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BIOMARKER DETECTION

Signals from single molecules

> the recognition of a particular sequence is rather like our ability to spot a high-altitude airplane with strobe lights slowly flying across a starry sky

Mutated DNA strands — even those with only a change in a single nucleobase — can be the cause of serious health problems. Quantifying these mutant DNA strands is particularly challenging when they are present in low concentrations in a patient's urine or blood samples and must be detected against a large background concentration of wild-type DNA. Now, writing in the *Journal of the American Chemical Society*, Nils Walter and co-workers report the development of an amplification-free method that they call single-molecule recognition through equilibrium Poisson sampling (SiMREPS).

"We were seeking a powerful tool to detect mutations that are cancer causing, or that make cancer harder to treat," explains Walter. "One such mutation is found in the epidermal growth factor receptor (EGFR), where a cytosine-to-thymine mutation in the DNA results in substitution of a threonine with a methionine in the protein. The ultimate result of this change is that certain classes of tyrosine kinase inhibitor (TKI) drugs no longer work."

Quantitative assays of DNA still rely heavily on polymerase chain reaction (PCR) amplification. However, errors in replication result from both the imperfect fidelity of the DNA polymerases used and from the temperature cycling necessarily employed in PCR. Even when these errors are eliminated in amplification-free methods, detection of single-nucleotide variants below 0.1% abundance is very difficult, because the signal-to-noise ratio is fundamentally limited by a small difference in free energy of binding DNA molecules.

Whereas most affinity-based detection methods rely on a snapshot of steady-state binding, SiMREPS follows the kinetics of binding over time. The binding kinetics differ between target and non-target binding. Therefore, observing the blinking that results from the repeated binding of fluorescent probes to surface-captured biomarkers provides a kinetic 'fingerprint'. This enables high-confidence identification and counting of single target molecules.

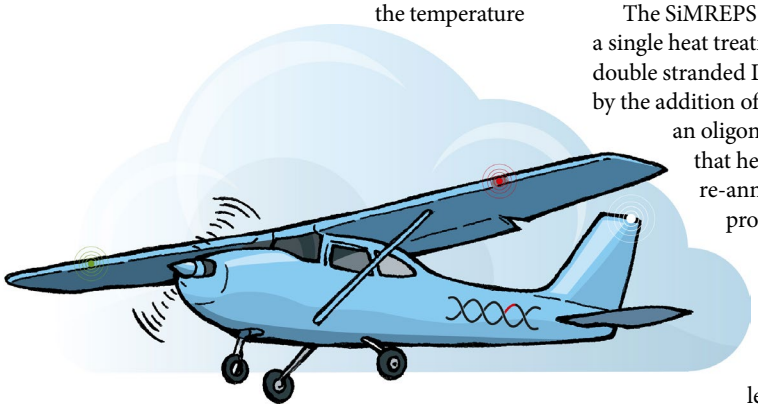
"In practice, we use a fluorescence microscope to observe a dark surface on which fluorescent probes bind more or less frequently to specific tiny spots, the blinking frequency of which reveals the binding event of interest with high confidence," explains Walter. "Conceptually, the recognition of a particular sequence is rather like our ability to spot a high-altitude airplane with strobe lights slowly flying across a starry sky."

The SiMREPS process begins with a single heat treatment to de-anneal double stranded DNA, followed by the addition of a large excess of an oligonucleotide (dT_{10}) that helps to prevent re-annealing. "Using this protocol, we were able to sensitively detect the drug resistant cytosine to thymine mutation in EGFR at the level of just one mutant molecule in a background of at least 1 million wild-type molecules," says Walter. "From a clinical perspective, early detection of this biomarker for drug-resistant cancer enables early prescription of 2nd and 3rd generation TKI drugs."

An unexpected consequence of Walter and co-workers' investigation is a recognition of the ease with which cytosine undergoes spontaneous deamination to uracil upon heat treatment. Uracil base pairs with adenine and becomes hard to distinguish from thymine. This spontaneous deamination, which would be amplified by the 35–40 heating cycles in a typical PCR reaction, would lead to a high false-positive rate in tests that rely on these methods. "Our SiMREPS technique avoids this problem as it requires only one, mild heating cycle. We can then treat the heat-melted DNA with a repair enzyme mix that removes almost all remaining uracils," says Walter.

It is hoped that the method can be further developed into a broadly applicable detection platform that can compete effectively with quantitative PCR and other single-molecule detection methods. "The SiMREPS technique is analyte-agnostic," explains Walter. "We have founded a company — aLight Sciences LLC — around this technology. So far we have published examples for detecting RNA and DNA biomarkers, and we are working on the quantification of several other types of analytes."

Stephen G. Davey



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ORIGINAL ARTICLE Hayward, S. L. et al. Ultraspecific and amplification-free quantification of mutant DNA by single-molecule kinetic fingerprinting. *J. Am. Chem. Soc.* **140**, 11755–11762 (2018)

FURTHER READING Johnson-Buck, A. et al. Kinetic fingerprinting to identify and count single nucleic acids. *Nat. Biotechnol.* **33**, 730–732 (2015)