Development of an HTRF-based High Throughput assay for the identification of natural ligands for AMP-activated protein kinase

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Introduction

AMP-activated protein kinase (AMPK) is a central cellular energy sensor and regulator [(1)]. To support the research activity on this target the Natural Bioactives and Screening department has the mission to screen natural products that could positively modulate the activity of this enzyme. A radiometric assay was originally developed for our research purposes. Upon activation, AMPK transfers a radiolabeled γ-phosphate (^{32}P) from an ATP to a positively-charged peptide substrate. The phosphorylated substrate binds to a phosphocellulose paper and the enzyme activity can be evaluated by counting the radioactivity[(2)] . Although very sensitive and accurate this methodology is not suitable for high-throughput screening (HTS) campaigns as it normally requires several manual steps and also consumes/produces a substantial amount of radioactivity and waste materials. In contrast, the Homogenous Time Resolved FRET technology (HTRF) is well adapted to HTS and automation: it helps to design simple assays which are homogeneous and usually only requires a few steps that can easily be automatized[(3)] . Two alternative assays from Cisbio based on this technology were evaluated: the HTRF® Transcreener® ADP and the HTRF® KinEASE™ STK-S1, and an assay was developed and validated up to a proof of concept screen which was performed on a set of 1000 samples.

Assay Selection

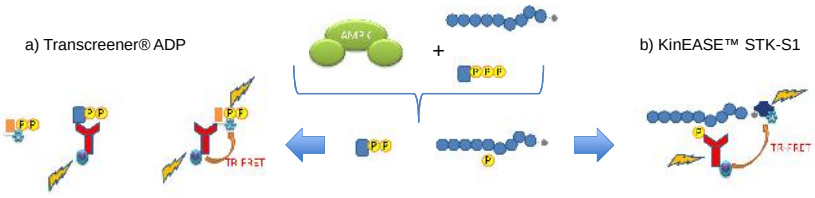


Figure 1: Two distinct approaches based on HTRF technology are used to Monitor AMPK activity: a) Quantification of ADP produced by competition using Transcreener® ADP kit; or b) direct quantification of a substrate phosphorylation using KinEASE™ STK-S1 kit

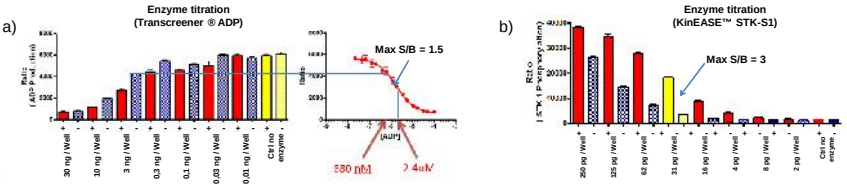


Figure 2: Various concentrations of enzyme are tested in presence (+) or in absence (-) of 30 µM AMP. Reaction is monitored either with a) Transcreener® ADP kit, or b) with KinEASE™ STK-S1 kit. The results are reported using the ratio signal (665/620):

Assay development

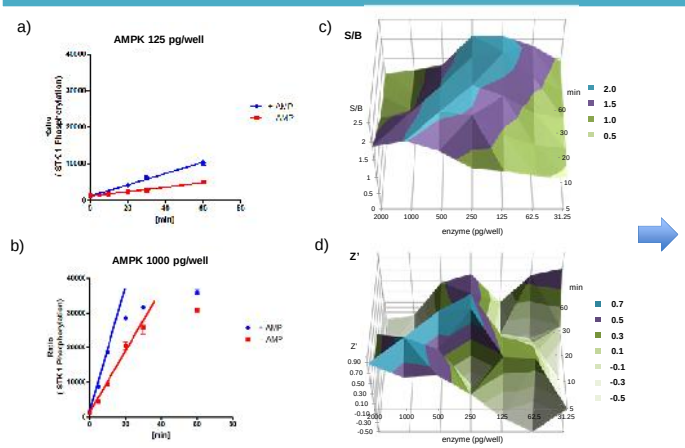


Figure 3: The influence of the reaction duration on substrate phosphorylation is measured in presence or in absence of 30 µM AMP using 125pg/well (a) or 1000 pg/well (b) of AMPK. Signal to background (c) and Z' factor (d) are then calculated on a broader condition test.

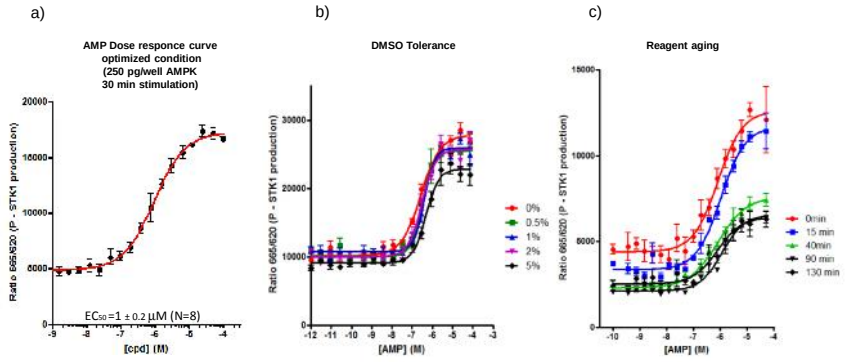
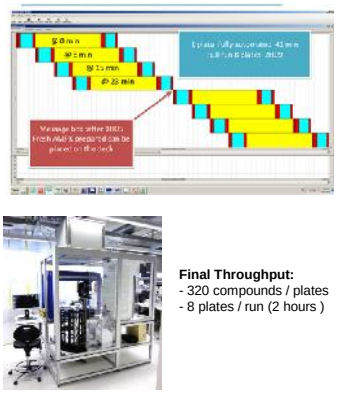


Figure 4: a) AMP titration is performed first in optimized conditions; b) The effect of % DMSO on AMP titration is evaluated; c) Finally the stability at room temperature of the various reagents is estimated by starting the experiment after 5 different waiting times.

Automation



Final Throughput:
- 320 compounds / plates
- 8 plates / run (2 hours)

→ **Up to 10 000 samples /day**

Figure 5: Automation on the PerkinElmer® Staccato platform. TR-FRET signal is measured off-line using Biotek® SynergyNeo

Proof of concept screening

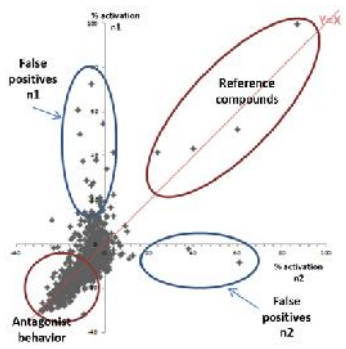


Figure 6: Comparison of n1 Vs n2 % of activation on a proof of concept screening of 1000 samples

Conclusion

- Transcreener® ADP kit allows a full flexibility on substrate selection and can be used as a universal readout for all type of kinases
- Direct phosphosubstrate quantification using KinEASE™ STK-S1 requires less enzyme and gives a better S/B ratio (***Figure 2***)
- The enzyme concentration and the reaction duration are two parameters that greatly influence kinase reaction (***Figure 3a,3b)***
- Optimal assay conditions can be obtained by co-optimization of enzyme concentration and reaction duration (***Figure 3c,3d)***
- AMP EC50 determined in the optimized conditions of 1 ± 0.2 µM ***(Figure 4)*** matches the one obtained on the original radiometric assay
- DMSO up to 2% has no impact on AMPK enzymatic reaction. Samples will be tested at a final concentration of 1% DMSO.
- After 15 min at room temperature the enzyme activity is reduced. Freshly prepared enzyme must be used for each run on the robotic platform.
- Automation of this assay enabled to reach a throughput of up to 10000 samples/ day ***(Figure 5).***
- Proof of concept screening results from 1000 compounds: (***Figure 6)***
  - No hits were identified,
  - 14 samples were false positives
  - 17 to 20% of the samples presented an antagonist behavior
  - 5.6 % presented interference with the HTRF technology

References:

1) AMP-activated protein kinase: a target for drugs both ancient and modern.*Chem Biol2012 Oct 26;19(10* Hardie DG1, Ross FA, Hawley SA.
2) Mechanism of action of compound-13: an α1-selective small molecule activator of AMPK. *Chem Biol 2014 Jul 17;21(7):866-79.* Hunter RW[1], Foretz M[2], Bultot L[3], Fullerton MD[4], Deak M[3], Ross FA[5], Hawley SA[5], Shpiro N[6], Viollet B[2], Barron D[3], Kemp BE[7], Steinberg GR[4], Hardie DG[5],Sakamoto K[8].
3) Development of a HTRF® Kinase Assay for Determination of Syk Activity. *Curr Chem Genomics. 2008; 1: 20–26.* Christopher Harbert,* Jeannette Marshall, Sharon Soh, and Krista Steger