Padlock probe ligation-based rolling circle amplification (RCA) can distinguish single-nucleotide variants, which is promising for the detection of drug-resistance mutations in, e.g., Mycobacterium tuberculosis (Mtbo). However, the clinical application of conventional linear RCA is restricted by its unsatisfactory picomolar-level limit of detection (LOD). Herein, we demonstrate the mechanism of a nicking-enhanced RCA (NickRCA) strategy that allows several polymerases to act simultaneously on the same looped template, generating single-stranded amplicon monomers. Limiting factors of NickRCA are investigated and controlled for higher amplification efficiency. Thereafter, we describe a NickRCA-based magnetic nanoparticle (MNP) dimer formation strategy combined with a real-time optomagnetic sensor monitoring MNP dimers. The proposed methodology is applied for the detection of a common Mtbo rifampicin-resistance mutation, rpoB 531 (TCG/TTG). Without additional operation steps, an LOD of 15 fM target DNA is achieved with a total assay time of ca. 100 min. Moreover, the proposed biosensor holds the advantages of single-nucleotide mutation discrimination and the robustness to quantify targets in 10% serum samples. NickRCA produces short single-stranded monomers instead of the DNA coils produced in conventional RCA, which makes it more convenient for downstream operation, immobilization or detection, thus being applicable with different molecular tools and biosensors.