

A sensitive homogeneous enzyme assay for euchromatic histone-lysine-N-methyltransferase 2 (G9a) based on terbium-to-quantum dot time-resolved FRET

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Abstract

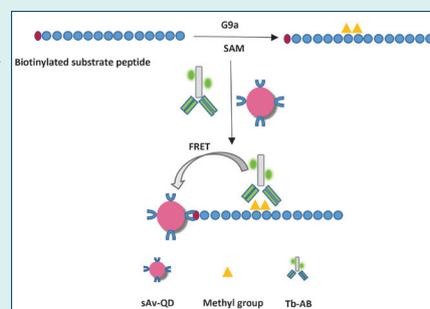
Introduction: Histone modifying enzymes include several classes of enzymes that are responsible for various post-translational modifications of histones such as methylation and acetylation. They are important epigenetic factors, which may involve several diseases and so their assay, as well as screening of their inhibitors, are of great importance. Herein, a bioassay based on terbium-to-quantum dot (Tb-to-QD) time-resolved Förster resonance energy transfer (TR-FRET) was developed for monitoring the activity of G9a, the euchromatic histone-lysine N-methyltransferase 2.

Overexpression of G9a has been reported in some cancers such as ovarian carcinoma, lung cancer, multiple myeloma and brain cancer. Thus, inhibition of this enzyme is important for therapeutic purposes.

Methods: In this assay, a biotinylated peptide was used as a G9a substrate in conjugation with streptavidin-coated ZnS/CdSe QD as FRET acceptor, and an anti-mark antibody labeled with Tb as a donor. Time-resolved fluorescence was used for measuring FRET ratios.

Results: We examined three QDs, with emission wavelengths of 605, 655 and 705 nm, as FRET acceptors and investigated FRET efficiency between the Tb complex and each of them. Since the maximum FRET efficiency was obtained for Tb to QD705 (more than 50%), this pair was exploited for designing the enzyme assay. We showed that the method has excellent sensitivity and selectivity for the determination of G9a at concentrations as low as 20 pM. Furthermore, the designed assay was applied for screening of an enzyme inhibitor, S-(5'-Adenosyl)-L-homocysteine (SAH).

Conclusion: It was shown that Tb-to-QD FRET is an outstanding platform for developing a homogenous assay for the G9a enzyme and its inhibitors. The obtained results confirmed that this assay was quite sensitive and could be used in the field of inhibitor screening.



Introduction

Epigenetics is the study of changes in the regulation of gene activity and expression, independent of gene sequences. An important epigenetic factor is post-translational modifications of histones, such as methylation, acetylation and phosphorylation, which are controlled by various histone-modifying enzymes (HMEs)

such as histone methyltransferases, acetyltransferases and phosphoryltransferases.¹ One of the histone methyltransferases (HMT) is euchromatic histone-lysine-N-methyltransferase 2 (EHMT2), also known as G9a, which catalyzes histone H3 lysine 9 mono- and dimethylation, usually associated with transcriptional gene silencing.²

It has been found that the initiation and growth of



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cancer not only depend on genetic mutations but also on epigenetic misregulations. For example, it has been reported that G9a is overexpressed in a number of cancers such as ovarian carcinoma, lung cancer, multiple myeloma, and brain cancer.¹⁻³ This can be attributed to the inhibition effect of G9a on tumor suppressor genes, which arises from enhanced methylation levels in H3 tails as a result of elevated G9a levels. It has also been reported a decrease in the activity of G9a could inhibit cell proliferation in several cancer cell lines. This fact has been attributed to autophagic death through an imbalance in the serine-glycine biosynthetic pathway.³ So, improvement of methods for HME assays and investigation of small molecules as their inhibitors have attracted great attention.^{4,5} Up to now, various compounds have been studied and introduced as G9a inhibitors. They are classified into H3 peptide substrate-competitive inhibitors and S-adenosylmethionine (SAM) cofactor-competitive inhibitors.⁶ Study on the ways to inhibit and control the activity of such enzymes and development of new inhibitors, selective toward subsets of HMTs or even a single HMT are an important and active field of research that can open new horizons for therapeutic interventions.^{7,8}

Various methods including mass spectrometry,^{9,10} radioisotope-based methods,^{11,12} enzyme-linked immunosorbent assays (ELISA),¹³ fluorescence,^{14,15} and colorimetric¹⁶ methods (based on measurement of enzymatic reaction by-products) have been reported to study histone methyltransferases as well as their inhibitors.

Förster resonance energy transfer (FRET) is a nonradiative energy transfer process in which the excited donor transfers its energy to a ground state acceptor through a dipole-dipole interaction.¹⁷ Due to the unique properties of FRET-based assays and the possibility of removing interfering background luminescence in the time-resolved (TR) fluorescence methods, TR-FRET has been known as a sensitive fluorescence method in various bioassays. One of the best donors for TR-FRET biosensing is luminescent terbium complexes (LTCs) owing to their sharp and distinct emission bands, stability, and very long excited-state lifetimes (in the millisecond range).¹⁸ Hildebrandt and coworkers¹⁹ applied LTCs as a donor with quantum dots (QDs) as an acceptor in TR-FRET. They indicated that QDs are suitable acceptors for lanthanide ions because of their strong absorption that has a very large spectral overlap with several emission bands of lanthanides.

Even though TR-FRET-based epigenetic enzyme assay using Tb-to-green fluorescent protein (GFP) and Eu-to-XL665 pairs^{20,21} have been already reported, to the best of our knowledge, terbium-to-quantum dot (Tb-to-QD) conjugate has not yet been used for this kind of assay. A steady-state FRET-based bioassay was also reported for the detection of the histone acetyltransferase activity using QDs as donors with dye as an acceptor.²² Particular

advantages of QDs such as the broad absorption spectra with large extinction coefficients, sharp and size-tunable fluorescence spectra, and chemically accessible surface area make them excellent choices as acceptors for FRET-based assays.¹⁷ Considering these facts, herein, we developed a rapid and sensitive homogenous enzyme assay for G9a on the basis of Tb-to-QD TR-FRET. The designed assay was applied for G9a inhibitor screening.

Materials and Methods

Materials

Human G9a with N-terminal GST tag expressed in Baculovirus infected Sf9 cell expression system was obtained from BPS Bioscience (San Diego, USA). Its concentration was 0.1 mg/mL and its MW was 74.6 kDa. It was aliquoted and stored at -80 °C. The working solution of the enzyme was prepared freshly by dilution with assay buffer. Anti-H3K9me2 monoclonal antibody (raised in Mouse against histone H3, dimethylated at lysine 9) with a concentration of 50 µg/50 µL was purchased from BPS Bioscience. Biotinylated Histone H3 (1-21) peptide with the sequence of H-ARTKQTARKSTGGKAPRKQLAGGK (Biotin)-NH₂, was obtained from AnaSpec Inc (Fremont, USA). The stock solution (500 µM) was prepared in ultrapure water, aliquoted and stored at -20°C. Working solution (1.0 µM) was prepared daily by diluting 1.0 µL of stock solution to 500 µL with assay buffer. S-(5'-adenosyl)-L-methionine chloride (SAM) as cofactor was obtained from Sigma (USA). Its stock solution (30 mM) was made in 5 mM H₂SO₄/10%v ethanol, aliquoted and stored at -80°C. The working solution (300 µM) was prepared just before use by mixing 1.0 µL of stock solution with 99 µL of assay buffer. S-(5'-adenosyl)-L-homocysteine (SAH) was also obtained from Sigma and a 25-mM stock solution was prepared in DMSO, aliquoted and stored at -20°C. Poly-L-lysine (0.01%) was obtained from Sigma and used for stopping the enzymatic reaction. Streptavidin (sAv)-coated QDs (with emission wavelengths of 605, 655 and 705) with a concentration of 2.0 µM were obtained from Life Technologies (ThermoFisher Scientific). Tris-HCl buffer (pH 8.5) containing 0.1% BSA was used as assay buffer in all experiments.

Instruments and equipment

Absorption spectra were recorded by a Lambda 35 UV/Vis System (PerkinElmer). PL decay curves were obtained by an EI fluorescence plate reader (Edinburgh Instruments) by the use of 4000 detection bins with 2 µs integration time and excitation at 337.1 nm with a nitrogen laser (20 Hz, VSL 337 ND, Spectra-Physics). Optical bandpass filters (Semrock) were (494 ± 10) nm for the Tb detection, (660 ± 7), (607 ± 5) and (707 ± 13) nm for the QD650, QD605 and QD705 detection channels, respectively.

Terbium bioconjugation

The conjugation of antibody (AB) with a terbium

complex (Tb) was done after the exchange of AB buffer to carbonate (100 mM, pH 9.0) using Zeba desalting column (7K MWCO, 0.5 mL). Subsequently, 1.04 μL of Tb-NHS (N-Hydroxysuccinimide) (8 mM in DMF) was added to the obtained AB in a centrifuge tube. It should be noted that the Tb/AB ratio was kept at 50. After briefly vortexing, the tube was wrapped in a foil and incubated in an Intelli mixer at 30 rpm for 4 hours. The obtained Tb-AB was purified with Zeba spin desalting columns (7K MWCO), by washing with 100 mM Tris buffer (pH 7.4). These columns contain a high-performance resin, which effectively retains small molecules and passes only larger compounds. To ensure all excess Tb is separated from Tb-AB conjugate, two rounds of purification was performed. The final volume of labeled AB was made to 150 μL with Tris buffer and its absorption spectrum was recorded by Lamda 35 Spectrophotometer.

General procedure for G9a enzyme assay

The mixture of 10 μL of assay buffer, 10 μL of G9a with various concentrations (the final concentrations were 0.02, 0.04, 0.08, 0.15 and 0.3 nM), 10 μL of peptide (400 μM) and 10 μL of SAM (100 μM) was incubated in a 96-well black microplate for 40 min. Afterward, 10 μL of detection mixture (containing 0.0005% poly-L-lysine, 0.01 μM Tb-AB and 2.5 nM sAv-QD705) was added into each well and incubated the microplate for more 1 hour. Lastly, PL decay curves of Tb donor and QD acceptor were measured in a time window from 0-8 ms. The time window from 100 to 900 μs was used for the calculation of TR-FRET ratio by the following equation: $\text{FRET-Ratio} = I(\text{ChA})/I(\text{ChD})$.

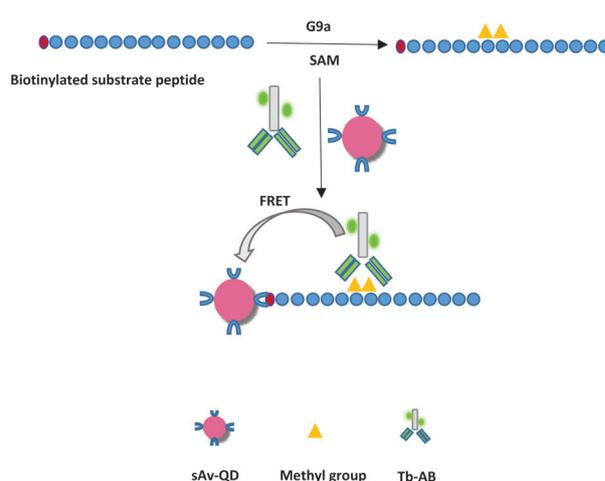
Procedure for inhibitor screening

The inhibitor screening procedure was almost the same as the previous procedure except that 10 μL of inhibitor with various concentrations in assay buffer (containing 2% DMSO) was first pre-incubated with 10 μL of G9a (0.6 nM) for 10 minutes. Final concentrations of inhibitor was 0.01, 0.1, 1.0, 10.0 and 100 μM . Afterward, the procedure was continued by adding peptide, SAM, then detection mixture according to the prior section.

Results

Principle of the bioassay

In this study, a rapid and sensitive homogenous enzyme assay was developed for G9a on the basis of Tb-to-QD time-resolved FRET. This enzyme acts on histone H3 and methylates its lysine 9 location. The amount of methylation is controlled by the activity of the enzyme. The overall strategy for assay was to use a Tb-conjugated antibody and biotinylated peptide together with streptavidin-coated QDs as a FRET donor and acceptor, respectively. We used biotinylated Histone H3 (1-21) peptide as a substrate. After enzymatic reaction with G9a and cofactor (SAM), which leads to the dimethylation of lysine 9 location on the substrate, the Tb-AB conjugate and sAv-QDs were added



Scheme 1. Principles of the G9a assay

and FRET ratio was measured, which was proportional to dimethylated peptide concentration and so to G9a activity. The illustration of the principles of this assay is shown in Scheme 1.

Conjugation of Tb to antibody

The most common technique for AB labeling involves the use of a chemical group that reacts with $-\text{NH}_2$. Due to the especially nucleophilic property of primary amines, they are suitable targets for conjugation with reactive groups including NHS Esters. Here a macrocyclic ligand-based Tb complex [23] activated by NHS was used for random labeling of AB. A high excess of Tb-NHS was used with respect to AB (50 molar excess). The absorption spectrum of purified Tb-AB conjugate is shown in Fig. S1 (Electronic Supplementary Material, ESM). The peak at 340 nm is due to Tb and the one at 280 nm is ascribed to AB. This confirms the binding of Tb to AB.

FRET pairs

In order to achieve suitable sensitivity in the enzyme assay, three different acceptors including QD605, QD655 and QD705 with the same Tb donor were tested. A macrocyclic ligand-based Tb complex²³ with high brightness was applied as a FRET donor. Each FRET pair was used for enzyme assay according to the general procedure. The obtained PL decay curves are shown in Fig. 1. The sensitization can be seen in the decay curves of the QD detection channels; the long-lifetime parts of these decay curves remarkably increase by adding G9a and SAM. These results show that FRET occurs with all pairs and so, Tb-to-QD FRET can be applied for the G9a assay. Control experiments with only Tb-AB and only QD-Peptide showed the background of Tb PL (spectral crosstalk in the QD detection channels) and directly excited QD PL. Furthermore, FRET efficiencies for the three mentioned FRET pairs were calculated using both Tb donor quenching and QD acceptor sensitization. All

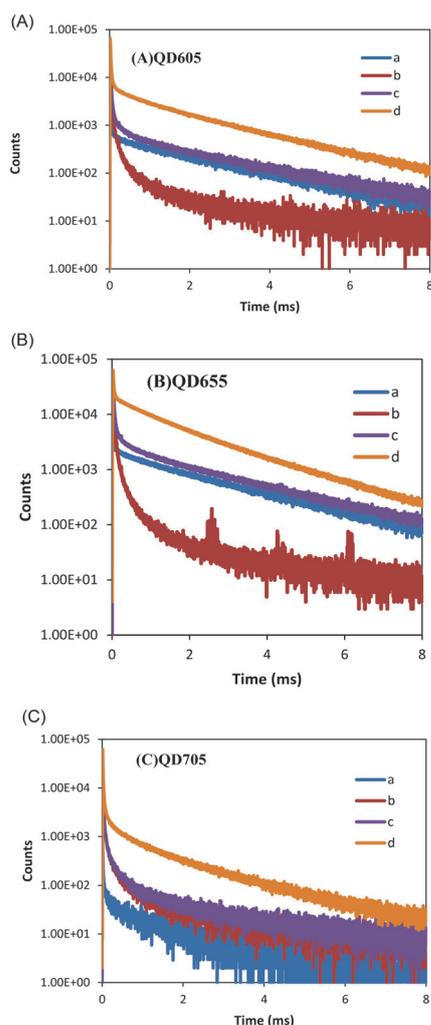


Fig. 1. PL decay curves of G9a assay obtained from acceptor channel for (A) QD605, (B) QD655 and (C) QD705. In each graph, (a) QD + peptide; (b); Tb-AB (c) Tb-AB + QD + peptide; (d) Tb-AB + QD + peptide + G9a + SAM.

fitting was performed by FAST software (Edinburgh Instruments). The results are given in Table 1. According to the obtained results, QD705 produced higher FRET efficiency and FRET ratio than the other QDs, so this QD was used for subsequent experiments.

G9a assay

The effects of several parameters, such as peptide and SAM concentration and reaction time, were examined to obtain the best condition for assaying the activity of G9a. It is known that the G9a enzymatic reaction takes place at an alkaline solution.²⁴ We chose pH 8.5 which is reasonably close to physiological pH, and at the same time, the enzymatic reaction is fast enough at this condition. The amount of H3(1-21) peptide on the enzymatic reaction was studied at a constant amount of G9a and saturating amount of SAM. The results are graphically shown in Fig. 2A. As can be seen, the methylation saturation is obtained above 100 nM of the peptide substrate. So this

Table 1. The FRET efficiencies using donor quenching and acceptor sensitization

QD	η FRET (D)%	η FRET (A)%
605	55	40
655	50	43
705	54	62

concentration of peptide was chosen for enzyme assay in the next experiments. The other factor that should be taken into account in the G9a enzymatic reaction is the concentration of cofactor, SAM, which provides the required methyl group for methylation reaction. As can be seen in Fig. 2B, above 20 μ M, the reaction and so the FRET signal reaches a plateau. Based on this result, the amount of SAM was adjusted in 20 μ M for subsequent works.

Moreover, a time course of methylation using peptide substrate H3(1-21) was monitored in the presence of SAM. The results are shown in Fig. 2C. As can be seen, the signal increased sharply up to 40 min and after that,

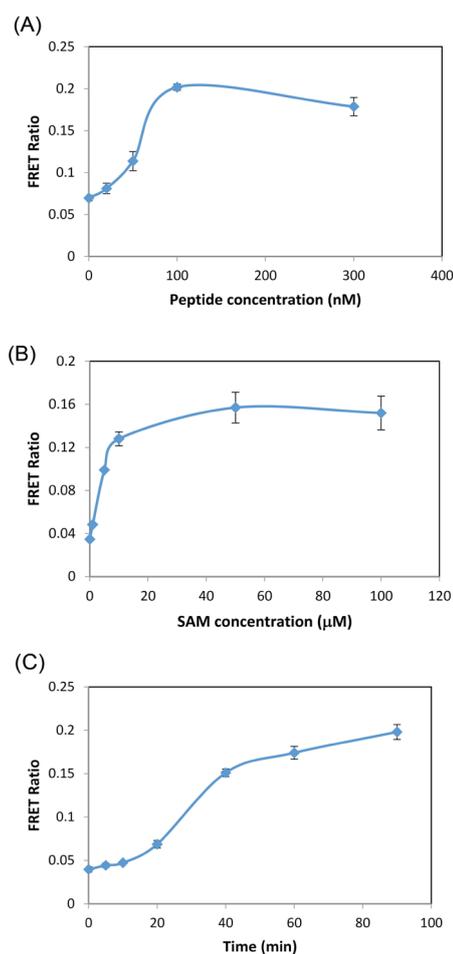


Fig. 2. (A) Effect of amount of peptide on the enzymatic reaction. Conditions: G9a, 0.15 nM; SAM, 50 μ M; incubation time, 40 min; Tb-AB 2 nM; QDs, 0.5 nM; (B) Effect of SAM concentration on the enzymatic reaction. Conditions: H3(1-21) Peptide, 100 nM, other condition same as (A); (C) Progress curve for G9a reaction. Conditions: same as (B).

the rate of reaction slowed down, possibly because of the formation of SAH.

At last, under the optimum conditions, a set of experiments was performed to investigate the relationship between FRET signal and concentration of G9a. An increasing concentration of recombinant G9a was added to the reaction mixture. The reaction was allowed to proceed for 40 min and then the FRET ratios were measured. The obtained results are shown in Fig. 3. As can be seen, FRET signal increases proportionally with the concentration of enzyme up to about 0.3 nM. At concentrations higher than this amount, the reaction reaches a steady state. There is a linear relationship between G9a concentration and signal in the range of 20–150 pM (Fig. 3 inset). The performance of the method is comparable with²⁵ or better than²⁶ some previously reported methods for G9a assay. Additionally, compared to the radioisotope-based method, which suffers from radioactive wastes and laborious steps, our method is simple and sensitive, which can be also applied for kinetic studies.¹²

We also investigated the selectivity of the enzyme assay by applying nonspecific peptide substrate (H4 (1–22), H3 (22–44)), co-enzyme (acetyl coenzyme A) and enzyme (GC5, SET7/SET9). As demonstrated in Fig. S2 (ESM), the signals from nonspecific compounds are comparable with that of control (contains no G9a) and so the method has good selectivity for G9a. In summary, the results confirmed that Tb-to-QD time-resolved FRET can be used for bioassay of G9a with satisfactory results.

Enzyme inhibition

In order to evaluate the possibility of application of the assay for inhibitor screening the effect SAH,²⁷ as an inhibitor, on the enzymatic reaction was examined. For this purpose, G9a was pre-incubated with various concentrations of inhibitor (in DMSO) before initiating reaction by adding peptide and SAM. Then, the designed FRET assay was exploited for the detection of enzymatic reaction products. As can be seen in Fig. 4, the sigmoidal dose-response curve was obtained for the inhibitor and IC₅₀ was calculated to be about 0.5 μM for SAH.

Discussion

The presented results show that an efficient FRET occurs between Tb and QDs after their conjugation with dimethylated peptide. QDs with an emission wavelength of 705 nm gives the highest FRET efficiency. This is due to the higher overlap between the absorption spectrum of QD705 and the fluorescence spectrum of Tb. In addition, at this wavelength, the background signal would be lower, which can be considered as another advantage. The use of time-gated detection also leads to a low background signal. Compared to methods like ELISA, our assay is much simpler because it is a homogeneous assay and does not need any separation step. One important advantage

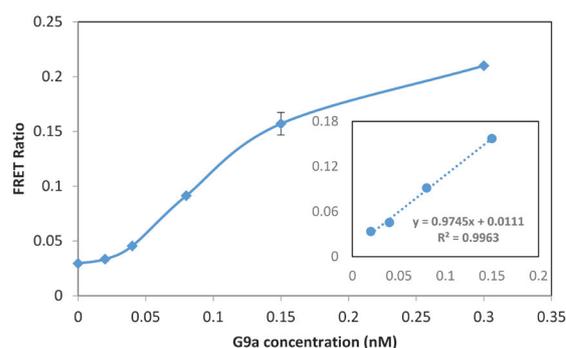


Fig. 3. G9a titration curve and corresponding calibration curve (inset). Conditions: H3(1-21) peptide, 100 nM; SAM, 50 μM; incubation time, 40 min; Tb-AB 2 nM; QDs, 0.5 nM.

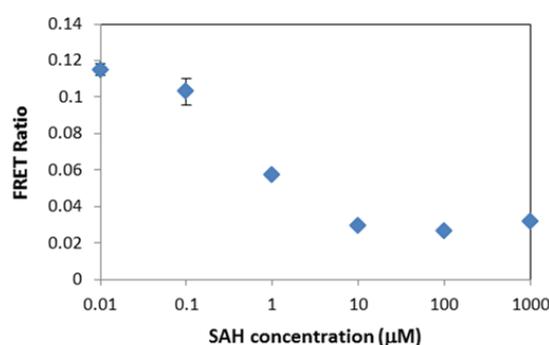


Fig. 4. Inhibition curve for SAH; Conditions: G9a, 0.15 nM; H3(1-21) Peptide, 100 nM; SAM, 10 μM; DMSO, 2%; incubation time, 40 min; Tb-AB 2 nM; QDs, 0.5 nM.

of using QDs as FRET acceptors is the possibility of performing multiplex assays. Currently, our group is working in this direction.

Study on the inhibition of epigenetic enzyme activity plays an important role in drug discovery and may open new ways in treatments of cancer.^{28,29} Indeed, the ultimate goal of this study was developing a high-throughput screening method for inhibitors. Therefore, in order to show the applicability of the G9a assay for studying the enzyme inhibition, SAH, which is a by-product of HMTs enzymatic reaction, was chosen as a well-known inhibitor of G9a, which acts through competition with SAM in the enzymatic reaction.³⁰ The results confirm that the method is suitable for inhibitor study.

Conclusion

We here demonstrated that Tb-to-QD TR-FRET can be applied as a sensitive tool for assay of G9a and subsequently for screening of its inhibitors in a simple homogeneous format. Terbium complex served as an efficient energy donor and streptavidin-coated ZnS/CdSe core-shell QDs acted as acceptors. The FRET efficiency was more than 50% and enzyme concentrations as low as 20 pM could be detected. We anticipate that this approach can be used as

Research Highlights

What is the current knowledge?

✓ Time-resolved FRET is a selective and powerful method for bioassay.

✓ QDs are bright acceptors in FRET-based sensors.

What is new here?

✓ Tb-to-QD time-resolved FRET was used for sensitive detection of G9a.

✓ A fast and homogeneous bioassay was developed for G9a and its inhibitors.

a versatile sensing platform for the development of assays for other epigenetic enzymes by changing the substrate peptide and corresponding antibody. These assays would be suitable for inhibitor screening and drug-discovery applications.

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Ethical statement

None to be declared.

Competing interests

There is no conflict of interests to be reported.

Authors' contribution

MA: Supervision, experiments design, data analysis, study consultation, writing and reviewing. TH: Experiments design, data analysis, draft preparation. NH: Provision of study materials and equipment, study consultation, writing and reviewing.

Supplementary Materials

Supplementary file 1 contains electronic supplementary material.

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