

This study presents a rapid, low-cost nanobioarray-based DNA hybridization method for detecting clinically relevant EGFR point mutations (T790M, L858R, and L861Q) associated with tyrosine kinase inhibitor resistance or sensitivity in non-small cell lung cancer. By combining optimized hybridization conditions with a gold nanoparticle-assisted wash, the authors demonstrate clear discrimination between wild-type and mutant EGFR sequences using both synthetic targets and PCR-amplified genomic DNA. Overall, the approach offers a faster alternative to conventional DNA sequencing for routine EGFR mutation screening and has potential clinical utility. However, while the topic is important and timely in the field of oncology, several sections of the manuscript require further revision and clarification before it can be considered for publication in Biomedicines.

- The study primarily uses synthetic oligonucleotides and commercial genomic DNA. Have the authors considered validating the method using clinical NSCLC patient samples to better demonstrate its real-world applicability?

The clinical samples were the genomic DNAs purchased from Horizon Discovery: HD709 (50 ng/μL) for the wild-type EGFR gene, and HD802 (50 ng/μL) for the mutated one, see lines 83-85.

- Please clarify the analytical sensitivity of the assay. What is the minimum mutant allele frequency that can be reliably detected?

The PCR products used in Fig 7 (volume of 1 μL) was 25 nM or $25 \times 10^{-9} \times 10^{-6} = 25$ femtomoles or 1.5×10^4 copies.

According to the information provided by Horizon Discovery, the allele frequencies of L861Q, L858R and T790M in HD802 are 12.5%

- How does the assay perform in the presence of closely related EGFR mutations or non-target SNPs? Additional data or discussion regarding potential cross-hybridization would strengthen confidence in the assay's specificity.

The assay results for wild-type and mutated EGFR are depicted in Fig 7.

- Please provide information on inter-assay and intra-assay variability. Were experiments repeated on different days or with different nanobioarray chip batches, and how consistent were the resulting differentiation ratios?

The experiments were repeated at least 3 times. The errors in DR are ± 0.2 .

- The choice of hybridization temperature (47 °C) and AuNP wash conditions appears to be critical for assay performance. Could the authors clarify how these parameters were optimized and whether the assay is robust to small variations in experimental conditions?

The temperature of 47 °C has been optimized in previous reports, refs 14, 16.

- While a 16-channel nanobioarray chip is demonstrated, please discuss the potential scalability of the platform, particularly for multiplexed detection or higher-throughput clinical applications.

The chip used here consisted of 16 channels. Other chips that contains 96 channels for high-throughput tests have been previously reported, see refs 12, 16.

- The manuscript states that the method can be completed within one day. A more detailed breakdown of the workflow steps and associated time requirements would help readers better assess the feasibility of clinical implementation.

The workflow has been depicted in Fig 1, as well as in figures in previously reports, e.g. refs 11, 13, 14.