

TECHNOLOGY originally developed by the UK Defence Science and Technology Laboratories (Dstl) at Porton Down for battlefield based bio-warfare agent detection, has formed the basis of a new rapid bacterial detection assay. Researchers at The Wellcome Trust Sanger Institute have used it to examine the growth kinetics of *Salmonella serovar typhi* containing different mutations associated with resistance to the fluoroquinolone group of



Adenylate Kinase Assay may provide Key to more Rapid Analysis of Bacterial 'Fitness'

Acolyte Biomedica develops proprietary diagnostic systems for clinical microbiology.

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antibacterial agents.

The assay, *RapiTECT GN* from Acolyte Biomedica, uses an Adenylate Kinase (AK) mediated bioluminescence detection system as a means of quantifying the number of bacteria in a liquid sample. AK is a constitutive enzyme that catalyses the conversion of ADP to ATP and is found in all bacteria (and other living cells). The assay method involves the addition of a bacterial lysis reagent to release the AK into the surrounding matrix. ATP is then generated by the AK enzyme following the introduction of an excess of ADP. Firefly luciferase/luciferin is added to the sample, light is emitted in the presence of the ATP and the photon emission is measured using a luminometer. Although certain aspects of the assay may be recognisable to those who already perform traditional ATP bioluminescence assays, the sensitivity of this method is improved due to the equilibrium reaction being artificially driven to

overproduce ATP by the addition of an excess of ADP (Fig. 1). Moreover, unlike traditional ATP assays AK levels are constant and do not fluctuate with metabolic state, providing a more reproducible, quantifiable signal which closely correlates with cell numbers. These features enable small changes in the microbial population to be detected at a very early stage of the growth cycle.

The target enzymes for the fluoroquinolone antibacterial agents are topoisomerases. Several mutations in topoisomerase enzymes have

been linked to the development of low level resistance both *in vivo*, and high level resistance *in vitro*. It is possible that high level resistance is counter selected *in vivo* after the antibiotics are withdrawn. Point mutations were introduced into the GyrA, and ParC subunits of *S. typhi* to generate isogenic mutants with high and low level resistance. The AK detection assay was then used to compare the growth rate of 24 strains containing different mutations, further experiments are also planned to measure the time-kill

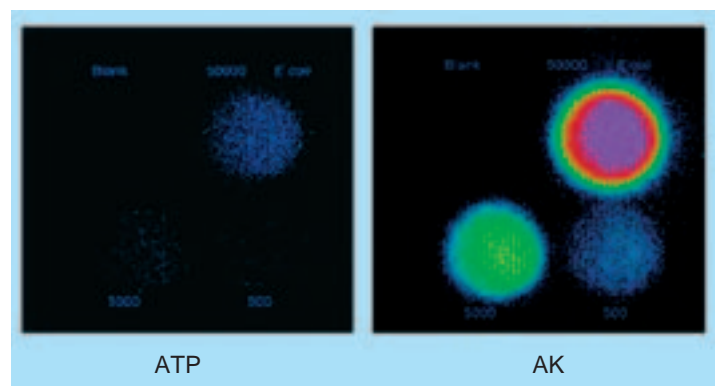


Fig. 1. - Comparison of traditional ATP and AK bioluminescence

properties of fluoroquinolones against each mutant. As the effects of the combination of mutation and the exposure to fluoroquinolone on cell size and clumping was unknown, the use of optical density was not considered appropriate. Furthermore the counting of bacteria down to 10^2 CFU/ml from an inoculum of 10^6 CFU/ml is necessary to establish bactericidal activity. This cannot be achieved by optical density measurement. It was on this basis the AK detection assay was assessed for suitability for this purpose.

An attenuated strain of *S. typhi* was used as the background for the introduction of mutations into target enzymes. The strain is dependent on aromatic amino acids (aro) and will grow only if these are added to the media. Using a Thermo LuminoSkan single tube luminometer the growth curve in Isosensitest broth (Oxoid, UK) plus aro supplement, was followed by RLU for the susceptible parental strain. This was carried out using the standard AK assay protocol from *Acolyte Biomedica*. In order to assess the reliability of the method this was compared to a plate count of colony forming units (CFU) calculated using a $20\mu\text{L}$ surface drop method on LB agar plus aro supplement. The results showed good correlation ($R^2 = 0.993$) between CFU and RLU over a growth curve from 10^5 to 2×10^6 CFU/mL (Fig. 2).

Two low level and two high level topoisomerase mutants were then tested in triplicate using the AK assay alone. The slopes of the resulting growth curves were calculated using *Prism4 for Windows* (Graphpad software Inc.). The results indicate that the susceptible parental strain behaved more reliably than the mutants, so it was concluded that the variation was not methodological.

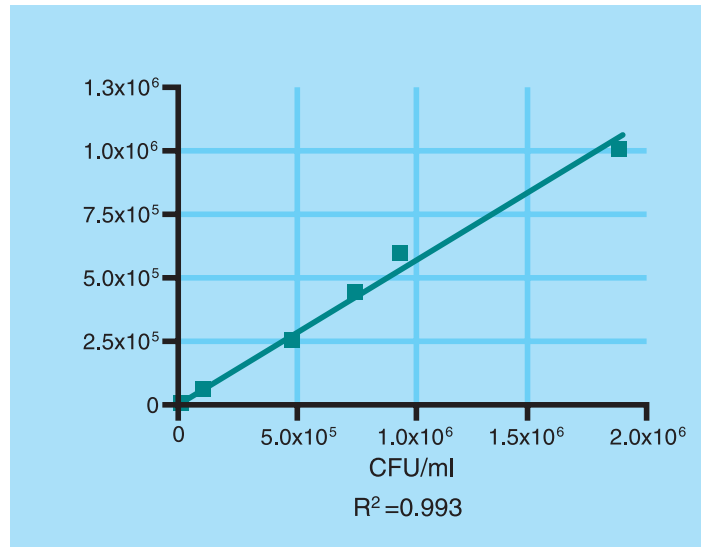


Fig. 2. – Correlation between CFU and AK assay RLU for attenuated strain of *S. typhi*

Although variation was seen there was a detectable, but not statistically significant, difference in the growth rate of the strains. More repeats using very carefully standardised conditions are necessary before definite conclusions can be drawn.

The measurement of growth rate has been applied to the analysis of bacterial cultures since bacteriology began. Growth rate has been used as a surrogate marker for virulence and as a phenotype representing the fitness of bacterial isolates. Techniques for the measurement of growth rate; colony counting and measurement of optical density, however are fraught

with problems. For example: Contamination of slow growing organisms such as *Mycobacterium tuberculosis*, clumping, or filament formation on exposure to different test conditions effects the estimation of bacterial numbers by both CFU or measurement of optical density, in unknown ways. Automated instrument assays for measuring growth rates by measurement of the activity of a constitutively expressed enzyme, such as AK, has several advantages. These include a high level of sensitivity (10 organisms) which would allow the accurate quantification of live bacteria down to levels found

towards the end of standard time-kill assays. Starting with an inoculum of 10^6 bacteria a four fold reduction in live count (normally considered to represent killing) would require the estimation of bacteria at 10^2 per ml. This is impossible by optical density measurements and very time consuming by culture as it requires numerous dilutions and replicates for reliable data. Full automation of growth curves, including killing curves, would be of immense benefit in several fields of bacteriology. Whilst this is not available with the current assay format (as it is necessary to sacrifice the cultures in order to count bacteria) multisampling of the experimental culture vessel using multi-channel pipettes for sampling and an automated luminometer allows the use of 96 well microtitre plate format. The ability of the AK assay to accurately measure small changes in microbial biomass mitigates the need for numerous sample testing points over a time course.

For the estimation of bacterial numbers in cultures over a wide range detection of AK has exciting possibilities. Further testing of this assay for the detection of survival of bacteria after exposure to antibiotics is currently underway. Moreover as AK is present in all living cells this technology can be adapted to provide an equally rapid, accurate means of detecting and enumerating any prokaryotic and eukaryotic cell types. This presents numerous possibilities within bioengineering, pharmaceuticals and cell culturing, replacing many of the lengthy, laborious techniques currently being used.



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