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A methylation-switchable conformational probe for sensitive and selective detection of RNA demethylase activity†

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We describe a novel methylation-sensitive nucleic acid (RNA) probe which switches conformation according to its methylation status. When combined with Differential Scanning Fluorimetry technique, it enables highly sensitive and selective detection of demethylase activity at a single methylated-base level. The approach is highly versatile and may be adapted to a broad range of RNA demethylases.

N-methylation of nucleic acid bases has been a subject of intense chemical and biological interest because of its critical roles in several key cellular processes, such as epigenetic gene regulation, RNA metabolism and cellular reprogramming.[1] In recent years, it has become apparent that the methylation status of nucleic acid is dynamically regulated by a complex interplay between DNA/RNA methyltransferases[2] and demethylases,[3] which add and remove 'methyl marks' from nucleic acid bases, respectively. It is also increasingly clear that dysregulation of these enzymes may underlie the pathogenesis of a range of human diseases,[4] hence there is currently tremendous interest in their mechanistic and inhibition studies.

One major class of DNA/RNA demethylase is the AlkB family of iron- and 2-oxoglutarate (2OG)-dependent dioxygenases, which includes *Escherichia coli* AlkB, and nine human homologues, ALKBH1-8 and FTO (fat mass and obesity-associated protein) (Fig. 1a).[5] The activity of AlkB demethylases is extremely challenging to assay, in part due to the small size and chemical inertness of the methyl group they remove. Consequently, despite significant efforts by various groups,[6-10] there is, at present, a lack of sensitive and high-throughput technique for their analysis. Current limited assays rely primarily on indirect approaches, such as the analysis of demethylation co-products (NADPH, FADH, formaldehyde) through coupled assays,[6] or the analysis of demethylated products following their digestion with restriction enzyme.[7] However these methods suffer from false positives/negatives that are typically associated with coupled reactions. They also require multiple steps which are laborious, time-consuming, and generally give poor reproducibility. Although direct detection of DNA/RNA demethylation has been achieved using radio-labelled substrates,[8] MALDI mass spectrometry,[9] and capillary electrophoresis,[10] these techniques lack throughput and/or sensitivity, which severely hinders the study of this important class of enzymes.

Recent studies have demonstrated that nucleic acid base methylation can induce a major overall conformational change in certain sequence contexts.[11] For instance, 5-methylcytosine has been shown to induce B- to Z-transition in some DNAs,[11a] while N^6-methyladenosine (m6A) could induce duplex-hairpin conversion in certain RNA sequences.[11b] Inspired by these interesting observations, we envisaged that (de)methylation-induced conformational change could provide a basis for the design of 'conformational probe' useful for sensing methylation change. To the best of our knowledge, this concept has not previously been applied to the analysis of DNA/RNA methylation, and, to date, there is no report of assay method which is based on methylation-sensitive probe.

Herein, we describe a novel detection strategy for RNA demethylase activity, which combines the discriminatory power of dynamic conformational probe with the sensitivity of Differential Scanning Fluorimetry (DSF) technique (Fig. 1b). In this approach, a structurally dynamic oligonucleotide that changes secondary structure according to its methylation status is employed as conformational probe. By design, when the probe is methylated, it preferentially adopts a hairpin conformation. However, on selective removal of methylation by a demethylase, the probe undergoes spontaneous and rapid transformation to a duplex conformation. Such a major switch from hairpin to duplex structure is accompanied by profound

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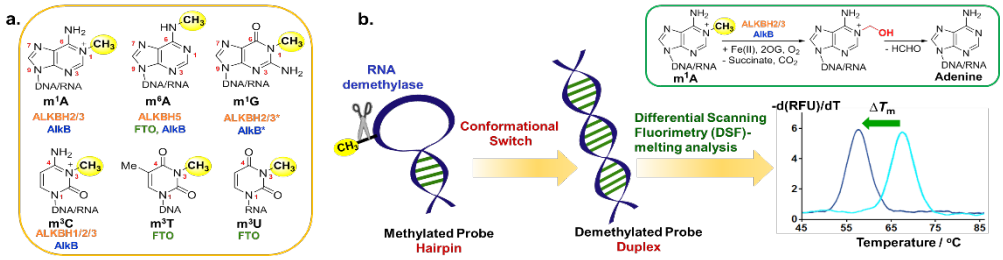


Fig. 1 (a) *N*-methylation on DNA/RNA that are selectively demethylated by the AlkB subfamilies (* weak activity). (b) The conformational probe-DSF strategy. The conformational probe is highly responsive to its methylation status. Removal of the methyl group by RNA demethylase triggers a dramatic switch from hairpin to duplex structure. This causes a profound change in the T_m of the probe, which can be sensitively detected *via* DSF technique. Inset shows the oxidative demethylation of m¹A by the m¹A-demethylases.

change in a number of biophysical properties, notably the melting temperature (T_m) of the probe, which can be accurately measured using DSF-based melting analysis. DSF is a simple and inexpensive technique that has been widely used for the detection of SNPs and protein-ligand interaction in 'thermal shift assay'.[12] However, to date, DSF (and the related high-resolution melt analysis)[13] has not been applied to the analysis of RNA secondary structure. As we shall demonstrate, DSF is a powerful technique for monitoring methylation-induced RNA conformational change. When used in combination with a conformational probe, it has the sensitivity to detect changes in RNA down to a single methylated base.

To implement the proposed strategy, we design a 'conformational probe' that is structurally-responsive to methylation changes. In this study, we used an m¹A-containing probe with a view of developing a high-throughput assay against m¹A-demethylases ALKBH2 and ALKBH3, which are potential therapeutic targets for cancer.[4] Preliminary studies of a small library of palindromic RNAs led us to identify r(CGCG**m¹A**AUUCGCG) as our model m¹A-probe (Table S1, ESI†). It consists of two terminal 'CGCG segments' and a middle 'AAUU segment' (underlined), where m¹A resides (for chemical synthesis, see ESI†). Due to its self-complementary nature, it can inherently adopt a bimolecular duplex, by engaging in intermolecular base pairing, and a monomolecular hairpin, by folding back on itself (Fig. 2a). We reasoned that since m¹A impairs Watson-Crick base pairing, the probe likely favours hairpin conformation, where m¹A can be stably localised within the 'loop' of the hairpin.

To verify our probe design, we performed DSF-based melting analysis of m¹A-probe. In this method, the melting profile was obtained by monitoring the fluorescence intensity of an RNA intercalating dye (SYBR Green I) at 520 nm as a function of temperature. The fluorescence spectrum showed a monophasic, sigmoid transition, implying that the probe exists predominantly as a single conformation under our experimental conditions (Fig. 2b). Moreover, the T_m of m¹A-probe is relatively constant over a 100-fold concentration range (Fig. 2c), suggesting that the probe likely adopts a hairpin conformation, as anticipated. In contrast, the melting profile of its demethylated counterpart r(CGCG**A**AUUCGCG) shows a concentration-dependent T_m, which is indicative of a duplex structure (Fig. 2b and 2c). The above results were confirmed by circular dichroism (CD)- and UV-melting analyses, which gave melting profiles that are highly consistent with those derived from DSF analysis (Fig. S1 and S2, ESI†). In addition, the CD spectra of m¹A- and demethylated probes clearly reveal characteristics that are consistent with B-like hairpin and A-form duplex, respectively (Fig. 2d).

To obtain additional insights into their conformations, we performed ¹H NMR analysis of the probes. The imino spectrum of m¹A-probe revealed only four NH peaks at 4 °C, likely suggesting a hairpin structure in which the stem contains four C·G base pairs, and the loop consists of four unpaired **m¹A**AUU nucleotides (Fig. 2e). The demethylated probe, however, showed six imino peaks, implying a fully base-paired duplex structure (Fig. 2f). We are aware that for more vigorous proof of probe conformations, other methods such as 2D NMR analysis are required. Nevertheless, our combined data clearly demonstrate that the conformation of the probe is highly dependent on its methylation status. It further suggests that demethylation of m¹A-probe may induce a hairpin-duplex

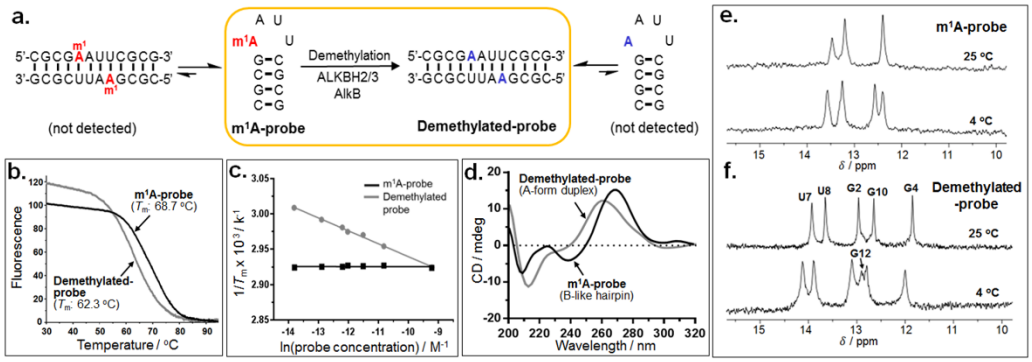
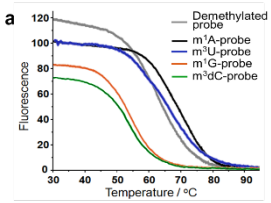


Fig. 2 Verification of m¹A-probe design. (a) The m¹A- and demethylated-probes were found to exist predominantly as hairpin and duplex conformations, respectively, as demonstrated by their (b) DSF-based melting profiles, (c) van't Hoff plots, (d) CD spectra, and (e,f) imino ¹H NMR spectra. Assignment of imino peaks for the demethylated-probe was based on literature data.[14] The terminal C·G base pair was not detectable at 25 °C for both probes, likely due to 'fraying'.



b.

	Sequence	Preferred conformation	T_m (°C) (at 5 μM)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol/K)	$\Delta G°_{310}$ (kcal/mol)
Demethylated-probe	r(CGCGAAUUCGCG)	Duplex	62.3 ± 0.2	-108.8 ± 1.2	-300.0 ± 3.4	-15.8 ± 0.2
m^1A-probe	r(CGCGm^1AAUUCGCG)	Hairpin	68.7 ± 0.3	-46.9 ± 0.9	-137.0 ± 2.1	-4.3 ± 0.1
m^3U-probe	r(CGCGAAUm^3UCGCG)	Hairpin	66.5 ± 0.2	-44.3 ± 0.7	-130.4 ± 1.7	-3.8 ± 0.2
m^1G-probe	r(CGCm^1GAAUUCGCG)	Hairpin	54.6 ± 0.3	-36.8 ± 1.5	-112.3 ± 3.6	-2.0 ± 0.1
m^3dC-probe	r(CGCGAAUUm^3dCGCG)	Hairpin	53.9 ± 0.1	-34.7 ± 1.3	-106.1 ± 2.1	-1.8 ± 0.1

c.

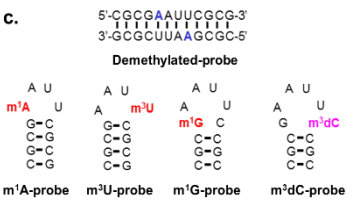


Fig. 3 Conformational probes investigated in this study. (a) The T_ms were measured at 5 μM strand concentration (in 10 mM sodium phosphate buffer, pH 7.0 containing 150 mM NaCl) using DSF-melting analysis. (b) $\Delta H°$ and $\Delta S°$ were derived from 1/T versus ln(concentration) plots (for duplexes) and from α versus T plots by curve fitting (for hairpins), assuming a two-state process. (c) Probes containing m1A, m3U, m1G and m3dC likely adopt a hairpin structure, as their T_ms are independent on strand concentration (Fig. S11-S13, ESI†).

transformation, consistent with our design strategy. Notably, only one set of NMR signals was observed for m^{1}A- and demethylated-probes, suggesting that they exist almost exclusively as single conformation in solution and do not co-exist as other secondary structures. The absence of the duplex form of m^{1}A-probe also implies negligible Hoogsteen base pairing between m^{1}A and U in this RNA sequence context. This result is interesting because Hoogsteen base pairs have been observed between m^{1}A and T in DNA duplexes.[15]

The thermodynamic parameters derived from DSF-melting experiments further revealed that the observed hairpin-duplex conversion is primarily an enthalpy-driven process ($\Delta\Delta H°$ = -61.9 kcal/mol), and despite the large entropy cost ($\Delta\Delta S°$ = -163.0 cal/mol/K), the demethylated duplex is significantly more stable than the m^{1}A hairpin ($\Delta\Delta G°_{310}$ = -11.5 kcal/mol) (Fig. 3, S3 and S4, ESI†). We appreciate that m^{1}A can potentially undergo base-catalysed Dimroth rearrangement to yield m^{6}A,[16] nevertheless, in our DSF analyses, the T_m of m^{1}A-probe was highly reproducible, suggesting little or no degradation under our experimental conditions. Our combined results, therefore, established m^{1}A-probe as a stable, methylation-responsive probe suitable for reporting demethylase activity.

We next examine if m^{1}A-probe could be recognised and demethylated by ALKBH2. In a typical conformational probe-DSF assay, the enzyme (50 nM) was incubated with m^{1}A-probe (10 μM) in the presence of co-substrate 2-oxoglutarate (10 μM), Fe(II) (10 μM), and L-ascorbate (200 μM) in 50mM HEPES buffer (pH 7.0) at 4 °C for various time intervals, after which the reaction was quenched and product formation was analysed using DSF-melting analysis. The negative first derivative plots are shown in Fig. 4a-e. In the absence of enzyme, only one melting peak **S** corresponding to the denaturation of m^{1}A-probe substrate was detected. However, after a 5-minute incubation with ALKBH2, a distinct biphasic melt transition was observed. The new melting peak **P** corresponds to the dissociation of the demethylated-probe, as verified by HPLC-based analysis (Fig. S5, ESI†). Consistent with our detection strategy, there was a large difference in T_m (ΔT_m = 9.9 °C) between substrate and product despite only a single methyl group change, presumably due to demethylation-induced hairpin-duplex conversion. This allows a resolution of their melting peaks. By comparison, a methyl mark difference between otherwise identical nucleotide sequences usually produces insignificant T_m shifts.[13]

As the reaction progressed, the product showed a time-dependent increase in T_m. Detailed analysis revealed an excellent linear relationship between product T_m and ln(product concentration), hence, the T_m of product provides a direct indication of demethylase activity (Fig. 4h-i). Analytical sensitivity tests further showed that DSF has the sensitivity to reliably detect serially diluted product as little as 100 nM (Fig. S6, ESI†). In addition, enzyme titration studies with substrate at 10 μM indicated that activity can be measured at ALKBH2 concentrations as low as 10 nM (Fig. S7, ESI†).

To evaluate the affinity and specificity of m^{1}A-probe towards the m^{1}A-demethylases, we determined the kinetic parameters for demethylation of m^{1}A-probe, m^{1}A-ssRNA and m^{1}A-dsRNA by ALKBH2, ALKBH3 and AlkB (Table 1). A comparison of the k_{cat}/K_m values reveals that m^{1}A-probe is an excellent substrate for ALKBH2, with efficiency that is comparable to that of m^{1}A-ssRNA and only slightly lower than m^{1}A-dsRNA (Table 1, Fig. S8 and S9, ESI†). This result suggests that ALKBH2 does not exhibit strict conformational requirement for substrate recognition. Intriguingly, m^{1}A-probe can also be efficiently demethylated by ALKBH3 and AlkB, despite their strong preference for single-

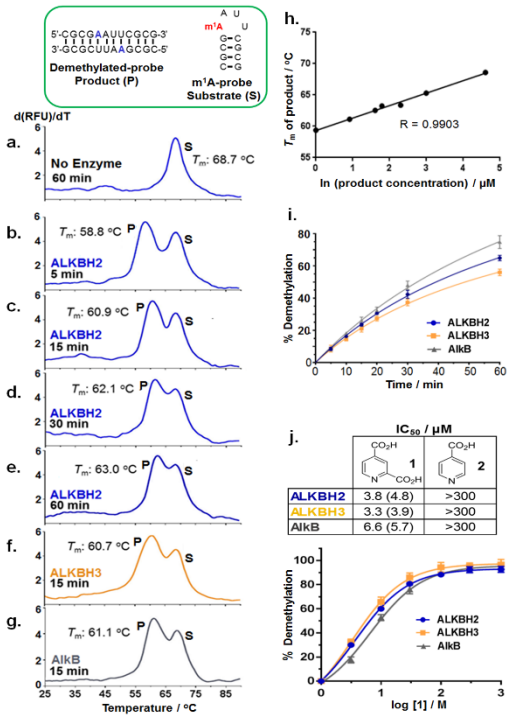
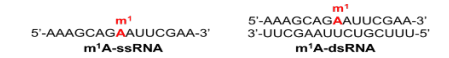


Fig. 4 Conformational probe-DSF assay of AlkB demethylase activity. The negative first derivative plots show melting profiles of reaction mixtures (a) in the absence of enzyme, and after incubation with (b-e) ALKBH2, (f) ALKBH3 and (g) AlkB. (h) There is excellent linear relationship between product T_m and ln(product concentration). (i) Time course analyses of m^{1}A-probe (10 μM) incubated with various m^{1}A demethylases (50 nM). (j) Enzyme inhibition curves of **1** against ALKBH2/3 and AlkB, as determined by DSF-based assay. The IC_{50}s are comparable to those derived from HPLC-based assay (in parentheses). Errors represent S.D. of three replicates.

Table 1 Kinetic analyses of m^{1}A demethylation by m^{1}A-demethylases.



ALKBH2	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}μM^{-1})
m^{1}A-probe	1.09 ± 0.14	3.67 ± 0.35	3.37
m^{1}A-ssRNA	0.95 ± 0.11	4.07 ± 0.15	4.30
m^{1}A-dsRNA	0.30 ± 0.07	2.19 ± 0.05	7.36
ALKBH3	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}μM^{-1})
m^{1}A-probe	1.47 ± 0.08	3.13 ± 0.22	2.13
m^{1}A-ssRNA	1.12 ± 0.16	3.56 ± 0.31	3.18
m^{1}A-dsRNA	6.60 ± 0.19	2.57 ± 0.27	0.39
AlkB	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}μM^{-1})
m^{1}A-probe	1.30 ± 0.12	4.10 ± 0.29	3.16
m^{1}A-ssRNA	1.44 ± 0.25	3.75 ± 0.12	2.61
m^{1}A-dsRNA	3.56 ± 0.24	2.25 ± 0.18	0.63

stranded substrates (Fig. 4f-i and Table 1). In fact, ALKBH3 and AlkB exhibit ~5-fold greater activities for m^{1}A-probe than for m^{1}A-dsRNA. To our knowledge, this is the first report demonstrating that m^{1}A-demethylases, in general, are able to recognise and accept substrates with hairpin structures. Importantly, m^{1}A-probe also displayed remarkable selectivity for m^{1}A demethylases, as demonstrated by the lack of product formation when incubated with AlkB subfamily members ALKBH5 and FTO, both of which are specific for m^{6}A with negligible activity towards m^{1}A substrates (Fig. S5, S10, ESI†).

Encouraged by these promising results, we proceeded to investigate the use of conformational probe-DSF assay for inhibitor screening. We initially profiled 2,4-pyridine dicarboxylic acid **1**, a known 'pan-inhibitor' of AlkB subfamilies,[17] for activity against the m^{1}A demethylases (Fig. 4j). In this high-throughput screen, the melting profiles of up to 96 samples were recorded simultaneously on a Real-Time PCR machine (for inhibition assay conditions, see ESI†). Our assay was able to successfully identify **1** as a potent inhibitor against all AlkB enzymes tested. We observed a gradual decrease in product T_m with increasing concentrations of **1**, and the determined IC_{50} values are comparable to those obtained using a HPLC-based assay (Fig. 4j). Moreover, in a negative control experiment, isonicotinic acid **2** (which lacks the 2-carboxylic acid group that is critical for activity) showed poor inhibition towards all AlkB enzymes, hence validating this assay as a reliable screening method for identifying m^{1}A demethylase inhibitors.

To further explore the utility of our conformational probe approach, we generated analogues of demethylated-probe containing other physiologically relevant *N*-methylation, namely N^3-methyluridine (m^{3}U), N^1-methylguanosine (m^{1}G) and N^3-methyldeoxycytosine (m^{3}dC) (Fig. 3). Remarkably, the introduction of these modified bases triggered a similar duplex-hairpin conversion, as determined by DSF-melting analyses (Fig. S11-S13, ESI†). This again amplifies the T_m difference between the methylated and demethylated species, which enables their excellent discrimination (Fig 3a). Hence, our general design for conformational probe is highly adaptable, and could easily be modified for the study of a range of *N*-methylated bases.

In conclusion, we have provided proof-of-principle that a novel strategy exploiting methylation-induced conformational change and DSF analysis can enable highly sensitive and selective detection of RNA demethylase activity. This is demonstrated by the development of the first conformational probe-DSF assay which is generally applicable to all currently known m^{1}A demethylases. The developed strategy provides a simple, direct and label-free detection, without recourse to radioactive or expensive techniques. It requires only small (nanomolar) amount of proteins and is amenable to high-throughput format. Hence, we anticipate that this assay shall be of widespread interest with regard to its potential use in a broad range of demethylation studies, such as the identification of novel demethylases, and the discovery of therapeutic leads or selective probes for AlkB isoforms. Importantly, the detection strategy outlined here is highly versatile and may, in principle, be adapted to the study of a range of RNA demethylases, and, more widely, other RNA-modifying enzymes.

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Notes and references

1 (a) Y. Fu, D. Dominissini, G. Rechavi and C. He, *Nat Rev Genet*, 2014, **15**, 293; (b) K. D. Meyer and S. R. Jaffrey, *Nat Rev Mol Cell Biol*, 2014, **15**, 313; (c) H. Grosjean, *Fine-Tuning of RNA Functions by Modification and Editing*, Springer Heidelberg, 2005; (d) B. S. Zhao and C. He, *Genome Biol*, 2015, **16**, 43; (e) S. C. Wu and Y. Zhang, *Nat Rev Mol Cell Biol*, 2010, **11**, 607.

2 (a) H. Grosjean, *DNA and RNA Modification Enzymes: Structure, Mechanism, Function and Evolution*, CRC Press, 2009; (b) J. A. Law and S. E. Jacobsen, *Nat Rev Genet*, 2010, **11**, 204; (c) Y. Motorin and M. Helm, *RNA*, 2011, **2**, 611.

3 C. J. Schofield and R. P. Hausinger, *2-Oxoglutarate-Dependent Oxygenases*, Royal Society of Chemistry, 2015.

4 (a) F. Lyko, R. Brown, *J Natl Cancer Inst*, 2005, **97**, 1498; (b) J. Fischer, L. Koch, C. Emmerling, J. Vierkotten, T. Peters, J.C. Bruning, U. Ruther, *Nature*, 2009, **458**, 894; (c) M. Camp, B.F. Eichman, *Mol Cell*, 2011, **44**, 343.

5 (a) B. Sedgwick, *Nat Rev Mol Cell Biol*, 2004, 5, 148; (b) B. I. Fedeles, V. Singh, J. C. Delaney, D. Li and J. M. Essigmann, *J Biol Chem*, 2015, **290**, 20734.

6 T. W. Roy and A. S. Bhagwat, *Nucleic Acids Res*, 2007, **35**, 147.

7 (a) G. Jia, C. He et al. *Nat Chem Biol*, 2011, **7**, 885; (b) J. D. W. Toh, Y. G. Gao, E. C. Y. Woon, et al. *Chem Sci*, 2015, **6**, 112; (c) M. Ma, H. P. Harding, S. O'Rahilly, D. Ron and G. S. Yeo, *Biochemical J*, 2012, **444**, 183; (d) B. Chen, C. G. Yang et al. *J Am Chem Soc*, 2012, **134**, 17963.

8 (a) S. C. Trewick, T. F. Henshaw, R. P. Hausinger, T. Lindahl, B. Sedgwick, *Nature*, 2002, **419**, 174; (b) D. H. Lee, T. R. O'Connor et al. *J Biol Chem*, 2005, **280**, 39448.

9 J. C. Delaney et al. *Nat Struct Mol Biol*, 2005, **12**, 855.

10 E. C. Y. Woon, C. J. Schofield et al. *J Med Chem*, 2012, **55**, 2173.

11 (a) M. Behe, G. Felsenfeld, *Proc Natl Acad Sci U S A* 1981, **78**, 1619; (b) R. Micura, W. Pils, C. Höbartner, K. Grubmayr, M. O. Ebert and B. Jaun, *Nucleic Acids Res*, 2001, **29**, 3997; (c) C. Roost, S. R. Lynch, P. J. Batista, K. Qu, H. Y. Chang and E. T. Kool, *J Am Chem Soc*, 2015, **137**, 2107; (d) J. D. Engel and P. H. von Hippel, *J Biol Chem*, 1978, **253**, 927.

12 F.H. Niesen, H. Berglund,M. Vedadi, *Nat Protoc*, 2007, **2**, 2212.

13 C. M. Rodriguez Lopez, B. Guzman Asenjo, A. J. Lloyd and M. J. Wilkinson, *Anal Chem*, 2010, **82**, 9100.

14 S. H. Chou, P. Flynn and B. Reid, *Biochemistry*, 1989, **28**, 2422.

15 (a) E. N. Nikolova, E. Kim, A. A. Wise, P. J. O'Brien, I. Andricioaei, H. M. Al-Hashimi, *Nature*, 2011, **470**, 498; (b) L. Lu, C. Yi, X. Jian, G. Zheng and C. He, *Nucleic Acids Res*, 2010, **38**, 4415; (c) H. Yang, Y. Zhan, D. Fenn, L. M. Chi and S. L. Lam, *FEBS Lett*, 2008, **582**, 1629.

16 J. D. Engel, *Biochem Biophys Res Commun*, 1975, **64**, 581.

17 N. R. Rose, E. C. Y. Woon, et al. *J Med Chem*, 2012, **55**, 6639.