay was applied in our LSD1 inhibitor screening experiment [16, 41, 42].

2.5. FDH Coupled Assay for LSD1

Formaldehyde dehydrogenase (FDH) is able to oxidize formaldehyde into formic acid with NAD$^+$ restored to NADH [43]. NADH formation can be quantified by the measurement of its absorbance ($\lambda = 340$ nm) or more sensitively by fluorescence intensity with excitation at 330 nm and emission at 460nm [44]. As LSD1 demethylation occurs via an oxidation reaction with formaldehyde [1], the generated formaldehyde can be evaluated with NAD$^+$ quantification experiment, and then the enzymatic activity of LSD1 can be determined by measuring the production of NADH when the demethylation reaction is coupled with the FDH assay.

Up to the present, FDH coupled assay has been used in the determination of LSD1 activity in several papers [2, 40], and was applied for the screening of LSD1 inhibitors [45]. The FDH coupling assay can be used to evaluate the demethylase activity of all histone lysine demethylases as they all generate formaldehyde during the demethylation reaction (Fig. 3). Furthermore, this method does not require the histone methylation-specific antibody comparing to the following HTRF/Alpha based model, hence, it is widely used in the demethylation assay. However, false reactions from conjugated enzymes can lead to false positive and negative result, which require additional experiment for further confirmation.

2.6. HTRF/Alpha Based LSD1 Inhibitor Screening Model

Homogeneous time resolved fluorescence (HTRF) is widely used in cell and biochemical experiments, and can be applied to the research of proteins and proteins, proteins and peptides, proteins and DNA /RNA interactions in different stages of drug studies. The HTRF technique mainly uses a europium-labeled antibody against the substrate or the demethylated product as a donor, and another fluorophore, such as ULight in LANCE Ultra, as the acceptor [15, 43]. When the donor europium chelate is excited at 320 or 340 nm, energy in the form of FRET elicits fluorescence emission at 665 nm from a nearby streptavidin tagged ULight acceptor bound to biotinylated peptide substrate [43]. Currently, this technique has been used for high-throughput screening of LSD1 inhibitors (Fig. 4A, B) [15]. Using europium cryptate-labeled antibody as a donor, streptavidin-tagged fluorophore acceptors bind to the biotinylated peptide substrate. If the methylation state recognized by the europium-labeled antibody is in close proximity to the acceptor, in this case allophycocyanin (APC), FRET occurs upon donor excitation. The loss of FRET signal indicates demethylation of LSD1 [15, 43].

To date, some commercialized kits also use this method for LSD1 inhibitor screening because of its low background and high reproducibility. More importantly, it reduces nonspecific signals to negligible levels and achieves an extremely high signal-to-noise ratio that greatly exceeds the sensitivity achieved by radioisotopes [43]. However, this technique also has some drawbacks. Firstly, as the protein needs to be labeled, it may change the protein conformation, which may lead to some unexpected false-positives or false-negatives. It is emphasized that this technology is susceptible to quenching of singlet oxygen compounds [15]. Thence, HTRF technique is convenient for screening LSD1 inhibitors, but additional methods are needed to confirm it further.

Amplified luminescent proximity homogeneous assay (ALPHA) technology (PerkinElmer, MA, USA)

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Fig. (3). Byeproducts quantification method for high-throughput screening of LSD1 inhibitors.
has attracted extensive attention in many fields such as basic medical research, clinical testing and new drug development because of its advantages in sensitivity, real-time, fast and convenience [46-48]. Alpha is a bead-based system in which ‘donor’ beads excited by a laser transfer energy in the form of singlet oxygen to ‘acceptor’ beads within 200 nm, inciting emission of a luminescent signal [49]. For the histone demethylase assays, a biotinylated histone H3-derived peptide is always applied as substrate. In the first step, the enzyme modifies the biotin labeled substrate in the presence of the required cofactor to generate the biotin labeled reaction product; the reaction product is then captured by streptavidin (SA) donor beads and anti-mark acceptor beads. Irradiation of complexed partners at 680 nm initiates in SA donor beads the production of singlet oxygen \((^1O_2)\) molecules that reach the acceptor beads in proximity to generate a cascade of energy transfer reactions culminating in an amplified light emission detected at 615 nm. Then the amplified light can be monitored with plate reader. Currently, analysis of LSD1 using ALPHA has already been reported (Fig. 4A, B) [50].

ALPHA technology is also a very popular method due to its uniformity, convenience and simplicity. It provides higher sensitivity, wider dynamic range, and more sensitive detection limit of interactions between low binding substances. Of course, it is not a perfect technology and also has some shortcomings. Similar to HTRF method, it requires specific antibody-coated beads, and is susceptible to quenching of singlet oxygen compounds [43]. Then, it may lead to false-positive or false-negative results. Nevertheless, ALPHA method is only available from PerkinElmer, accessibility of the commercial kit should also be considered for the screening.

2.7. Scintillation Proximity Assay for LSD1

Scintillation proximity assay (SPA), widely regarded as the "gold standard" for radio-high-throughput screening, is a radio-isotopic technology format and has been applied to cell adhesion molecule binding, protein-peptide interaction, protein-DNA interaction and cell biochemical assays [51, 52]. With this method, the beads in the SPA have a scintillator that, when stimulated, emits light, stimulates when the radiolabeled molecules interact and bind to the surface of the bead. This interaction will trigger the emission of light from the bead, which will produce the energy conversion of the radioactive decay that releases the photon, which can be detected by using a photomultiplier tube such as a scintillation counter or a CCD imager [52]. Simultaneously, scintillation proximity assay for the lysine KDMs was established as reported [53]. Biotin-labeled methylated peptides are demethylated by a KDM, and subsequently a protein methyltransferase KMT7 is added to methylate the peptide product with 3H labeled SAM (S-(50-adenosyl)-L-methionine) after KDMs is quenched by heat shock. In this manner, KDMs activity is monitored by the incorporation of a 3H-methyl group into the target peptide (Fig. 5).

This method requires no separation step and is not sensitive to the fluorescence interference from the candidate compound. More importantly, this assay requires both the writer and eraser of the histone modification, and additional optimization for this assay is necessary for the two steps reaction.

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**Fig. (4).** HTRF/Alpha based LSD1 inhibitor screening model. A, Work flow of HTRF/Alpha based LSD1 inhibitor screening model; B, Principle of HTRF/Alpha based LSD1 inhibitor screening model.
Fig. (5). Work flow of scintillation proximity assay for LSD1.

Table 1. Evaluation of the platforms used in recent high-throughput screening campaigns for inhibitors of lysine demethylase.

<table>
<thead>
<tr>
<th>Types</th>
<th>Apparatus requirement</th>
<th>Costs</th>
<th>High-throughput</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass spectrometry</td>
<td>Mass spectrometer</td>
<td>High</td>
<td>No</td>
<td>Lable free; Direct readout of product formation</td>
<td>Low throughput</td>
</tr>
<tr>
<td>Luminol coupled assay</td>
<td>Plate reader</td>
<td>Low</td>
<td>Yes</td>
<td>Lable free; High sensitivity</td>
<td>Compound that may interact with H2O2 is not applicable</td>
</tr>
<tr>
<td>Amplex red coupled assay</td>
<td>Plate reader</td>
<td>Low</td>
<td>Yes</td>
<td>Lable free; High sensitivity</td>
<td>Not available for compound with auto-fluorescence and/or quenching; Compound that may interact with H2O2 is not applicable</td>
</tr>
<tr>
<td>4-aminoantipyrine coupled assay</td>
<td>Plate reader</td>
<td>Low</td>
<td>Yes</td>
<td>Lable free</td>
<td>Poor sensitivity; Compound that may interact with H2O2 is not applicable</td>
</tr>
<tr>
<td>FDH coupled assay</td>
<td>Plate reader</td>
<td>Low</td>
<td>Yes</td>
<td>Lable free</td>
<td>Compound that may interact with HCHO is not applicable</td>
</tr>
<tr>
<td>HTRF/Alpha based assay</td>
<td>Plate reader</td>
<td>Medium</td>
<td>Yes</td>
<td>High sensitivity</td>
<td>Requires labeled antibodies; susceptible to auto-fluorescence and/or quenching by compounds</td>
</tr>
<tr>
<td>Scintillation proximity assay</td>
<td>Scintillation counter</td>
<td>Medium</td>
<td>Yes</td>
<td>High sensitivity</td>
<td>Heat required; Additional enzyme reaction required</td>
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</table>

CONCLUSION

As the substrate of LSD1 is H3K4me1/2, inactivation of LSD1 may lead to the accumulation in some cell lines. Nevertheless, different from other histone demethylase, inactivation of LSD1 does not lead to the accumulation of global H3K4me1/2 in some cell lines [54], quantification of H3K4me1/2 in cells, either use plate reader or high content analysis, is not applicable for LSD1 inhibitor screening. In some cases, accumulation of H3K4me1/2 occurs around some specific promoters or enhancers, which suggest that chromatin immunoprecipitation- quantification polymerase chain reaction (ChIP-qPCR) have to be applied [37, 55, 56]. But ChIP-qPCR cannot be applied for inhibitor screening. Besides, some indirect inhibition to other targets may also lead to the downregulation of LSD1 expression or activity, so cell based assay is not applicable for LSD1.

As stated above, there are several kinds of assays that can be applied for HTS of LSD1 inhibitors. Each method has its pros and cons (Table 1). Normally, two or more assays are needed for LSD1 inhibitor screening, and the method should be similar in the principle of the assay. In order to exclude the false positive results, only one byproduct quantification assay should be chosen, such as luminol coupled assay, amplex red coupled assay and 4-aminoantipyrine coupled assay, then FRET based assay, including ALPHA and HTRF, or substrate quantification assay, including mass spectrometry and scintillation proximity assay, can be utilized. Our lab used amplex red based assay for the first round screening due to its advantages in its short
screening time, which can be completed within 1 hour, low costs and sensitivity. Then we applied HTRF for the second round confirmation, which performs higher costs than amplex red assay with less false positive results. Finally, some label free techniques, such as Monolith NT.AbbLabelFree instrument from NanoTemper and BLI from Pall were applied for further confirmation [16, 57]. Hence, no matter which method is used for LSD1 inhibitor screening, pros and cons of the method should be evaluated carefully (Table 1) depending on the experience of the researcher, and additional evaluation should be applied to compensate the shortage of the first round screening and exclude some false positive results. Finally, molecule interaction should also be evaluated to have some enzyme kinetic parameters.

LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>KAT</td>
<td>Histone acetyltransferases</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>HMT</td>
<td>Histone lysine methyltransferase</td>
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<tr>
<td>KDM</td>
<td>Histone lysine demethylase</td>
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<td>FAD</td>
<td>Flavin adenosine dinucleotide</td>
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<tr>
<td>JMJC</td>
<td>Jumonji C</td>
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<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
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<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
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<tr>
<td>BLI</td>
<td>Bio-layer interferometry</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
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<tr>
<td>TR-FRET</td>
<td>Time-resolved fluorescence resonance energy transfer</td>
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<tr>
<td>LSD1</td>
<td>Lysine demethylase 1</td>
</tr>
<tr>
<td>SAMDI</td>
<td>Self-assembled monolayer desorption/ionization</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>DHBS</td>
<td>3,5-Dichloro-2-hydroxybenzene-sulfonic acid</td>
</tr>
<tr>
<td>4-AA</td>
<td>4-Aminoantipyrine</td>
</tr>
<tr>
<td>FDH</td>
<td>Formaldehyde dehydrogenase</td>
</tr>
<tr>
<td>HTRF</td>
<td>Homogeneous time resolved fluorescence</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>ALPHA</td>
<td>Amplified luminescent proximity homogeneous assay</td>
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<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SPA</td>
<td>Scintillation proximity assay</td>
</tr>
<tr>
<td>SAM</td>
<td>S-(5-adenosyl)-L-methionine</td>
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<tr>
<td>ChIP-qPCR</td>
<td>Chromatin immunoprecipitation-quantification polymerase chain reaction</td>
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CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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