Real-Time Monitoring of ATP Depletion Suitable for HTS

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Summary

Kinases, ATPases and aminoacyl tRNA synthetases are examples of enzymes that can be measured by ATP depletion assays. The disappearance of ATP can be measured either after a pre-determined reaction time or continuously monitored in real-time. In both cases the firefly luciferase reaction can be employed. Setting up the real-time assay as a first order reaction measuring the rate constant of the ATP depletion has several advantages:

- The rate constant can be calculated from measurements of the light signal performed at any two points in time and is unaffected by variations in initial ATP concentration and by library compounds inhibiting luciferase.
- The assay can be miniaturised and is highly suitable for HTS.
- Deviation from first order kinetics is easily detected by monitoring the signal over time.
- Measuring ATP depletion as a first order reaction, i.e. at low ATP, gives a higher sensitivity, which means less enzyme consumption.
- A half-time of 10 h of the blank (no enzyme except luciferase) allows reaction times from minutes to hours depending on enzyme activity.
- The assay is linear over three orders of magnitude with a z =0.96.
- These properties make the assay very easy to set up.

Assay Principles

- Negligible ATP consumption in luciferase reaction
- Luciferase activity unchanged during measurement
- Linear range of luciferase reaction, i.e.[ATP]≤1 µM
- Real-time measurement of ATP depletion by having luciferase and luciferin present during ATP degrading reaction:

  ATP + substrate → ADP+substrate-Pi

  ATP+D-luciferin+O_2 → AMP+PPi+oxyluciferin+CO_2+light
- First-order reaction kinetics, i.e.

  1) [ATP]<<Km for ATP degrading reaction

  2) [ATP]<<[substrate]
- Light readings in presence of ATP degrading activity normalised by dividing with corresponding readings in absence of ATP degrading activity
- The first order rate constant, k=[ln(I_1)-ln(I_2)]/(t_2-t_1), is used as a measure of ATP degrading activity, where I_1 and I_2 are normalised light emission at times t_1 and t_2.

Effect of ATP Concentration

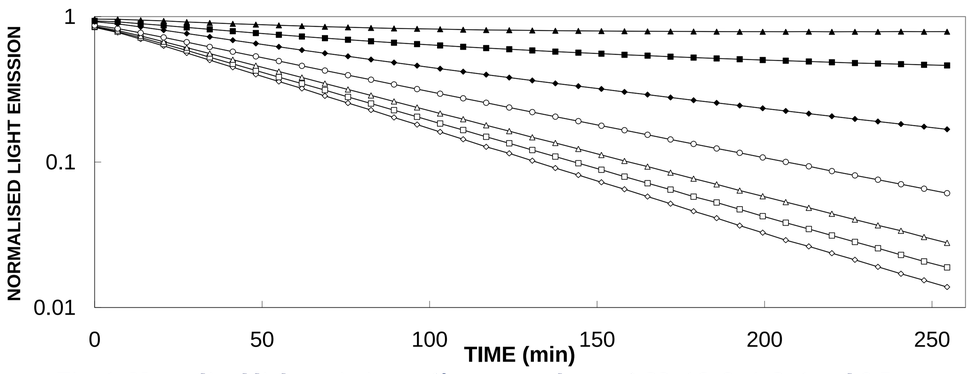

Fig. 1: Normalised light emission at (from top to bottom) 32, 16, 8, 4, 2, 1 and 0.5 µM ATP. PKA (0.125 ng/µL) with Kemptide (23.5 µM) as substrate.

Effect of Luciferase Inhibitors

As many as 3% of substances in compound libraries may inhibit luciferase (Auld D S *et al.* J Med Chem 2008, 51, 2372-2386). Such compounds are unlikely to be scored as kinase inhibitors, when the light is read only once, because inhibition of both the protein kinase and the luciferase will have opposite effects on the light signal, potentially resulting in false negatives. If on the other hand the light is read more than once so that a decay rate can be calculated and used as a measure of kinase activity, the effect of luciferase inhibition cancels out. This is illustrated in Fig. 2 with an assay of protein kinase A (PKA).

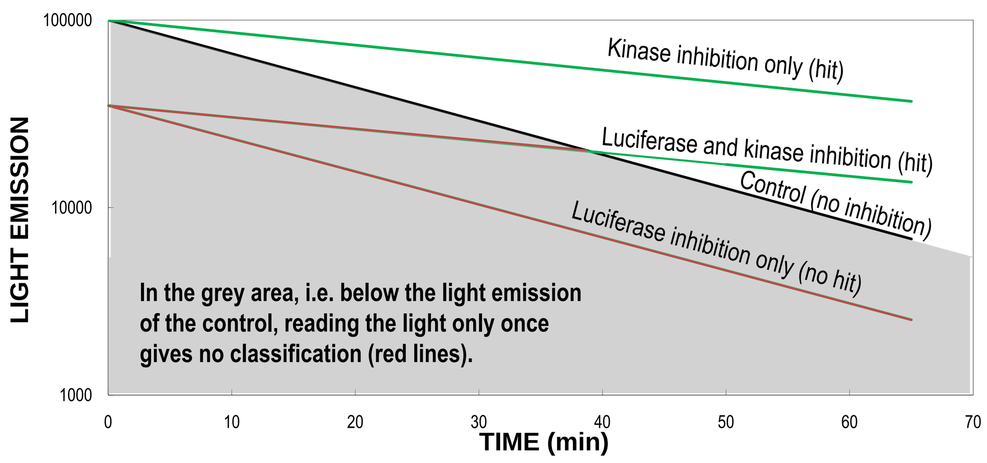

Fig. 2: Under 1st-order reaction conditions the normalised light emission plotted on a log scale is a straight line and the rate constant can be measured from two light readings, I_1 and I_2,at any two points in time, t_1 and t_2, as k =[ln(I_1) -ln(I_2)]/(t_2 -t_1). The measured rate constant for the kinase reaction is independent of inhibition of luciferase.

Z´ as High as 0.96

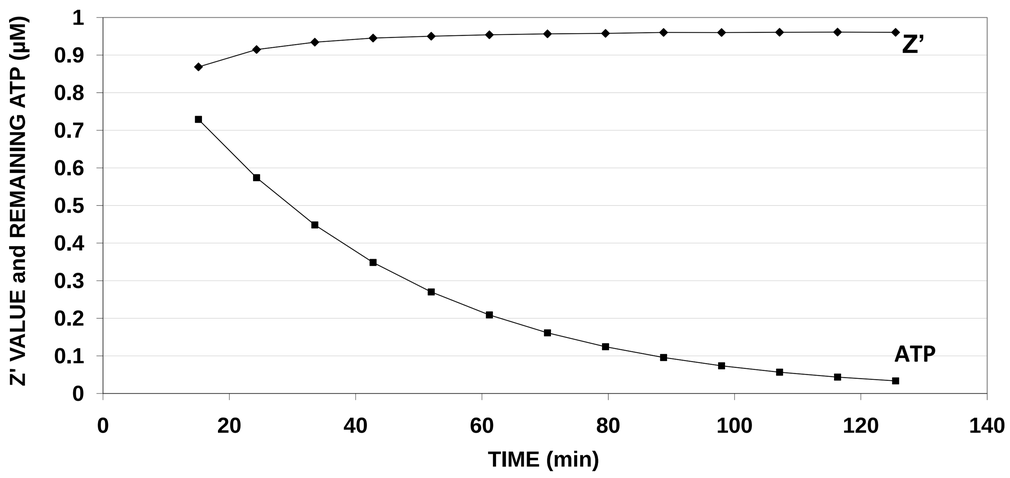

Fig. 3: Z' value and remaining ATP as a function of time. 40 blanks (no PKA) and 40 samples (with PKA) were measured. An average blank curve was used to calculate individual normalised blank and sample curves from which Z' values were calculated. A maximum Z' (0.96) is obtained already when ATP has gone down one order of magnitude.

Standard Curve for PKA

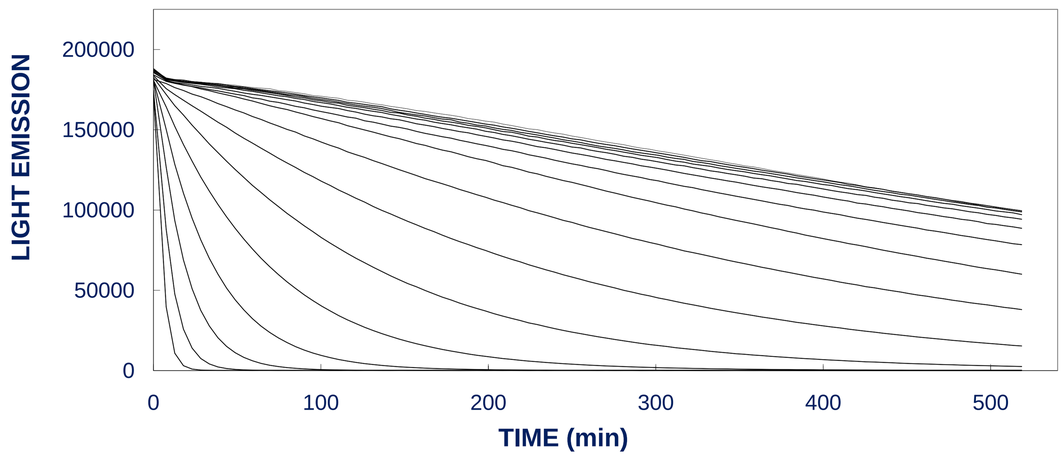

Fig. 4A: Raw data for PKA standard curve (0.007-107 ng/well).

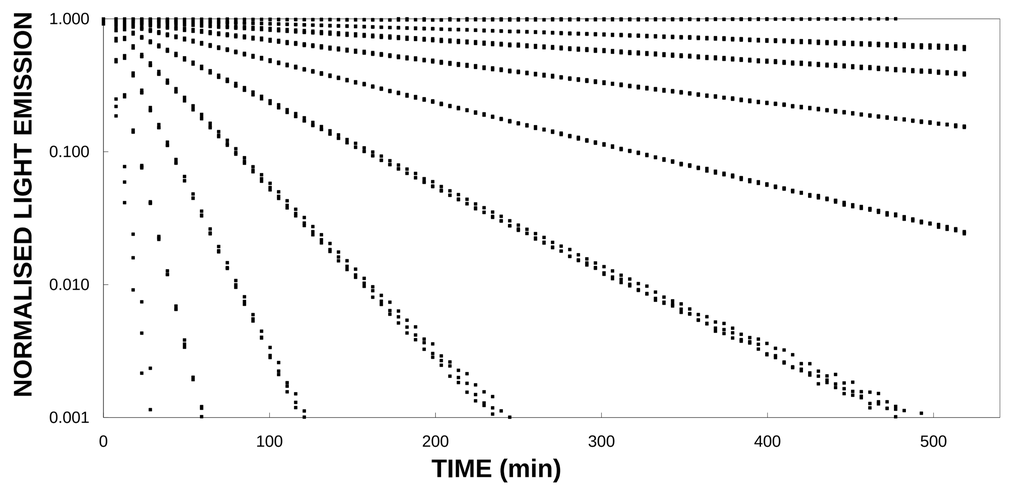

Fig. 4B: Same data as in 4A but normalised and shown on a logarithmic axis.

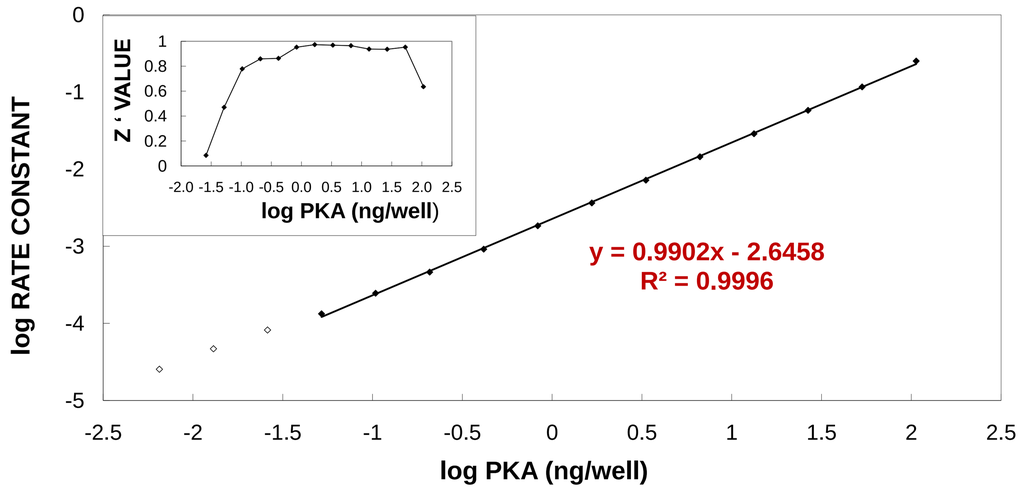

Fig. 4C: Same data as in 4A and B but 1st order rate constants calculated and plotted versus amount of PKA in a double logarithmic plot. The three lowest concentrations were not included in the regression analysis.

Analytical Characteristics

- First-order rate constant of ATP consumption is measured making the assay suitable for all kinases and all types of substrates
- Variations in ATP and luciferase activity cancel out in the calculations and do not affect assay results
- Linear range covers over three orders of magnitude
- Z' as high as 0.96
- High kinase activity measured in minutes
- Low kinase activity detectable by measuring for hours
- Easy to set up (only one substrate to optimise and wide linear range makes it easy to choose the best kinase level for HTS)
- All competitive and non-competitive inhibitors detected
- Kinase RR Kit is a Real-Time Reaction Rate assay of any kinase activity
- In HTS the light emission is measured twice, which obviates problems with classification resulting from luciferase inhibition
- In non-HTS further advantages can be gained by reading the plate repeatedly
- No radioactivity, no antibodies, no conjugates and linear rather than sigmoidal standard curve

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