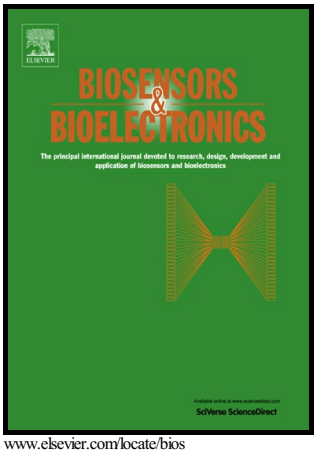

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Multiplex PCB-based electrochemical detection of cancer biomarkers using MLPA-barcode approach

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ABSTRACT

Asymmetric multiplex ligation-dependent probe amplification (MLPA) was developed for the amplification of seven breast cancer related mRNA markers and the MLPA products were electrochemically detected via hybridization. Seven breast cancer genetic markers were amplified by means of the MLPA reaction, which allows for multiplex amplification of multiple targets with a single primer pair. Novel synthetic MLPA probes were designed to include a unique barcode sequence in each amplified gene. Capture probes complementary to each of the barcode sequences were immobilized on each electrode of a low-cost electrode microarray manufactured on standard printed circuit board (PCB) substrates. The functionalised electrodes were exposed to the single-stranded MLPA products and following hybridization, a horseradish peroxidase (HRP)-labelled DNA secondary probe complementary to the amplified strand completed the genocomplex, which was electrochemically detected following substrate addition. The electrode arrays fabricated using PCB technology exhibited

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an excellent electrochemical performance, equivalent to planar photolithographically-fabricated gold electrodes, but at a vastly reduced cost (>50 times lower per array). The optimised system was demonstrated to be highly specific with negligible cross-reactivity allowing the simultaneous detection of the seven mRNA markers, with limits of detections as low as 25 pM. This approach provides a novel strategy for the genetic profiling of tumour cells via integrated “amplification-to-detection”.

KEYWORDS: Barcodes. Multiplex ligation-dependant probe amplification system (MLPA). PCB genosensor arrays. Electrochemical detection. Breast cancer markers

1 INTRODUCTION

It has been shown that circulating tumour cells (CTCs) have prognostic value in metastatic breast cancer patients and a plethora of methods and technologies have been developed to isolate, enumerate and analyse CTCs, which are defined as cancer cells that have detached from the primary tumour site and entered the peripheral blood circulation. Isolation and enumeration of CTCs may be highly important not only for early detection of metastatic disease early but also for monitoring disease progression. Furthermore, molecular characterization of the CTCs is of great importance. Genetic profiling using a limited set of genetic markers has potential as a rapid and cost-effective molecular diagnostic tool for analysing CTCs (Kvastad et al., 2015).

Multiplex ligation-dependent probe amplification (MLPA) has garnered huge importance in molecular diagnostics due to its' accuracy, robustness, low cost, relative simplicity and high multiplexability for up to 40-50 different target sequences in one reaction (Schouten et al., 2002; Sellner and Taylor, 2004). MLPA exploits specifically designed MLPA probes (55-80 mer) consisting of two or three oligonucleotides (right and left hybridization oligonucleotides, and in

some cases also a spanning oligonucleotide), containing target-specific sequences and universal PCR primer sequences. In the presence of a complementary target sequence, the MLPA probes hybridize next to each other and are subsequently ligated, followed by classic exponential amplification using a single primer pair. Amplified products vary in length, typically between 130 and 480 bases, depending on the probe length and are analysed using capillary electrophoresis (Schouten et al., 2002). In the absence of a complementary target sequence, ligation will not occur and amplification of the complete complex of the two MLPA probes does not take place. MLPA is dependent on length-based discrimination of the products, which requires the use of capillary electrophoresis, and also limits the number of probes within a single reaction to 40-50 probe pairs. To analyse a higher numbers of targets, array based MLPA assays has been reported where selective DNA tag sequences were incorporated into the MLPA probes (Rooms et al., 2011; Yan et al., 2011; Zeng et al., 2008), with detection being based on the hybridization of the tag sequence to a surface-immobilized DNA probe, with fluorescence read out.

In recent years, electrochemical detection has been shown to have great potential as an alternative to fluorescence for genetic analysis, as it is characterised by high sensitivity, ease of use, low cost, rapid response, low power requirements and compatibility with integration in microsystems (Ghindilis et al., 2007; Wang, 2002).

The use of Printed Circuit Board (PCB) technology in the biosensor field has recently emerged as an alternative to standard photolithographic techniques for electrode array microfabrication (Bhavsar et al., 2009; Li et al., 2013; Salvo et al., 2014), as PCB technology offers low cost mass production, not requiring clean room facilities. Clinical Micro Sensors Inc. (now GenMark Diagnostics, Inc.), reported on the first use of PCB platform in molecular diagnostics (Farkas, 1999; Umek et al., 2001), where they reported electrochemical sequence

specific detection of DNA via sandwich hybridization with a reporter probe containing ferrocene moieties. Gassmann and his colleagues (Gassmann et al., 2012) developed a PCB based DNA chip for amplification and electrochemical detection, while Tseng and his group (Tseng et al., 2014) recently described a PCB based electrochemical biosensor array for the quantitative detection of PCR amplicons using methylene blue as the redox indicator.

In this paper, we report the electrochemical detection of seven genes relevant to the molecular characterization of breast cancer cells amplified using MLPA incorporating unique barcode sequences using a novel low-density electrode array fabricated using standard printed circuit board (PCB). The CDH1 gene encoding for epithelial Cadherin 1 is a tumour suppressor gene, which expression has been implicated in cancer progression and metastasis. The CDH2 gene encodes for the protein Cadherin 2 and appears to be a potential breast cancer metastases marker. The CD24 and CD44 genes, the ratio “CD44 positive-to-CD24 negative” are of particular clinical relevance in breast cancer and are used to classify breast cancer cells with stem-like characteristics. The protein CD24 is involved in cell adhesion and found at the surface of most B Lymphocytes and differentiating neuroblasts, whilst CD44 is involved in cell-cell interactions, cell adhesions and expressed in a large number of mammalian cell types. The ERBB2 gene encodes for the protein HER2 (Human Epidermal Growth Factor Receptor 2), of which over-expression plays a major role in the development and progression of certain breast cancer types. E3 ubiquitin-protein ligase HUWE1 is an enzyme that is encoded by the HUWE1 gene, which has been identified as being overexpressed in breast, lung and colorectal cancers. Finally, the KRT19 gene encodes for the proteins Keratin, type I cytoskeletal 19 known to support epithelial cell integrity and that can be used as a reference marker due to its' high sensitivity in the diagnosis of disseminated breast cancer tumour cells.

The microarray consists of 64 individually addressable gold working electrodes sharing common reference and counter electrodes. Cancer genetic biomarkers were amplified by asymmetric MLPA and the ssDNA amplicons hybridized to capture probes complementary to each of the incorporated barcode sequences. The surface bound DNA duplexes were then hybridized with a secondary DNA probe labelled with HRP molecule and finally a precipitating TMB substrate for membranes was added and detected using fast electrochemical pulse amperometry. Assay conditions such as hybridization time and temperature were optimised and the specificity and sensitivity evaluated.

2 MATERIALS AND METHODS

2.1 Materials

Chemicals were purchased from Sigma Aldrich (Spain) unless otherwise stated. Ultrapure water was obtained from a Millipore purification system (Millipore, Spain).

2.2 Instrumentation

Electrochemical characterization of the electrode array was performed using a PGSTAT12 potentiostat (Metrohm AG, The Netherlands) using an external Ag/AgCl wire reference electrode and a platinum counter electrode. The PGSTAT12 was equipped with four MUX modules (Metrohm AG, The Netherlands) of sixteen channels each. The MUX module allows sequential interrogation of up to 64 working electrodes that share the same reference and counter electrode.

All electrochemical DNA detection assays were performed using a dedicated 64-channel measuring system. This system allows simultaneous amperometric measurements of all electrodes using the on-chip reference and counter electrodes. The study of the effect of

mixing on the hybridization efficiency was carried out using a Cavro™ syringe pump obtained from Tecan Systems (San Jose, CA, USA).

2.3 Electrode chip design

The electrode chip was designed “in-house” using AutoCAD software (Autodesk Inc, USA) and manufactured at Fineline GmbH (Hilden, Germany) using printed circuit board (PCB) technology. Its' design was based on a previous sensor chip prototype designed and manufactured “in-house” using the same technology (Salvo et al., 2014). The electrode array is a one-layer PCB of 1 mm thick fabricated using the classical FR-4 glass epoxy resin (30 μm Cu thickness) as rigid substrate and with a surface finish of 3 μm soft gold deposited on a nickel layer of approximately 4 μm. Soft gold surface finish refers to the electrolytic deposition of gold onto nickel-protected copper tracks from a gold deposition bath of 99.99% purity as per manufacturer's instructions. This PCB-based chip has a square shape with a side-length of 24.6 mm and consists of 64 individually addressable circular gold working electrodes of 300 μm diameter, sharing a common gold counter and common gold reference electrode, also circular and 250 μm of diameter. All electrodes are linked to connection pads, which are located at the edges of the PCB, through tracks of approximately 100 μm wide separated by a gap of 175 μm. The electrode arrays were insulated with green solder mask with openings to define the geometrical area of the connection pads, working, counter and reference electrodes, and to avoid exposure of the electrode tracks to fluids (Figure 1, A and B).

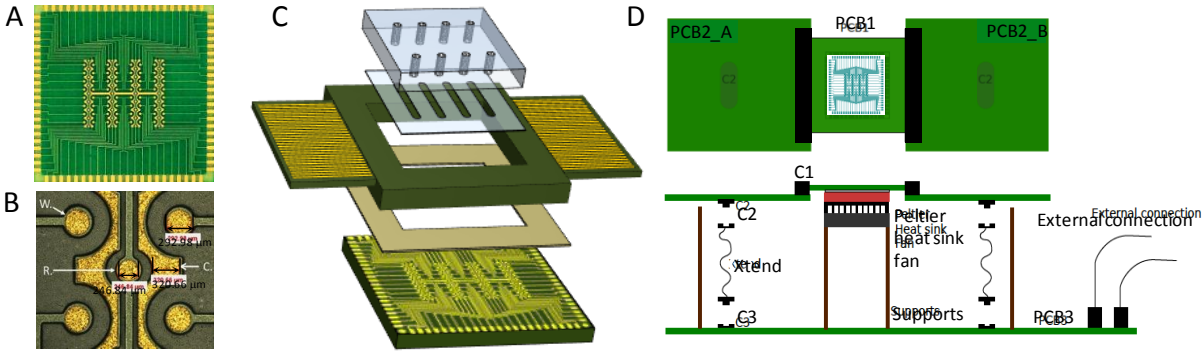

Figure 1 – Electrochemical detection platform. A) 64-electrode array realised on PCB; B) Magnified image of the PCB chip showing the working (W), counter (C) and reference (R) electrodes; C) Schematic representation of the sensor (1. PCB chip, 2. Anisotropic conductive adhesive tape, 3. PCB carrier, 4. Microfluidic channels of double-sided adhesive tape and 5. Laser machined PMMA gasket); D) Detection unit fully assembled.

2.4 Electrochemical detection unit set-up

In order to allow the connectivity between the electrode chip and the potentiostat, as well as the injection of samples to the array, a laboratory test set-up was designed, fabricated and assembled (Figure 1, C and D). The unit consists of the PCB sensor, four interface PCBs, and a series of connectors and cables coupled to the potentiostat. The PCB chip is first mounted onto a PCB carrier with the help of an anisotropic conductive double-sided adhesive gasket (3M, USA). This PCB carrier slots through edge connectors into two interface PCBs, which can be connected to either the commercial PGSTAT12 potentiostat or the dedicated measuring prototype system. To allow the addition of samples to the sensor, simple microfluidic flow cells were fabricated using a Fenix CO_2 laser (Synrad Inc., USA) to cut and drill 2 mm thick poly(methylmethacrylate) (PMMA) sheets and define the microfluidic channels in 100 µm thick medical grade double-sided adhesive gasket (Adhesives Research Ltd., Ireland).

2.5 Oligonucleotide sequences

DNA probes designed in-silico and synthetic single stranded DNA (ssDNA) were purchased as lyophilized pellets from Biomers.net or MWG Operon GmbH (Germany) and reconstituted in

Rnase and Dnase-free water. The details of the sequences used can be found in the supplementary information (SI).

2.6 Barcode design

In-house barcode generation software to generate unique barcodes composed of only purines or pyrimidines (Costea et al., 2013) was exploited. Only unique barcodes with no significant hybridization with each other or any adapters used in the experiments were considered. The uniqueness of barcodes was determined based on how many base changes, insertions or deletions (edit distance) were required to convert one barcode to another. A relatively high edit distance (6) to ensure unique mapping after sequencing was chosen.

Random DNA sequences of 23 bases in length and composed of only purines or pyrimidines were generated. Not more than three mono- di- or tri-mer repeats were included. Only those with 45% to 65% GC content were taken further. Sequences with low complexities (e.g. containing repeats) were removed using the Lempel-Ziv (LZ) compression algorithm (Ziv and Lempel, 1977). Sequences with low complexity were compressed better due to their poor information content, whereas those with higher complexity were compressed less, leading to higher compression scores. Only sequences with compression score greater than 11 were selected. This was done empirically based on the number of sequences eliminated. Only sequences that were at least 6 Levenshtein edit distance to each other were considered, i.e. even if five errors per barcode were introduced during experiment or sequences, all the barcodes were uniquely mapped. Levenshtein distance is the minimum number of changes (insertions, deletions, substitutions) to convert one string to another (Levenshtein, 1966). We paired barcodes to gene-specific primers if no significant hybridization formed between them (deltaG >= -5 kcal/mol at 50.0 C). Sequences that hybridize to each other were also discarded (maximum Tm = 50.0) (Costea et al., 2013).

2.7 Asymmetric multiplexed ligand dependant probe amplification (MLPA)

MLPA primers and probes were designed and quality tested by MRC-Holland according to their standardized protocols. A universal primer pair (primer X and Y) was used to amplify all ligated probes by PCR, (Primer pair obtained from MWG, Supplementary Information). A 2.5 µl of a positive quality control sample containing a mixture of 10 nM of each cancer biomarker template was used. An initial incubation of 5 min at 98°C was followed by the addition of 1.5 µl of a mix containing 0.75 µl MLPA Buffer (MRC-Holland, Amsterdam, the Netherlands) and 0.75 µl of a solution containing 3 fmol of each target-specific oligonucleotide. The mixture was incubated at 95°C for 60 s to denature the probes, after which hybridization took place at 60°C for 1 h. Subsequently, ligation of MLPA probes was performed by adding 8 µl of a mix containing 1.5 µl ligase buffer A (MRC-Holland, Amsterdam, the Netherlands), 1.5 µl Ligase Buffer B (MRC-Holland, Amsterdam, the Netherlands), 1 µl Ligase-65 (MRC-Holland) and 4 µl of water. Ligation was performed for 4 minutes at 54°C followed by an incubation of 5 min at 98 ºC.

Finally, single stranded DNA amplicons were generated by asymmetric multiplex amplification, which was performed by addition of 8 µl of a mix containing 1.2 units SALSA Polymerase (MRC-Holland, Amsterdam, the Netherlands), 4 nmol dNTPs, 20 pmol of Cy3-labeled forward primer Y, 3 pmol of unmodified reverse primer X and 4 µl of Q-solution (Qiagen, Hilden, Germany). Asymmetric amplification consisted of 35 PCR cycles (30 s 95°C, 30 s 60°C, and 60 s 72°C). After the final cycle of the PCR, the samples were held at 72°C for 10 min (Figure 2, A and B).

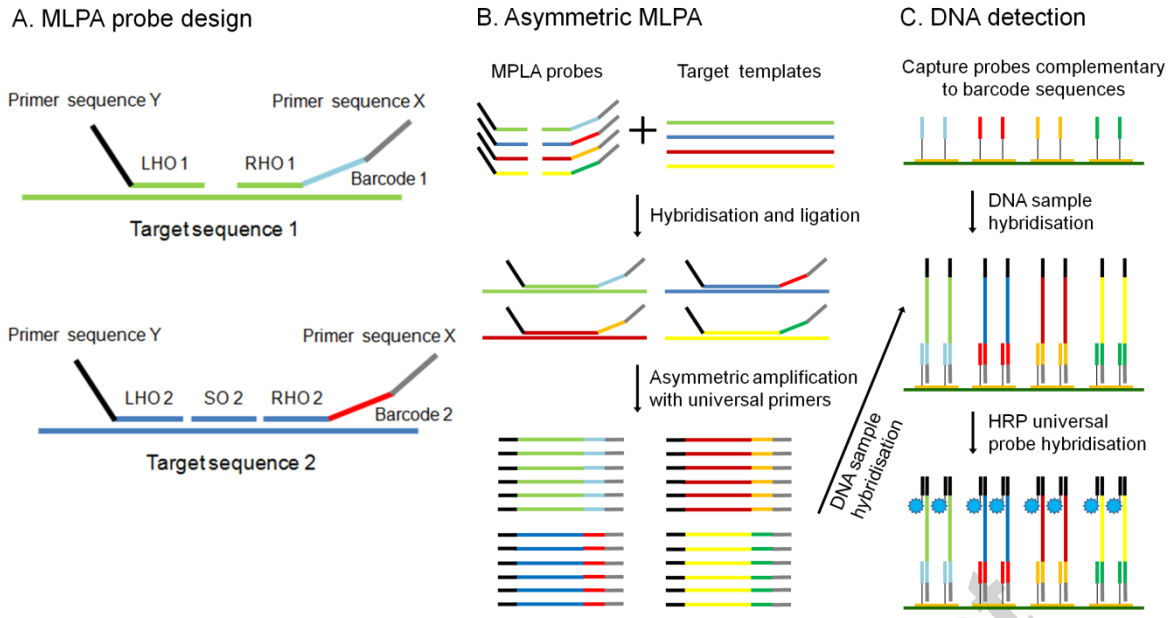

Figure 2 – A) Design of the MLPA probes. The MLPA probe mix consists of either two or three oligonucleotides: a left hybridization oligonucleotide (LHO) consisting of a target-specific sequence and a universal primer sequence Y, a right hybridization oligonucleotide (RHO) consisting of a target-specific sequence, a unique barcode and a universal primer sequence X. Some of the probes also had a sequence-specific spanning oligonucleotide (SO) to increase specificity. B) Schematic representation of the asymmetric MLPA process showing the hydridization and ligation of the MLPA probes to the single strand MLPA samples and the asymmetric amplification with universal primers. C: Schematic layout of the electrochemical detection using unique barcodes and a universal reporter probe labelled with HRP enzyme.

2.8 Electrode chip functionalization and assay

Electrode arrays were successively sonicated for 5 minutes in deionised water, acetone and isopropanol and blow dried in a stream of compressed air before being plasma treated for 20 minutes (5 SSCM O_2/ 5 SSCM Ar) using a Orion-8-HV sputter (AJA Internacional Inc., USA). Cleaned arrays were subsequently immersed in a 1 mM ethanolic solution of carboxylate-terminated aromatic dithiol (Sensopath Ltd., USA) overnight. Following extensive washing with ethanol, the arrays were immersed for 30 minutes in a solution of 200 mM EDC and 50 mM NHS prepared in deionised water, rinsed with water and dried in a stream of compressed air. DNA probes were dissolved to a concentration of 10 µM in 50 mM sodium phosphate (pH 8.5) printing buffer and spotted onto individual electrodes by contact printing using an XActII

microarrayer (LabNext Inc., USA). The modified arrays were incubated overnight in a wet chamber containing a solution of saturated NaCl to achieve approximately 75% relative humidity. Following rinsing with deionised water, the arrays were blocked with ethanolamine (50 mM, 0.1M Tris buffer pH 9), rinsed with deionised water and stored at 4ºC until use. Negative controls consisted of unspotted sensors blocked with ethanolamine.

Approximately 10 µL of the diluted ssDNA MLPA products were injected into the microfluidic set-up and incubated for 60 minutes at 37 ºC. Channels were subsequently flushed with 100 µL of Tris buffer pH 8.0 containing 1 M of NaCl (hybridization buffer) before injecting 20 µL of HRP-labelled secondary probe prepared at a concentration of 10 nM in hybridization buffer and left to incubate for 30 minutes at 37 ºC, before flushing the microfluidics with 100 µL of hybridization buffer (Figure 2.C). The presence of the HRP label was measured by fast amperometry following injection of 20 µL of TMB Enhanced HRP membrane substrate (Diarect AG, Germany) and measuring the reduction current derived from the reduction of the HRP-oxidized TMB at -0.2 V (vs. internal reference).

Data were processed using a Visual Basic macro running under MS Excel to treat the current traces recorded at the 64 electrodes. Initially, the current response at 500 ms was used as hybridization signal. Limits of detection were taken as the concentration value corresponding to the averaged current response of the negative control sensors over the entire concentration range plus three times the average standard deviation.

3 RESULTS AND DISCUSSION

3.1 Electrochemical characterization of the PCB-electrode array

The cost associated with the fabrication of sputtered planar electrode arrays typically escalate due to the many lithographic steps that need to be realised in a clean room environment. In this process, expensive glass or silicon wafers are first coated with a photosensitive lift-off resin, photopatterned by exposure to UV through a photomask followed by the development of the pattern, metal coated and finally dipped in a suitable lift-off solvent to reveal the metal pattern. In a final step, the electrode tracks are insulated by spin-coating another resin that is further photopatterned and developed to create openings at the electrode active sites and their connections. The resulting wafer is finally carefully diced to release individual sensor chip. This type of process is widely accepted and results in well-defined electrodes with sub-micrometers resolution. However, the associated cost is high and incompatible with inexpensive clinical diagnostics, as well. Thus, for biosensors to truly impact on the medical device industry and move from research laboratories settings to the point-of-care and deliver valuable information on a patient's conditions, the fabrication costs have to be considerably decreased without jeopardising the quality of the data generated. Metal micro-patterning based on current printed circuit board fabrication techniques, is an interesting approach for the low-cost mass fabrication of electrochemical sensor arrays. A copper-clad FR4 substrate can be photopatterned and etched, and structures with dimensions as low as 50 μm can be routinely achieved. The copper layer can subsequently be coated with thin layers of gold, silver or nickel either via electrolytic or electroless metal deposition techniques. Using PCB technology, electronic components can be directly integrated on the same device, the thermal conductivity can be adjusted, the electrical conductivity is excellent (<1 ohm cm^{-2}) and flexible substrates such as Kapton can also be used, demonstrating the versatility and maturity of the technology.

However, the quality of the biosensor metal surface is of crucial importance, as the controlled immobilization of specific biological or synthetic receptors at their surface is required. Cleanliness, metal contamination and surface roughness are factors that will affect the

orientation and density of the immobilized receptors, as well as possibly leading to elevated electrochemical background noise. To address those pitfalls, the PCB arrays were manufactured with a surface finish of high purity 3 µm soft-gold electroplated on a 4 µm thick nickel layer, which in turn, was electrodeposited on a 30 µm thick single copper clad FR4 substrate. The nickel layer acts as a physical barrier limiting the solid state diffusion of copper into the soft-gold layer and thus preventing the further copper oxidation at the electrode surface. In an initial attempt, an electroless nickel immersion gold (ENIG) process (Milad and Mayes, 1998) was used to coat the copper layer with gold. This approach resulted in highly contaminated and electrochemically unstable electrodes (data not shown), whilst the soft-gold PCB electrodes exhibited electrochemical behaviour comparable to those of polished polycrystalline electrodes. As seen in Figure 3.A, the voltammogram of a single 300 µm diameter electrode presents a well defined gold oxide region centred at 1.1 V and a sharp reduction peak centred at 0.733 V as can be expected from a pure gold layer, showing no contamination of the gold surface by the underlying copper and nickel layers. Furthermore, three oxidation waves were measured at 1.035 V, 1.115 V and 1.216 V. These features indicate the polycrystalline nature of the gold surface and can be attributed to the low-index crystallographic planes Au(111), Au(100) and (110) for which the stability of an adsorbate is known to differ depending on the surface crystallographic orientation of the gold substrate in the order Au(1 1 1) < Au(1 0 0) < Au(1 1 0) (Arihara et al., 2003; Strutwolf and O'Sullivan, 2007).

The real-surface area, as described by Trassati et al. was calculated from the charge required to reduce the oxide layer and estimated to 5.16 10^{-3} cm^2, i.e. roughness factor of 7.3 (Trasatti and Petrii, 1991). In a final test, we immersed the electrodes in a 5 mM ethanolic solution of 3-mercaptopropionic acid (3-MPA) to assess the ability of the electrolytic gold surface to support the formation of high quality self-assembled monolayers. As can be seen in Figure 3.B, the SAM coating insulated the electrode surface. The bare electrode exhibited well defined oxidation and reduction peaks in the presence of 5 mM $K_2Fe(CN)_6$ centred at 0.218 V and 0.138

V of 1.489 µA and -1.413 µA in intensity, respectively. Following immobilization of the alkanethiol SAM these peaks were shifted and suppressed indicating the efficient blocking of the electrode by the 3-MPA. The intensity of the oxidation wave positioned at 0.294 V decreased slightly to 1.117 µA. However, the reduction wave was considerably more affected being shifted to 0.007 V and broadened to a current maximum of -0.853 µA. The SAM was finally electrochemically desorbed in degassed NaOH to estimate the electrode surface coverage. Following three cycles of desorption, the reduction peaks seen at -0.869 V, -1.068 V and -1.103 V had effectively disappeared, indicating the efficient removal of the SAM from the electrode surface (Figure 3.C). A total reduction charge of 50.32 nC was measured, which translated into a surface coverage of 1.091 10^{-10} mol cm^{-2}, in agreement with results previously published on the deposition of 3-MPA SAM on polycrystalline gold electrodes (Henry et al., 2009a). Electrochemical impedance spectroscopy was used to further confirm the quality of the SAM (Figure 3.D). Upon functionalization of the electrode, the resistance to charge transfer (R_{ct}) increased significantly from 3209.18 for the bare electrode to 81073.7 ohm, and the surface coverage calculated as (1 - õ) = R_{ct0}/R_{ct} was estimated to be 96 % of the electrode surface.

The quality of the electrodes obtained by electrolytic soft-gold plating was therefore found equivalent to that of planar photolithographically fabricated gold electrodes. The results of the preliminary electrochemical characterization demonstrated the absence of contaminants, such as nickel, at the electrode surface as well as the ability of the fold PCBs to support well-organized SAMs of MPA. The cost of the electrode array was vastly reduced, with a cost of ca. 2€ per array as compared to >100€ per array as compared to photolithographically fabricated electrodes.

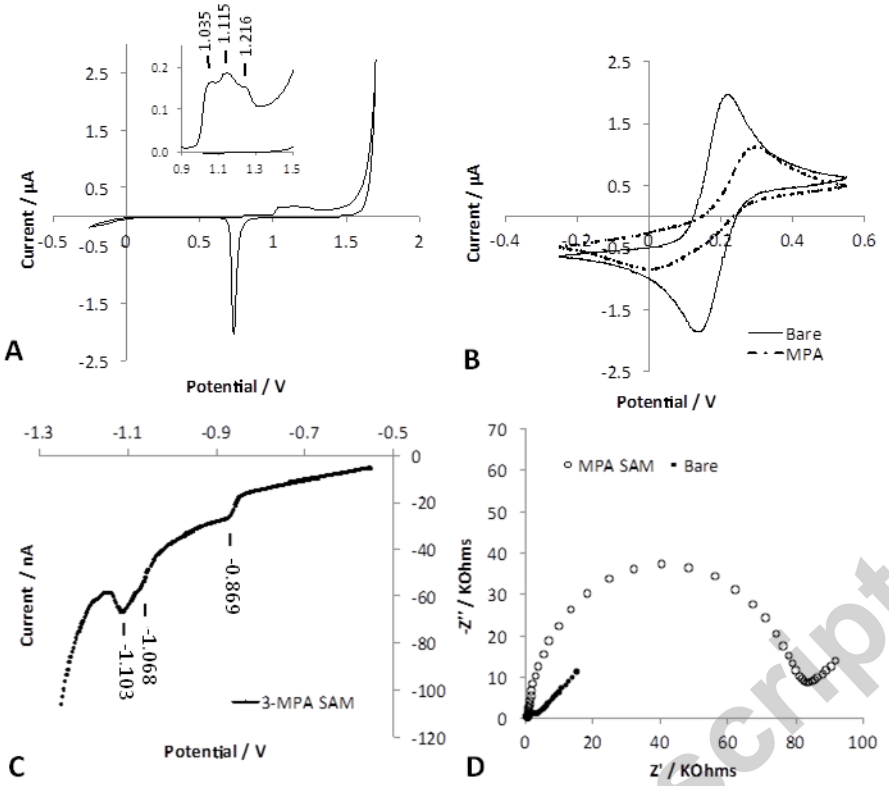

Figure 3 - A) Cyclic voltammetry in 0.5M sulfuric acid of a single electrode after O_2/Ar plasma treatment indicating the polycrystallinity nature of the 3 µm thick gold deposited; B) Cyclic voltammetry in 5 mM potassium ferricyanide prepared in 10 mM phosphate buffer (100 mV s^{-1}) for a bare and an MPA modified 300 µm in diameter electrode made on PCB; C) Reductive desorption of the MPA monolayer in 0.1M NaOH; D) Electrochemical impedance spectroscopy in 5 mM potassium ferricyanide (10^6 to 1 Hz, 5 mV sinusoidal excitation, at 0 V bias potential vs. OCP) for a bare and an MPA modified electrode.

3.2 Genosensor preparation and assay optimization

The sandwich assay format exploited offers very high sensitivity, as previously demonstrated (Henry et al., 2010). The target DNA present in a sample hybridizes to a short complementary DNA probe immobilized on the electrode surface and complementary to a short sequence of the targeted DNA sequence. In a second step, a HRP labelled secondary probe is introduced and hybridized selectively to a second region of the targeted DNA. Finally, the substrate TMB is used to reveal the presence of HRP, which is proportional to the concentration of target DNA

captured at the electrode surface (Figure 2.C). The system however suffered from relatively high RSD (relative standard deviation) values and high background signals.

The high RSD was reduced by automating several preparative steps, improving the sensor-to-sensor as well as chip-to-chip reproducibility (n=160, 14% RSD and n=5, 11% RSD respectively, 10 nM CD24). Physical cleaning methods, such as O_2/Ar plasma cleaning, were preferred over harsh chemical solutions such as piranha etching. It was also found that if the O_2 present in the plasma could effectively remove any organic materials left over from the microarray patterning process, the Ar plasma in turn helped generating a fresh gold surface at an etch rate of approximately 2 nm min^{-1} (Lewicka et al., 2011). The treated arrays were immediately functionalised with -COOH terminated bipodal PEG alkanethiol (Fragoso et al., 2008) and kept under vacuum until further use. The immobilized SAM served the dual function of protecting the array from environmental contamination as well as enabling the coupling of $–NH_2$ terminated DNA probes via EDC/NHS carbodiimide chemistry deposited onto individual electrodes using a microarray contact spotter.

The high background signal was found to originate from the active transport of the oxidized TMB (TMB_{ox}) during its' injection into the microfluidic cell (Figure 4.A). Theoretical calculations and molecular modelling ruled out the possibility of passive diffusion of TMB_{ox} to generate such a high background current in the experimental timeframe, as 295 and 374 seconds were required for the TMB_{ox} to migrate to the next electrodes 1.155 and 1.465 mm away, respectively (Supporting Information). However, due to TMB:HRP reaction kinetics and the fluid dynamics, the TMB_{ox} generated at one electrode can be actively transported to the next electrode even minutes after the TMB injection, resulting in high background current (Figure 4.C). To limit the transport of TMB_{ox} to adjacent electrodes, different means of either slowing down the reaction kinetics or forcing the precipitation of TMB_{ox} were investigated. Precipitating TMB formulations, which typically contain additives such as alginic acid, methyl

vinyl ether/maleic anhydride copolymer, dextran sulfate and/or carrageenan, can readily precipitate TMB_{ox} and are commonly used in immunohistochemistry and Western blotting. The use of precipitating TMB as an efficient electrochemical substrate has already been reported (del Río et al., 2014), and the precipitated TMB was found to conserve its' electroactivity, albeit producing lower currents. More importantly, it formed a stable electroactive precipitate at the electrode surface that could not be dissolved in aqueous buffer. Consequently, following the hybridization and HRP-labelling steps, the arrays were incubated for 5 minutes in precipitating TMB and flushed with 100 μL of Tris buffer before carrying out the electrochemical measurement (Figure 4.A).

Figure 4.C presents a comparison of the genosensor array measured under various conditions for a CDH1 sensor exposed to a concentration of 1 nM CDH1 (synthetic amplicon). Currents measured in conventional TMB substrate (ELISA) averaged 175.9 ± 11.8 nA and a low signal-to-background ratio (S/B) of 3.9. Performing the same assay but measuring in p-TMB considerably decreased the background signal, as well as the specific signal, although the S/B ratio was considerably improved to 10.2. Flushing the electrode array with Tris buffer was found to rinse any p-TMB poorly adsorbed at the electrode surface (Figure 4.A), further reducing the background current to 2.7 ± 0.3 nA whilst not affecting the intensity of the specific signal. Under those conditions the S/B was improved by a factor of 7.5. Finally, by comparing the raw data for a control electrode and a CDH1 positive electrode exposed to p-TMB and measured in Tris buffer, as presented in Figure 4.B, the final current value could be measured after 100 ms which corresponds to the time required by a control electrode to reach a steady-state response. Holding the electrode at 0 V for 10 ms followed by a potential step at -0.2 V for 490 ms results in the development of a small non-faradaic current, seen as a rapid return to baseline at the control electrode. The combination of precipitated TMB and rapid redox-cycling by the HRP at the electrode surface leads to the development of a large faradaic current that

rapidly decays and stabilizes. The measurement was therefore taken at 100 ms to limit background signals and conserve a high specific signal, with a S/B of 26.9.

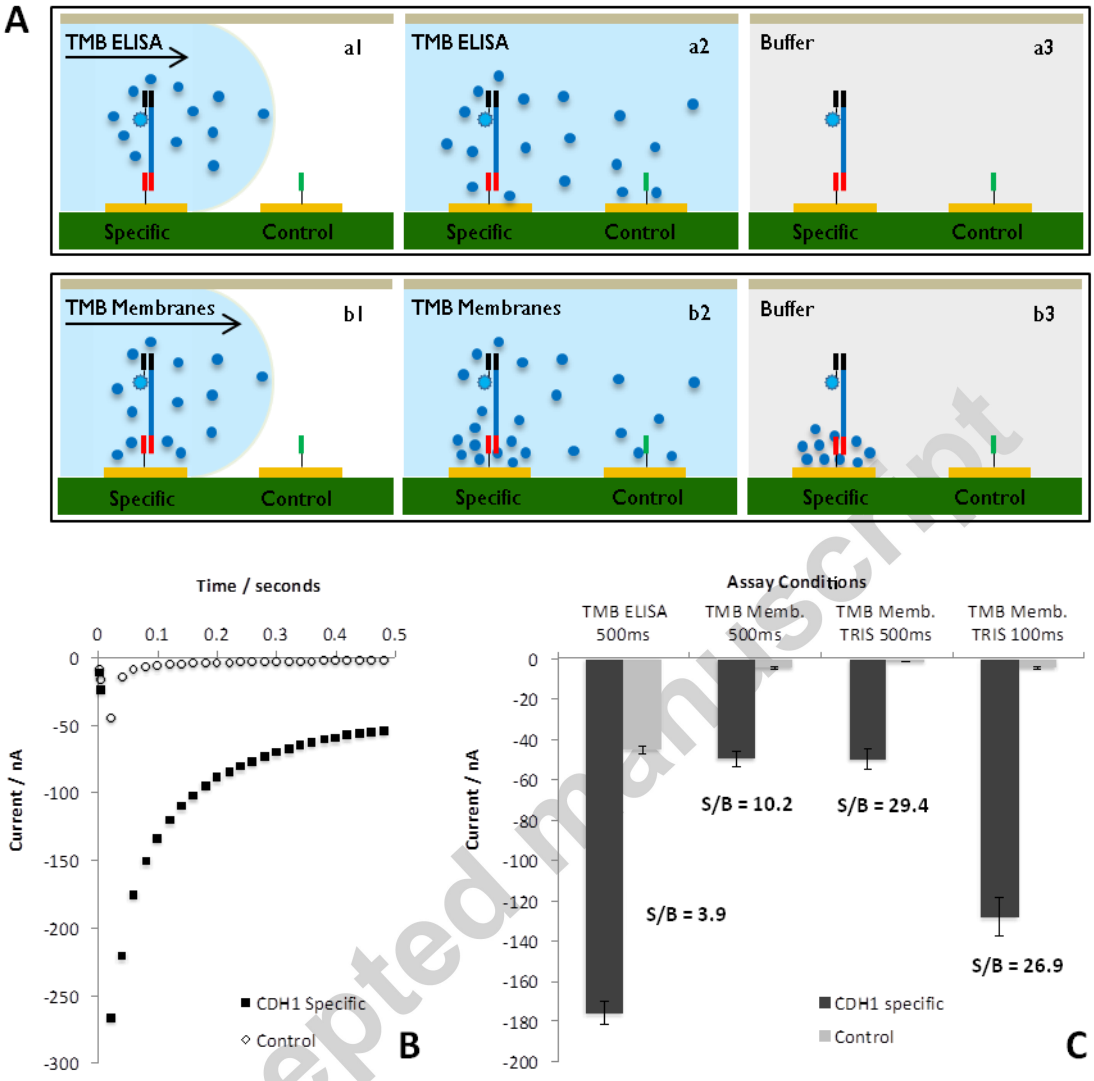

Figure 4. A) Schematic representation of the assay designed to suppress background signal. Illustrations a1 and a2, and b1 and b2 show the injection in the flowcell of TMB-ELISA and TMB membrane respectively. TMB ELISA leads to large background signal due to diffusion. Using precipitating TMB, a stable TMB coating forms onto the electrode. A buffer flush removes adsorbed TMB and decrease background signals. Representations a3 and b3 depicts the state of each system after flushing with buffer; B) Raw data measured in TMB-ELISA for a control and a CDH1 specific sensor exposed to 1 nM of CDH1 synthetic amplicon and subsequently HRP-labelled; C) Comparison of the signal measured under different conditions (TMB ELISA vs. precipitating TMB, at 500 ms and 100 ms).

Several aspects also have a remarkable influence on the output of solid phase hybridization assays. In order to further improve the assay performance, the effect x of factors such as temperature, time and mixing have were also evaluated. Carboxylate-modified PCB electrodes were functionalised with two genetic markers, CD24 and ERBB2, as previously described and each assessed with 1 nM synthetic ERBB2 target at various hybridization temperatures (room temperature (RT), 37 and 50 ºC) and times (2, 10, 30 and 60 min). The temperature was monitored via the use of a purpose-built temperature-controlled device that consisted of a heating element that was mounted beneath the electrode chip. This device allowed execution of constant-temperature experiments. Once set at the desired temperature, approximately 60 seconds were required to re-establish the experimental temperature upon injection of room temperature reagents. As presented in Figure 5.A, performing hybridization at 37 ºC considerably enhances hybridization efficiency, and for a hybridization of 30 minutes at 37 ºC, the current values measured were approximately 3.1 times higher than those obtained with hybridization at room temperature. At 37 ºC, the current readings for 30 and 60 min were very similar, indicating saturation of the probe-functionalised surface by target DNA. Performing the experiments at 50 ºC considerably reduced the sensitivity of the assay, as this temperature adds considerable stringency to the assay, which is translated into smaller signals. Increased hybridization temperatures and times did not have a significant impact on the background signals measured on both the control and the non-specific CD24 probe modified electrodes.

Hybridization depends on the diffusion of a DNA target from the bulk solution to a surface-bound DNA probe so an efficient DNA diffusion is required. To enhance sample diffusion at the electrode surface, the effect of mixing during both the target and the DNA-HRP hybridization steps was evaluated. In another array prepared as described above, a concentration of 1 nM of synthetic ERBB2 marker was injected into the microfluidic cell and allowed to react for 60 min at RT with and without mixing. The mixing was achieved by repeatedly passing the sample back and forth over the sensor surface using syringe pumps at withdrawing/dispensing flow rates of

0.8 µL/s. Following washing, a 10 nM solution of DNA-HRP was injected into the cell and left to react for 30 min at RT under static or mixing conditions. As outlined in Figure 5.B, higher current levels were obtained when mixing was implemented.

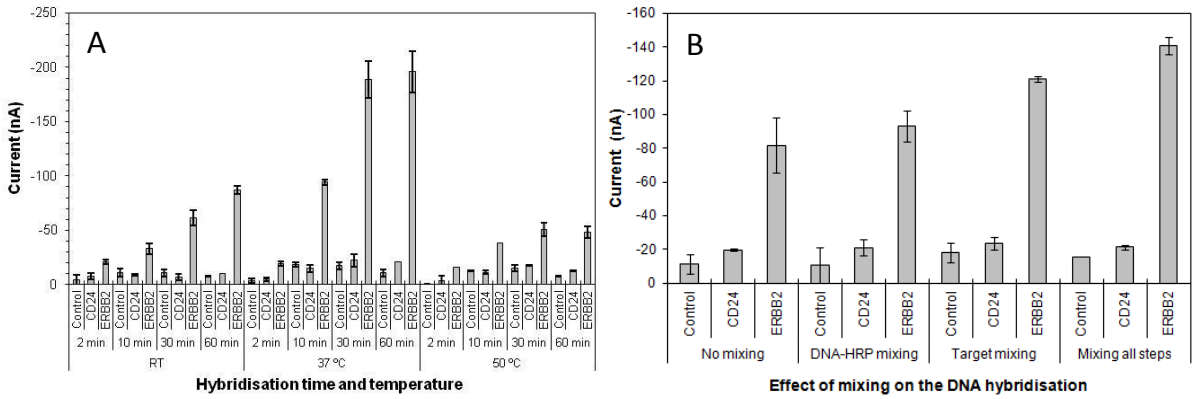

Figure 5. Effect of time, temperature (A) and mixing (B) on the DNA hybridization efficiency of the synthetic ERBB2 amplicon.

3.3 Assay performances and multiplexed detection of MLPA products

3.3.1 Assay performances using synthetic oligonucleotides

The sequence and composition of receptor DNA probes have a great impact on the specificity and sensitivity of the assay. Based on the optimised electrochemical assay, each probe was assessed individually for hybridization efficiency and possible cross-reactivity. The electrodes were exposed to known concentrations of their respective synthetic amplicons, prepared in hybridization buffer solution. Sensitivities vary for each of the genosensor, from 59.0 $nA \cdot nM^{-1}$ for HUWE1 to 171.5 $nA \cdot nM^{-1}$ for ERBB2. The limits of detection taken as the marker concentration equivalent to the current response recorded at a control sensor plus three times the standard deviation (n=8) were 53 pM, 29 pM, 258 pM, 161 pM, 36 pM, 25 pM and 122 pM for ERBB2, KRT19, CD24, CD44, CDH1, CDH2 and HUWE1, respectively (Supplementary Information).

To evaluate the specificity of the assay, a cross-reactivity study was carried out. The electrode arrays were functionalised with probes for the detection of each of the seven genetic markers, as already described. Subsequently, a concentration of 1 nM of each synthetic marker was injected individually to the sensor to test its' interaction with all the probes. As can be observed in the Figure 6.A, very low cross-reactivity was measured. The signals recorded for unrelated probes remain close to that recorded for the negative control sensors (i.e. coated with the carboxylate-terminated aromatic dithiol). All assay steps were realised at a constant temperature of 37ºC. Electrochemical measurements were carried out using the developed measuring system, which allows the simultaneous reading of the 64 working electrodes in less than 5 seconds. The variations in sensor sensitivity are attributed to the marker probe design. Whilst the designs were optimised in silico by adjusting the GC contents, melting temperature and reducing cross-reactivity between probes, the different sequences will lead to heterogeneous hybridization efficiencies. Both the low cross-reactivity and low detection limits observed, demonstrate the suitability of the barcode-based detection approach and its' ability to accurately assess the levels of DNA amplicons in unknown samples, making the developed assay suitable for detection of MLPA amplicons.

3.3.2 Analysis of MLPA product

To confirm the suitability of the barcode approach for the detection of MLPA amplicons, a positive control sample containing all seven gene markers was prepared. The generation of ssDNA was carried out by multiplexed asymmetric amplification, which preferably amplifies one strand of the DNA target, by limiting the concentration of one of the primers. A PCB sensor array was prepared for the simultaneous detection of the seven genetic markers. The ssMLPA sample was diluted three times in the hybridization buffer and injected onto the sensor chip via the microfluidics. In another microfluidic channel, a solution containing a mixture of all

synthetic markers at a 1 nM concentration was injected as a calibrator for quantification. All gene markers were successfully detected in the MLPA sample with current signals ranging from 151 to 214 nA while maintaining low current levels in the control electrodes (12 nA) (Figure 6.B). Using the current values measured in the reference channel, the concentration of each cancer marker present in the sample was estimated by interpolation. The concentrations were of 1.8, 2.5, 2.6, 2.9, 2.9, 3.4 and 2.1 nM for CD24, CD44, CDH1, CDH2, ERBB2, HUWE1 and KRT19, respectively.

This result shows the potential applicability of the MLPA-barcode-detection approach for genetic profiling. The system exhibited great possibilities for miniaturization and integration into a stand-alone module for the amplification and detection of DNA. Indeed, the barcode approach could be used to generate generic electrode array platforms for detection of different sets of MLPA products on different arrays, but using the same barcode sequences, highlighting the huge potential application of the developed approach.

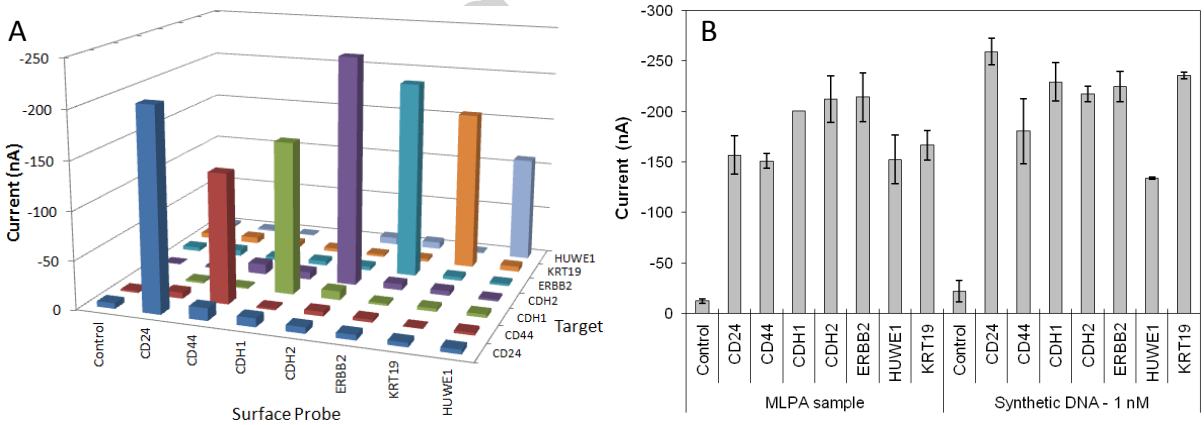

Figure 6. A) Electrochemical cross-reactivity study carried out for a breast cancer marker set (CD24, CD44, CDH1, CDH2, ERBB2, KRT19 and HUWE1). The PCB sensor was functionalized with each one of the thiolated DNA capture probes of the set, and then assessed for the detection of a concentration of 5 nM of each synthetic amplicons; B) Electrochemical detection of single stranded MLPA generated by asymmetric PCR. In the calibration channel, a mixture of each synthetic marker was injected at a concentration of 1 nM.

4. CONCLUSIONS

A method for the multiplex amplification and detection of seven genetic markers present in circulating breast cancer cells was reported. mRNA detection was based on DNA amplification exploiting asymmetric MLPA, which facilitates simultaneous amplification and ssDNA generation of multiple genes. The MLPA probes were specifically designed to incorporate a unique barcode sequence in each amplified gene, which was subsequently used for hybridisation to a surface-immobilised probe. A low-cost, low-density electrode array fabricated using standard PCB technology, was designed and fabricated, exhibiting excellent electrochemical properties as well as array-to-array and sensor-to-sensor reproducibility. The electrochemical measurement was optimised to considerably improve the signal-to-background ratio as well as enhancing hybridization via mixing, achieving limits of detection as low as 25 pM. High specificity was demonstrated, thus facilitating the simultaneous detection of seven gene markers. This approach provides a novel strategy for the multiplexed genetic profiling of tumour cells and the use of barcodes provides a generic platform for detection of other MLPA amplicon sets e.g. lung cancer / prostate cancer, incorporating the same barcodes and thus using the same surface-tethered probes on an electrode array.

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Highlights

- Multiplex amplification using MLPA incorporating unique barcode sequences for detection via hybridisation
- Cost-effective gold microelectrode arrays produced using printed circuit board technology
- Genetic profiling of circulating breast cancer tumour cells via quantitative detection of mRNA markers