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# A single tube system for the detection of *Mycobacterium tuberculosis* DNA using gold nanoparticles based FRET assay

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ABSTRACT

The global combat against MTB is limited by challenges in accurate affordable detection. In this study, a rapid, affordable, single tube system for detection of unamplified MTB16s rDNA was developed. Utilizing a AuNP based FRET system, this assay achieved a sensitivity and specificity of 98.6% and 90% respectively.

*Mycobacterium tuberculosis* (MTB) is a fatal communicable pathogen where 10.4 million people have fallen ill in 2015 (World Health Organization (WHO), 2016). Due to the low sensitivity of smear microscopy (positivity in general TB suspects is lower than 25%; Urbanczik, 1985) and prolonged culture, empiric treatment starts after presumptive diagnosis without species differentiation (Neonakis et al., 2008). The importance rises from the abundance in nontuberculous mycobacterium (NTM) infections that cause similar clinical symptoms but treated with different regimens (Hashemi-Shahraki et al., 2013; Worodria et al., 2011; S.M. Hwang et al., 2013). The development of new diagnostic tools of short turnaround time, cost-effectiveness and ability to differentiate between MTB forms is therefore highly warranted (Ditiu and Raviglione, 2011). In this study, a novel single tube MTB gold nanoparticles (AuNPs) based assay was developed for the identification of *M. tuberculosis* through targeting its 16s rDNA gene. Briefly, extracted DNA is added to the hybridization buffer containing the Cy3-labeled probe, followed by the addition of 40 nm AuNPs. In case of positive MTB samples, intense fluorescence is observed due to probe target DNA hybridization. In case of negative samples, the absence of target DNA forces the probe to adsorb on the AuNP surface quenching its fluorescence and enabling facile differentiation between MTB positive and negative samples (Fig. 1).

Sputum samples were collected from 72 *Mycobacterium* suspected patients and cultured on Löwenstein-Jensen agar. Reference bacterial strains included MTB *H37Ra* (positive) and *Mycobacterium smegmatis* (negative). Four *E. coli* cultures, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Enterobacter aerogenes* were used as negative controls. DNA extraction was performed using QIAamp DNA Mini kit (Qiagen Inc., Valencia, CA). To further confirm that the samples were *Mycobacterium* positive and to differentiate MTB from NTM, two pairs of primers for *rpoB* gene (BioBasic, Ontario, Canada) were used to analyze samples by multiplex PCR (Veriti, Applied Biosystems CA, USA) (Mokaddas and Ahmad, 2007). Gel electrophoresis indicated that 70 samples were MTB and had amplicons size of 235 bp and 2 samples were NTM (amplicons size: 36 bp). Culture results also indicated that 70 out of the 72 samples were *Mycobacterium*. Of the 72 samples, 13 samples were randomly selected and amplified by 16S-23S ITS primers (Mokaddas and Ahmad, 2007) and sequenced by primer walking (Macrogen, Seoul, South Korea). Forward and reverse sequences were then assembled by AlignX Vector NTI 11.5 software (Life technologies, Carlsbad, California, USA) and finally analyzed by the National Center for Biotechnology Information (NCBI) database. Amplicons of sequenced samples had size of 350 bp and sequencing indicated that only one sample was NTM (*M. kansasii*) whereas 12 samples were MTB in concordance with multiplex PCR.

AuNPs were synthesized by the Turkevich method (Turkevich et al., 1951), then concentrated by centrifugation at 17,968*g* (Sigma Laborzentrifugen, Osterode am Harz, Germany) for 20 min. The synthesized AuNP had a hydrodynamic diameter corresponding to 40 nm and a zeta potential of $-19$ mV as determined by Malvern Zeta sizer (3000HSA; Malvern Instruments Ltd., Malvern, UK). The AuNPs appeared spherical and unaggregated as determined by scanning electron microscope (SEM; LEO SUPRA 55; Carl Zeiss AG, Oberkochen, Germany) (Fig. S1). AuNP concentration in terms of number of AuNPs $ml^{-1}$ was also determined as detailed in Tammam et al. (2015).

To avoid false positive results, the optimal AuNP-probe ratio

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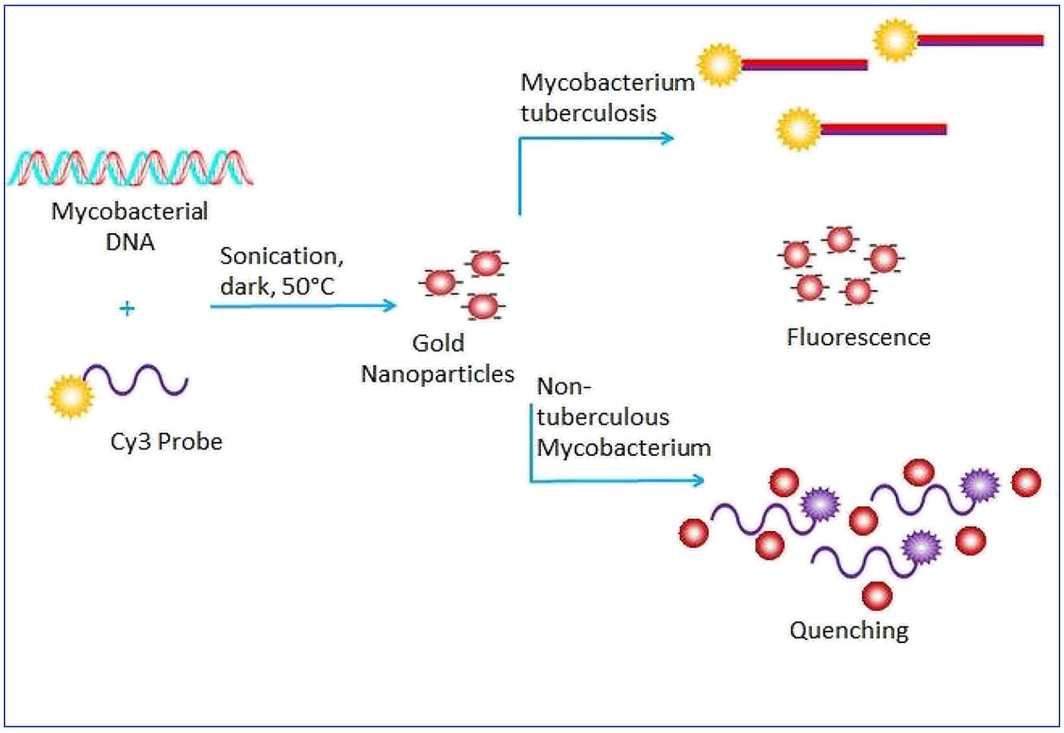


**Fig. 1.** Diagram of the AuNP-based assay for fluorometric detection of MTB. Extracted DNA is added to the hybridization buffer containing the Cy3-labeled probe, followed by sonication and annealing, then 40 nm AuNPs are added. In case of positive MTB samples, intense fluorescence is observed. In case of negative samples, the fluorescence is quenching by the adsorption of the Cy3-probe on the surface of AuNPs.

(AuNPs concentration sufficient to quench the Cy3-labeled probe) had to be determined. For such reason, increasing concentrations of AuNPs were added to the hybridization buffer (2.5 µl of 0.5 M NaCl, 1 µl of 1 µM Cy3-labeled probe, 6.5 µl nuclease free water) and fluorescence intensity determined at λexc. 544 and λem. 584 nm (BMG Labtech 413-3179). Due to adsorption of Cy3 labeled probe onto AuNPs, Cy3 fluorescence decreased with the increase in AuNP concentration, up to a value of $276 \times 10^{11}$ AuNPs $\mu l^{-1}$. Increasing AuNP concentration further did not lead to a decrease in fluorescence intensity indicating that this concentration of AuNPs is capable of fully adsorbing and quenching all the added Cy3-labeled probe (Fig. S2).

The probe, Cy3-5′-ACCACAAGACATGCATCCCG-3′ (BioBasic) (Hussain et al., 2013), was used for MTB DNA detection. DNA (2 µl) extracted from samples was added to hybridization buffer then fragmented in sonicator water bath (Sonorex digital 10p, Bandelin, Dusseldorf, Germany) at 50 °C for 10 min in dark. Samples were denatured for 3 min at 95 °C followed by annealing at 52 °C for 45 s (Applied Biosystems, USA). After 15 min, 40 µl of AuNPs were added and fluorescence intensity measured. All samples were tested in triplicates. The detection limit was determined using serial dilutions of MTB H37Ra. To avoid any base-line fluorescence from free probe (if any), results were normalized to negative controls.

Of the 72 *Mycobacterium* samples tested, the AuNPs based FRET assay yielded one false negative and of the 8 negative control tests, one false positive, translating to a sensitivity and specificity of 98.6% and 90%, respectively (Fig. 2A). The sensitivity and specificity of the FRET assay are higher than some PCR tests performed using large number of samples (sensitivity and specificity of PCR were 93% and 84%, respectively.) (Kivihya-Ndugga et al., 2004). When the MTB-AuNP assay was performed using a serial dilution of MTB H37Ra reference strain (DNA concentration: 40–0.3 ng/µl) a linear relationship was observed between relative fluorescence and DNA concentration from 40 to 10 ng/µl and 3 ng/µl was set as the assay's lower detection limit (Fig. 2B). The entire procedure was conducted in 1 tube, minimizing contamination and volume losses and the assay turnaround time was ≈ 15 min.

In MTB positive samples, fluorescence was observed due to probe target hybridization making the probe unavailable for AuNP binding while in negative samples (including NTM samples), the absence of target DNA forces the probe to be adsorbed on the AuNP surface quenching its fluorescence. The latter was based on the fact that ssDNA could efficiently bind to the surface of citrate AuNPs, since it can uncoil exposing its bases, whereas dsDNA (owing to its double-helix structure) presents its negatively charged phosphate backbone (Kim et al., 2006).

It is worth noting that other FRET based nanosensors have been used for detection of MTB DNA (S.H. Hwang et al., 2013; Shojaei et al., 2014). However, the reported approaches utilized amplified DNA and the nanoparticles used were toxic, costly or required complicated preparation methods. The proposed AuNP-based FRET MTB assay enables differentiation of MTB from non-MTB and NTM samples; an important requirement from a therapeutic perspective. Notwithstanding, testing a larger number of samples, including those representing other respiratory diseases, would be insightful. More importantly the proposed assay is estimated cost is 1.5–2 USD per test, which is cheaper than most of the molecular tests, as reported by the WHO, where the average cost of molecular testing of MTB for 1 sample is about 10–17 USD (UNITAID, 2013). In addition, recently, portable affordable fluorescence detectors have been developed, particularly those that rely on cell phones (Rajendran et al., 2014; Wargocki et al., 2015). Owing to their portability, these devices when combined with AuNP-based FRET MTB test will not only be of benefit in low resource setting but may enable MTB detection at point-of-care in rural off-track low access areas. This study has been approved by the Institutional Review Board of the American University in Cairo and the Egyptian Ministry of Health.

## Conflict of interest statement

Professor Hassan Azzazy is an inventor of patent applications for use of gold nanoparticles for detection of infectious agents. He is a co-founder of D-Kimia, LLC, a novel diagnostic solutions company. Other

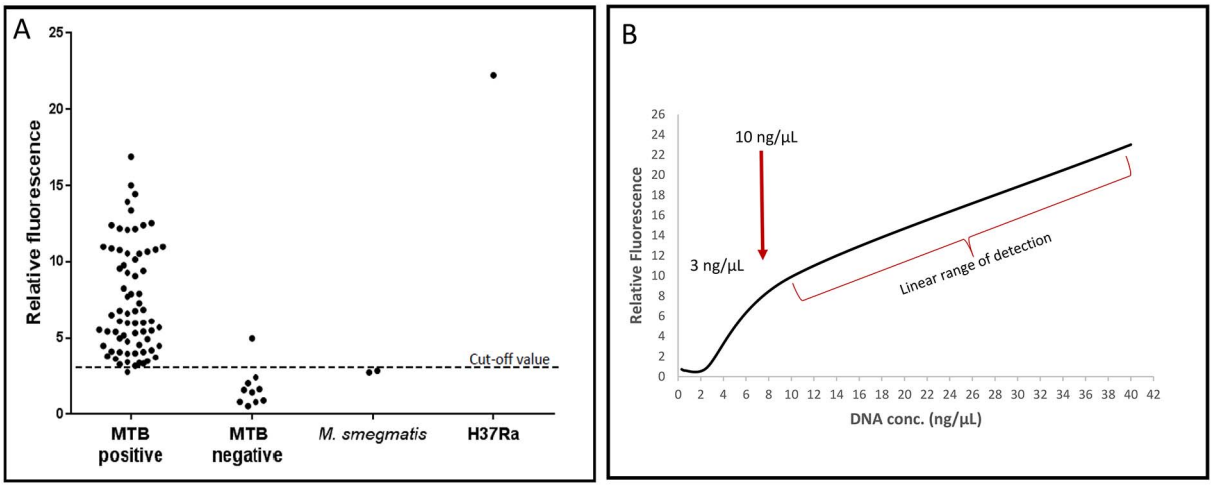


**Fig. 2.** (A) Identification of positive and negative MTB samples using the AuNPs based FRET assay. *M. smegmatis* is the negative control while the H37Ra is the positive control. In presence of the MTB DNA target, the Cy3-labeled probe hybridizes to the MTB 16s rDNA forming a stable dsDNA structure. So, the AuNPs become separated from Cy3-probe and fluorescence is emitted. In negative samples, due to the absence of target DNA, the probe is adsorbed on the AuNP surface quenching its fluorescence. The dotted line represents the proposed cut-off value which is 3-fold higher fluorescence than negative samples. (B) Determination of the limit of detection of the MTB AuNP based FRET assay. The MTB-AuNP assay was performed using a serial dilution of H37Ra reference strain (DNA concentration: 40–0.3 ng/µl) a linear relationship was observed between relative fluorescence and DNA concentration from 40 to 10 ng/µl. From 10 to 3 ng/µl, a fluorescence signal was observed, however,the relationship between the fluorescence signal and the DNA concentration was non-linear. As low as 3 ng/µl MTB DNA could be detected with a 3-fold higher relative fluorescence than blank.

authors declare no competing interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2017.06.001.

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