**Amperometric IFN-γ immunosensors with commercially fabricated PCB sensing electrodes**

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**Abstract**

Lab-on-a-Chip (LoC) technology has the potential to revolutionize medical Point-of-Care diagnostics. Currently, considerable research efforts are focused on innovative production technologies that will make commercial upscaling of lab-on-chip products financially viable. Printed circuit board (PCB) manufacturing techniques have several advantages in this field. In this paper we focus on transferring a complete IFN-γ enzyme-linked immune-sorbent assay (ELISA) onto a commercial PCB electrochemical biosensing platform, We adapted a commercially available ELISA to detect the enzyme product TMB/H2O2 using amperometry, successfully reproducing the colorimetry-obtained ELISA standard curve. The results demonstrate the potential for the integration of these components into an automated, disposable, electronic ELISA Lab-on-PCB diagnostic platform.

*Keywords:* PCB sensors; immunosensors; amperometric biosensor; Lab-on-PCB

1. INTRODUCTION

Electrochemical biosensors ([Ricci et al. 2012](#_ENREF_36)) are a promising alternative for Point-of-Care (POC) diagnostics ([Antiochia et al.](#_ENREF_1) ; [Henihan et al. 2016](#_ENREF_15); [Luka et al. 2015](#_ENREF_25); [Narayan 2016](#_ENREF_32); [Rajan et al. 2016](#_ENREF_35); [Sharma et al. 2015](#_ENREF_37); [Soper et al. 2006](#_ENREF_38); [Wang 2006](#_ENREF_46); [Whitesides 2006](#_ENREF_49); [Yager et al. 2006](#_ENREF_53)), combining simple, rapid, reliable and low-cost detection, while at the same time allowing integration in complete Lab-on-a-Chip (LoC) platforms. While extensive research efforts are focused on optimising electrochemical biosensor performance (sensitivity, specificity, reliability), their cost-effective integration into standardized, mass-manufactured POC platforms remains an open question ([Volpatti and Yetisen 2014](#_ENREF_44)). Printed Circuit Boards (PCBs) are currently re-emerging ([Merkel et al. 1999](#_ENREF_26); [Wego et al. 2001](#_ENREF_48)) as an attractive alternative addressing this issue, as they offer an affordable upscaling solution via the already mature and established PCB industry ([Wu et al. 2011](#_ENREF_51)). PCB manufacturing, is primarily aimed at consumer electronics applications, but has recently been explored as an alternative approach for integration of electronics and microfluidics: Lab-on-PCB platforms ([Aracil et al. 2013](#_ENREF_2); [Aracil et al. 2015](#_ENREF_3); [Miguel Moreno et al. 2009](#_ENREF_27)). Several LoC components have been demonstrated on PCBs ([Gassmann et al. 2015](#_ENREF_11); [Gong and Kim 2008](#_ENREF_13); [Guo et al. 2014](#_ENREF_14); [Moschou et al. 2014](#_ENREF_31); [Vasilakis et al. 2016](#_ENREF_43); [Wu et al. 2010](#_ENREF_50)), including chemical and biological sensors ([Jacobs et al. 2014](#_ENREF_17); [Li et al. 2013](#_ENREF_21); [Moreira et al. 2016](#_ENREF_28); [Moschou et al. 2016](#_ENREF_29); [Moschou et al. 2015](#_ENREF_30); [Prodromakis et al. 2011](#_ENREF_33); [Pu et al. 2016](#_ENREF_34); [Trantidou et al. 2013](#_ENREF_39); [Tseng et al. 2014](#_ENREF_41)). However, until now PCB immunosensors have not been demonstrated using electrodes manufactured with an industrial fabrication process.

The aim of this work is to address this point, demonstrating that an electrochemical sensing platform fabricated using solely industrial PCB manufacturing techniques can indeed be exploited for immunosensing. In this paper, we have used commercially fabricated gold plated PCB electrodes to develop an amperometric IFN-γ immunosensor. IFN-ɣ is a cytokine that is measured in clinical laboratories to aid in the diagnosis of TB infection ([Lalvani 2007](#_ENREF_20)). Currently available commercial assays are based on the principle that patients with TB infection have T cells that respond to restimulation with TB specific antigens (CFP-10, ESAT-6 and TB7.7) by producing IFN-ɣ. IFN-ɣ is usually detected by enzyme-linked immune-sorbent assay (ELISA, QuantiFERON-TB Gold In-Tube, Cellestis) or IFN-ɣ producing T cells quantified by enzyme-linked immunospot (T-SPOT, Oxford Immunotec). Both ELISA and T-SPOT tests are performed in centralized laboratories by highly trained personnel using complex equipment, whereas a use low cost point of care test by general healthcare workers is likely to improve health comes for individuals with latent TB infection. The T-SPOT is more expensive and labour intensive than than QuantiFERON cell isolation, cell quantification, cell dilution by healthcare scientist, overnight incubation and ELISA SPOT detection of released IFN-ɣ by individual cells using enzymatic colorimetric detection and spot counting. In contrast QuantiFERON test involves overnight incubation of blood tube containing TB specific peptides without further manipulation, followed by a semi-automated detection of secreted IFN-ɣ inside the blood tubes on bespoke ELISA automated bench top instrumentation using a traditional 96 well IFN-ɣ ELISA plate test and enzymatic colorimetric detection.

However, due to the cost of the IFN-ɣ ELISA test, samples are often batched for testing in diagnostic laboratories once or twice a week. There is, therefore, an unmet need to reduce the cost of the IFN-ɣ ELISA so that testing of individual samples becomes financially viable, which also paves the way to adapt the platform for POC testing. To achieve this, two major constraints of the traditional ELISA technology have to be overcome, firstly, the cost of the chemistry which is driven by the cost of the IFN-ɣ antibodies and standards and secondly, the reduction in the cost of the ELISA instrumentation in order to make it viable to run each sample independently if possible. In this study we approached these constraints in two ways. Firstly, we miniaturized the volume of the ELISA test requirement form a traditional 100uL volume per sample to a 10uL volume per sample thus achieving 1/10 reduction in costs compared to the traditional ELISA test. Secondly, we replaced the traditional complex photometric ELISA instrumentation with amperometric detection based on commercial PCB platforms, thus reducing the cost of ELISA detection instrumentation to a degree that is financially viable to mass produce and be used for individual ELISA sample testing. Furthermore, when PCB platforms technology is combined with microfluidics, the whole ELISA process can be fully automated on a Lab-on-PCB platform. To assess the effectiveness of this combination, we miniaturized a commercial IFN-γ ELISA assay and assessed its effectiveness by colorimetric detection. Subsequently this miniaturized ELISA was adapted onto a PCB gold surface platform and the complete ELISA process was replicated. The final step of the ELISA process was followed by amperometric measurements of the reduction of hydrogen peroxide to water by horseradish peroxidase associated with the ELISA antibody in the presence of chromogenic substrate 3,3',5,5'-Tetramethylbenzidine (TMB) which acts as a hydrogen donor. Our findings indicate PCB associated ELISA can be detected by amperometric measurements.

2. MATERIALS AND METHODS

ELISA antigen detection assays are typically quantified by optical absorbance measurements of the chromogenic substrate reacting with the enzyme-linked, antigen-specific detection antibody (colorimetric quantification). Horseradish peroxidase (HRP)-linked detection antibodies are most frequently exploited in ELISAs, owing to HRP’s stability, low-cost and ease in producing colored products when reacting with an appropriate substrate. 3,3’,5,5’-Tetramethylbenzidine (TMB) is the most commonly used chromogen for HRP; its oxidation by HRP/H2O2 produces a blue-colored complex, with its optical absorbance being proportional to the quantity of the HRP-linked antibody ([Fanjul-Bolado et al. 2005](#_ENREF_9)). An additional interesting feature of TMB is its electroactivity (TMBoxidized+2H++2e-→TMBreduced), with several groups having demonstrated its quantification via amperometry ([Gau and Wong 2007](#_ENREF_12); [Kim et al. 2013](#_ENREF_19); [Volpe et al. 1998](#_ENREF_45)). In this work an HRP/TMB-based ELISA assay is leveraged to quantify the amount of the target antigen IFN-γ, measuring the unstopped, coloured reaction product both via optical absorption and electrochemically via amperometry on PCB sensing electrodes.

2.1 Assay reagents

*2.1.1 96 and 384 well ELISA*

An IFN-ɣ sandwich ELISA (R&D DuoSet DY285, DuoSet Ancillary Reagent Kit 2 DY008) was performed on 96 well plates according to manufacturer’s recommendations. Briefly 100µl/well mouse anti-human IFN-ɣ capture antibody (4µg/ml) was incubated overnight at 4°C (Fig. 1a). Following a wash step, wells were blocked with 300µl 1% BSA (Sigma Aldrich A1595) for 1 hour at room temperature. After a further wash step, 100µl of standard prepared from doubling dilutions was added in triplicate to the plate (1000pg/ml-15.6pg/ml) and incubated at room temperature for 2 hours (Fig. 1b). The plate was then washed and 100µl biotinylated goat anti-human IFN-ɣ detection antibody (200ng/ml, preincubated for 1 hour with 2% normal goat serum) was added to every well and the plate incubated for 2 hours (Fig. 1c). The plate was washed and 100µl streptavidin HRP (diluted 1:40) added to every well for 20 minutes (Fig. 1d). After a final wash step, 100µl of TMB/H2O2 substrate was added to every well (Fig. 1e) and the reaction was stopped with sulfuric acid after 20 minutes. The ELISA was further performed on 384-well NUNC plates (Thermo Scientific 269390) as above but using 10µl of solution at each step before stopping the reaction with 5µl of sulfuric acid. Both plates’ optical absorption was read on a spectrophotometer (Promega GloMax Multi) fitted with a 450nm filter.

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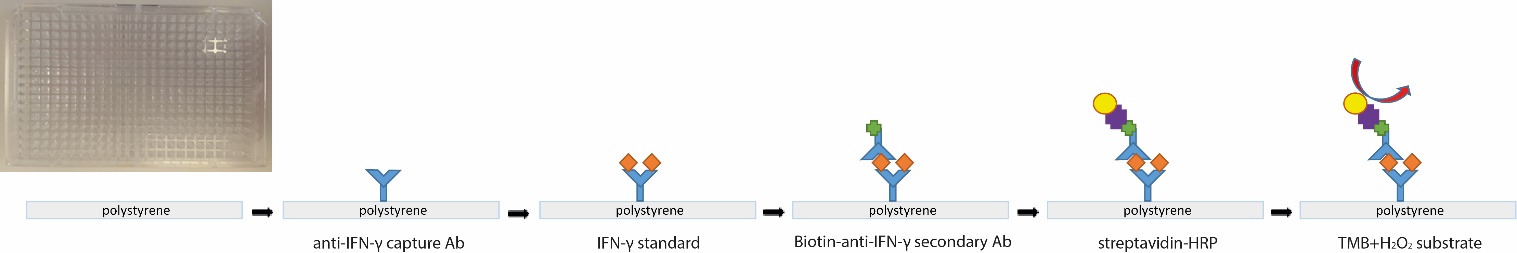


Figure 1. Schematic of IFN-γ ELISA assay followed in 96- and 384 well plates.

*2.1.2 PMMA/PCB well ELISA*

The aforementioned 10μL IFN-γ ELISA assay was further adapted on PCB gold-plated (MacDermidTM PCB electroless immersion plating, Newbury Electronics Ltd), rectangular surfaces (1 mm x 2 mm). In order to form 20μL microwells these surfaces, laser micromachined (Epilog Laser) PMMA sheets (Goodfellow Cambridge Ltd) were attached with double sided tape on top. The PMMA/PCB stack was placed in a heated press (Specac laboratory hydraulic press, P= 20kg, T= 80oC) for 1 hour, to ensure leak-tight adhesion of the two materials (Fig. 2).

10µl of anti-human IFN-ɣ cysteine terminated Fab (60µg/ml, AbD 23510, BIORAD) was incubated in each microwell for 60 minutes at room temperature (Fig. 2a). The wells were blotted on tissue paper, washed 3 times and then blocked with 10µl 1% BSA for 1 hour. After a further wash step, 5µl of the IFN-ɣ standard and 5µl of detection antibody (800ng/ml, preincubated for 1 hour with 2% normal goat serum) were added to the wells in duplicate and incubated for 1 hour (Figs. 2b and 2c). To assess specificity we added TREM-1 standard (Triggering receptor expressed on myeloid cells 1, 6000pg/ml or 3000pg/ml, R&D DuoSet DY1278B) instead of IFN-ɣ standard. The wells were washed and 10µl streptavidin HRP (diluted 1:20) added to every well for 20 minutes (Fig. 2d). After a final wash step, 10µl substrate was added to every well (Fig. 2e). Following a 20 minute incubation one set of duplicates was measured using amperometry and the second set was transferred into PCR tubes containing 5µl of stop solution, with the respective optical absorbance measured at 450nm on a nanodrop.

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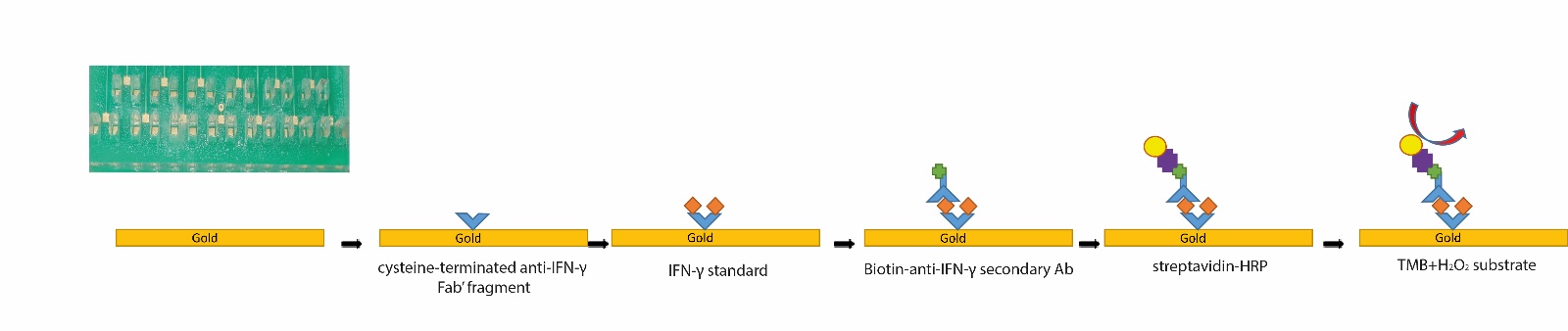


Figure 2. Schematic of IFN-γ assay followed in gold PMMA/PCB plates.

2.2 PCB sensing electrodes

Commercially fabricated double layer PCBs (Fig. 3) were exploited for the amperometric detection of the assay TMB/H2O2 product. The first layer of the PCB comprises integrated reference electrodes and 6 different diameter cylindrical, gold-plated microchambers (for this work Vchamber=1 μL). The 4.1 cm x 5.4 cm PCBs (Supplementary data, Fig. S1) were designed using standard PCB design software (Altium Designer 16®) and manufactured by Newbury Electronics Ltd. Both layers were patterned according to the design provided; the reference electrode layer was subsequently silver plated, while the sensing layer gold plated (MacDermidTM PCB electroless immersion plating). The two layers were attached together with low-flow prepreg adhesive films in an industrial heated press. Prior to use, the finished two-layer PCBs were sonicated in acetone for 15 minutes, cleaned with IPA and DI water and dried under N2, so as to remove any fabrication-induced impurities.

To create the AgCl electrode the two-layer PCBs were immersed for 30 min in a sodium hypochlorite NaOCl solution (NaOCl, Sigma-Aldrich, reagent grade, available chlorine 4.00%–4.99%), creating a the Ag/AgCl pseudo-reference electrodes at the bottom layer ([Moschou et al. 2015](#_ENREF_30)). UFL connectors were soldered to the sensing electrodes (working, counter, reference) for connection to a commercial potentiostat (PalmSens3). The potentiostat was connected to a laboratory laptop and data recorded via the PSTrace software package.

3. RESULTS

**3.1 Colorimetric results**

The electrochemical biosensor development started by adapting a standard IFN-γ ELISA to the requirements of the PCB platform to be exploited. The commercial ELISA (Fig. 1) was initially tested for its functionality in 384 well polystyrene plates, with the reagent volumes reduced 10 fold (10 μL wells as opposed to standard 100 μL), adapting to the much lower volumes compatible with microfluidic integration. The IFN-γ assay was successfully transferred onto 384-well plate format with the expected decrease in absolute absorbance (Supplementary data, Fig. S2) owing to the reduction of the total path length as predicted by the Beer Lambert law. The limit of detection (LOD) was calculated as the concentration corresponding to 3 times the standard deviation above the blank mean value and determined to be 28.22pg/mL.

The reduced volume assay was then transferred onto gold plated PCB platforms. Since the binding surface in this case comprises an immersion gold finish and not polystyrene, the capture antibody immobilization cannot rely on passive absorption. To this end the click-chemistry of bisulfide bond formation on gold was exploited ([Trilling et al. 2013](#_ENREF_40)), adding at the capture antibody (Fab’ format) non-antigen-binding end cysteine molecules (thiol-terminated).

The colorimetric assay was replicated with this adaptation in custom made 10 μL PMMA/PCB wells (Fig. 2) to verify its functionality. The stopped developed reaction product (TMB/H2O2 substrate) was measured via Nanodrop, clearly reproducing the expected ELISA standard curve (Fig. 3). TREM-1 standard was added to two wells (instead of IFN-γ) to verify the assay specificity. Measured optical absorption for both TREM-1 concentrations was lower than the lowest IFN-γ standard concentration, demonstrating that this assay demonstrates specificity for IFN-γ.



**Figure 3.** Optical absorbance standard curve for IFN-γ (TREM-1 standard illustrated as negative control).

**3.2 Amperometric results**

The unstopped developed reaction product (oxidized TMB/H2O2) ([Fanjul-Bolado et al. 2005](#_ENREF_9); [Kim et al. 2013](#_ENREF_19); [Lu et al. 2007](#_ENREF_24)) was then transferred onto the PCB integrated amperometric sensing platform (Fig. 3). 5 μL of sample was added to the working and counter electrodes to ensure simultaneous immersion of the underlying Ag/AgCl reference electrode. A -200 mV bias was applied between the working and reference electrode and the current flow between the working and counter electrodes was recorded for 60 seconds (Supplementary data, Fig. S3), for all 7 standard x2 dilutions and a blank negative control sample. All measurements were repeated 3 times and the average and standard deviation of each end-point current were calculated. The average value of each standard concentration was subtracted by the blank average and plotted against the nominal IFN-γ concentration (Fig. 4) to reproduce the ELISA standard curve. The respective TREM-1 measurements were also plotted in the same graph, demonstrating values lower than the lowest IFN-γ concentration and thus verifying the specificity of the acquired sensing signals towards only the targeted protein. Plotting together the colorimetry-derived and amperometry-derived standard curves (Supplementary data, Fig. S4) we see that the PCB biosensors seem to be reproducing the colorimetric curve very accurately for the IFN-γ concentration range studied, as already reported in literature for similar immunoassays ([Kim et al. 2013](#_ENREF_19); [Lu et al. 2007](#_ENREF_24)). The limit of detection (LOD) for the PCB biosensors was calculated as 126.75pg/mL.



**Figure 4.** PCB amperometric sensor derived standard curve for IFN-γ (TREM-1 standard illustrated as negative control).

Following the accurate reproduction of the ELISA standard curve, human serum samples spiked with a known IFN-γ concentration (1000pg/mL) were measured both colorimetrically and electrochemically via the PCB biosensors, in order to calculate the spike recovery of each method. The stopped sample’s absorbance was found to be 0.20 and the unstopped amperometric measurement 3.21 μA (average values, n = 3). Exploiting the previously obtained standard curves (Figs. 3, 4) as calibration curves, the corresponding IFN-γ concentration was calculated to be 1207pg/mL via colorimetry and 1219pg/mL via the PCB immunosensors (Fig. 5).



**Figure 5.** IFN-γ recovery in human serum samples for both colorimetric and amperometric detection.

**4. DISCUSSION**

In this study, we have adapted a commercially available IFN-γ assay and transferred it into 10μL wells. Using adapted capture antibodies (cysteine terminated Fab’ fragments) we have also shown that this assay can be transferred onto commercial PCB platforms; this result paves the way for automation of the complete immunoassay in a Lab-on-PCB platform, integrating microfluidics for accurate, μL-volume reagent delivery. We further demonstrated that the PCB-integrated amperometric biosensors produce similar standard curves to optical absorption measurements for IFN-γ concentrations ranging between 15-1000pg/mL, thus allowing the integrated electronic detection of IFN-γ on the same platform as the assay reaction and working towards a full electronic ELISA Lab-on-PCB system for the first time. The calculated LOD was almost 5 times larger for amperometric detection (LODcolorimetry=28.22pg/mL, LODamperometry=126.75pg/mL).

Several IFN-γ sensors have been presented in literature, based on colorimetric, optical or electrochemical approaches (Table 1). Optical detection schemes can provide similar or better LOD compared with our sensors ([Jeong et al. 2013](#_ENREF_18); [Zhu et al. 2016](#_ENREF_57)), but these are based on Surface Plasmon Resonance (SPR) and require elaborate optical detection apparatus, limiting their potential for POC applications. The lateral flow POC test demonstrated by Denkinger et al ([Denkinger et al. 2013b](#_ENREF_8)) features a much higher LOD, but does not provide quantification of IFN-γ. Electrochemical sensors (Table 1) are widely used for sensitive, quantitative POC systems, with excellent specificity; most papers demonstrate LOD that are lower than the colorimetric and amperometric methods used in this work. However, all these methods employ complex, custom assays optimized for electron transfer efficiency (nuclease cleavage-assisted target amplification ([Yan et al. 2013](#_ENREF_54)), MNP-IFN-γ-Au-CdS complexes ([Wang et al. 2016](#_ENREF_47)), HRP-Ab2-AuNP ([Zhang et al. 2016](#_ENREF_55)), P-Fc-A-IFN-γ ([Xia et al. 2015](#_ENREF_52)), MB-QD-Ab2 ([Huang et al. 2015](#_ENREF_16))). In this work, we demonstrate exploitation of a commercially available assay for commercialisable PCB-based sensing electrodes.

Based on previous analysis, it is to be expected that a more elaborate assay should result in improved performance (LOD, dynamic range, sensitivity). This suggests that further investigation is required on improving the assay sensitivity for 10μL sample volumes, along with the implementation of flow-enhanced detection, expected to achieve a more than 5-fold LOD improvement ([Kim et al. 2013](#_ENREF_19)). It is also likely that this method can be easily adapted to sense different protein biomarkers by changing the target-specific capture and detection antibody.

The presented PCB biosensors were also tested for protein recovery in human serum samples (CIFN-γ = 1000pg/mL), achieving 120% recovery. However, the standard deviation of the sensor measurements in serum was quite large compared to the colorimetric results; further work is required to increase the accuracy of the reported system via three routes: a) parallelization of standard curve and clinical sample sensing, avoiding drift ascribed to reference electrode AgCl dissociation and sensing surface saturation, b) sensing electrode arrays as opposed to single sensor measurement for each sample, so as to minimize measurement errors, c) microfluidic integration and automation of sample delivery to sensors, controlling accurately the analysed sample volume and avoiding possible reagent evaporation during analysis.

**Table 1**

Analytical performance of published IFN-γ detection systems.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Detection method** | **LOD (pg/mL)** | **Dynamic range (pg/mL)** | **Selectivity** | **Reference** |
| Immuno-chromatographic | 5000 | - | Serum | ([Denkinger et al. 2013a](#_ENREF_7)) |
| Fluorescence (FRET) | 85×103 | 85×103-1,714×103 | IL-2 | ([Tuleuova et al. 2010](#_ENREF_42)) |
| Electrochemi-luminescence | 0.03 | 0.1-500 | HIgG, Tf, BSA | ([Zhu et al. 2016](#_ENREF_15)) |
| Optical (LSPR) | 171.5 | 171.5-1,715 | - | ([Lin et al. 2016](#_ENREF_22)) |
| Optical (SPR) | 171.5 | 171.5-1,715×103 | Serum | ([Chuang et al. 2014](#_ENREF_6)) |
| Optical (LSPR) | 2 | 2-500 | - | ([Jeong et al. 2013](#_ENREF_18)) |
| Optical (SPR) | 565.8 | 5,143.7-5,709×103 | BSA, biotin, IgG, IL-1, TNF-α | ([Chang et al. 2012](#_ENREF_4)) |
| Electrochemical (SWASV) | 0.4 | 0.4-40 | Water, IP-10 | ([Wang et al. 2016](#_ENREF_47)) |
| Electrochemical (DPV) | 0.048 | 0.1-10×103 | BSA, AA, glucose, UA, IL-22 | ([Zhang et al. 2016](#_ENREF_55)) |
| Electrochemical (SWASV) | 0.34 | 1-500 | Serum | ([Huang et al. 2015](#_ENREF_16)) |
| Electrochemical (FET) | 1,400 | - | BSA, papain | ([Farid et al. 2015](#_ENREF_10)) |
| Electrochemical (SWV) | 19.5 | 171.5-171×103 | BSA, BHb, Thrb, IgG, PDGF-BB,  IL-6 | ([Xia et al. 2015](#_ENREF_52)) |
| Electrochemical (SWV) | 1,300 | 1,000-500×103 | BSA | ([Chen et al. 2014](#_ENREF_5)) |
| Electrochemical (DPV) | 1.1 | 1.7-12 | BSA, IgG, anti-IgG | ([Yan et al. 2013](#_ENREF_54)) |
| Electrochemical (DPV) | 5,000 | 5,600-5,143×103 | PDGF-BB, BSA, IgG, CEA, IL-6 | ([Zhao et al. 2012](#_ENREF_56)) |
| Electrochemical (SWV) | 1,000 | - | IgG, anti-IgG, BSA | ([Liu et al. 2010](#_ENREF_23)) |

**5. CONCLUSIONS**

In this paper we demonstrate the first amperometric immunosensors implemented on commercially fabricated Printed Circuit Boards (PCBs). IFN-γ was chosen as the target analyte in this instance, owing to this cytokine’s use in TB diagnosis. The commercially available IFN-γ ELISA assay was adapted to gold-plated PCB surfaces, immobilizing cysteine-terminated anti-IFN-γ Fab’ as capture antibodies. The TMB/H2O2 assay product was subsequently sensed via amperometry on PCB integrated electrodes, demonstrating excellent specificity with a limit of detection of 126.75pg/mL. Plasma recovery in human serum/IFN-γ spiked samples was similar both for standard colorimetry and amperometry, although amperometric results demonstrated increased variability. This issue is expected to be alleviated in the next generation fully integrated Lab-on-PCB ELISA chip which will feature assay parallelization, accurate reagent fluidic control and sensing electrode arrays. Future work includes improving the assay sensitivity to the clinical range of interest and testing the device with patient TB antigen specific IFN-γ clinical samples, towards a clinical diagnosis relevant POC device.

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**Figure Captions**

**Figure 1.** Schematic of IFN-γ ELISA assay followed in 96- and 384 well plates.

**Figure 2.** Schematic of IFN-γ assay followed in gold PMMA/PCB plates.

**Figure 3.** Optical absorbance standard curve for IFN-γ (TREM-1 standard illustrated as negative control).

**Figure 4.** PCB amperometric sensor derived standard curve for IFN-γ (TREM-1 standard illustrated as negative control).

**Figure 5.** IFN-γ recovery in human serum samples for both colorimetric and amperometric detection.

**Table 1.** Analytical performance of published IFN-γ detection systems.