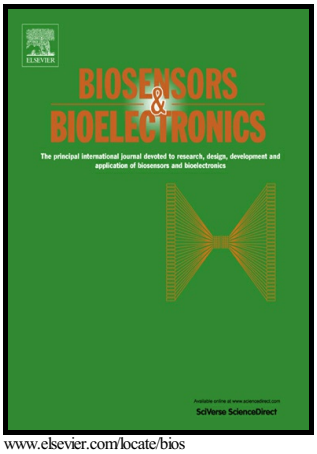

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An Interdigitated Electrode Biosensor Platform for Rapid HLA-B*15:02 Genotyping for Prevention of Drug Hypersensitivity

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Abstract

Prevention of life threatening hypersensitivity reactions to carbamazepine is possible through pre-treatment screening of the associated HLA-B*15:02 risk allele. However, clinical implementation of screening is hindered by the high cost and slow turnaround of conventional HLA typing methods. We have developed an interdigitated electrode (IDE) biosensor platform utilizing loop mediated isothermal amplification (LAMP) that can rapidly detect the HLA-B*15:02 allele. DNA amplification is followed by solid-phase hybridization of LAMP amplicons to a DNA probe immobilized on the IDE sensor surface, resulting in a change in sensor impedance. The testing platform does not require DNA extraction or post-amplification staining, achieving sample-to-answer in 1 hour and 20 minutes. The platform was tested on 27 whole blood samples (14 HLA-B*15:02 positive and 13 negative) with sensitivity of 92.9% and specificity of 84.6% when applying a cutoff of impedance change. Based on these characters the LAMP-IDE platform has potential to be further developed into point-of-care use to help overcome barriers in HLA-B*15:02 screening.

Keywords

Loop Mediated Isothermal Amplification; Interdigitated Electrodes; Impedance Sensor; Nucleic Acid Sensor; Point-of-care Diagnostics; Pharmacogenetics

1. Introduction

Adverse drug reactions (ADRs) account for >770,000 injuries or deaths per year (Agency for Healthcare Research and Quality 2001) in the US. Cutaneous reactions are a common form of immune-mediated ADRs ranging from minor maculopapular exanthema to severe reactions such as Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug-induced hypersensitivity syndrome (DIHS), which carry mortality rates of up to 30% (Svensson et al. 2001). Cutaneous ADRs are largely unpredictable and pose significant clinical and economic burden (Sultana et al. 2013).

Pharmacogenetics research, which examines the role of inter-individual genetic variations in drug response (Yeo et al. 2011), has recently identified a number of associations between the human leukocyte antigen (HLA) gene family and ADRs. Traditionally known for its role in immune response and various disease processes, the HLA gene is one of the most polymorphic in the human genome. Variation is most pronounced within the Class I HLA region, which encompasses the *HLA-A*, *HLA-B*, and *HLA-C* genes. To date, the International Immunogenetics (IMGT)/HLA database records 12,544 allele sequences within Class I, of which 4828 belong to the HLA-B group (Robinson et al. 2015).

One of the most prominent pharmacogenetics discoveries is the strong association between carbamazepine-induced SJS/TEN and the HLA-B*15:02 allele. This association was first found among the Han Chinese population (Chung et al. 2004; Man et al. 2007), and was subsequently reported in other Asian populations where the allele is prevalent, including Thai (Locharernkul et al. 2008), Malay (Chang et al. 2011), and Indian (Mehta et al. 2009). Carbamazepine is widely used globally (World Health Organization 2015) as a first-line treatment for epilepsy (Glauser et al. 2006) and for other indications such as neuralgia and bipolar affective disorder. The US Food and Drug Administration and other regulatory agencies recommend HLA-B*15:02 screening prior to administering carbamazepine in patients of high-risk Asian ancestry (U.S. Food and Drug Administration 2007).

Although pre-treatment HLA typing was demonstrated to be clinically beneficial for prevention of carbamazepine-induced SJS/TEN (Chen et al. 2014), implementation in routine practice has been hindered by several factors. Due to its highly polymorphic nature, the gold-standard clinical methods for conventional HLA genotyping include sequence based typing (SBT) (Erlich 2012), and commercial polymerase chain reaction (PCR) with sequence

specific primer (SSP) or sequence specific oligonucleotides (SSOP) (Karlin and Phillips 2014). These laboratory-based approaches have several limitations. First, they require expensive laboratory equipment such as thermal cyclers and fluorescence optical detection systems operated by skilled laboratory personnel. Second, although the PCR step may only take several hours, DNA extraction is required, and in clinical practice, HLA testing has long turnaround time (at least several days) starting from sample collection, transportation, to testing and reporting of results to the clinicians, thereby compromising effective treatment strategies. Given these constraints, it is not surprising that HLA-B*15:02 testing remains non-existent in most low-resource Asian countries. In settings that have implemented a screening policy, current platforms prove impractical when immediate decision on drug usage needs to be made (e.g. for seizure prevention), leading to extra clinic visits or hospital stay, and use of alternative medications which are not only more expensive, but may be less effective and may potentially lead to other unwanted side effects (Chen et al. 2016).

To reduce the time and cost for diagnostic HLA-B*15:02 testing, this study aimed to develop a 2-step rapid genotyping platform (Fig. 1) that can use crude blood samples and can be amenable for point-of-care applications and miniaturization. The first differentiation step involves a loop mediated isothermal amplification (LAMP) reaction that selectively amplifies HLA-B alleles. The second step involves hybridization of the LAMP amplicons with the DNA probes immobilized on the surface of the interdigitated electrode (IDE) sensor, which serves as the mono-allelic determinant of an HLA-B*15:02 LAMP amplicon. Positivity is indicated by changes in the electrical impedance of the sensor surface as a result of specific probe hybridization to complementary HLA-B*15:02 LAMP amplicons.

LAMP (Notomi et al. 2000) is an isothermal gene amplification method that has gained popularity due to its rapidity, sensitivity, and permissiveness toward the use of crude specimens such as saliva (Sriworarat et al. 2015), urine (Edwards et al. 2014), stool (Fernandez-Soto et al. 2016), and blood (Cheung et al. 2014) samples. LAMP amplicon hybridization for various diagnostic purposes have previously been performed through solid (Nakamura et al. 2007) and liquid phase (Deng et al. 2015; Yongkiettrakul et al. 2014) approaches using colorimetric, fluorometric, and electrochemical platforms. Attempts to integrate the LAMP reaction with electrical sensing have required post-hybridization labelling (Nakamura et al. 2007; Nakamura et al. 2009), which adds substantially to the cost of testing. In contrast, our label-free electrical impedance spectroscopy (EIS) approach relies solely on detection of signal impedance changes associated with affinity binding on the IDE

configuration. Other advantages of IDE sensors are their demonstrated sensitivity for detection of various biomolecules (Abeyrathne et al. 2016a; Abeyrathne et al. 2016b; Wang et al. 2017b), as well as their simple and low-cost fabrication through UV lithography. These qualities render the LAMP-IDE platform amenable for future miniaturization and integration into a point-of-care device, negating the need for expensive equipment for detection of the HLA-B*15:02 allele.

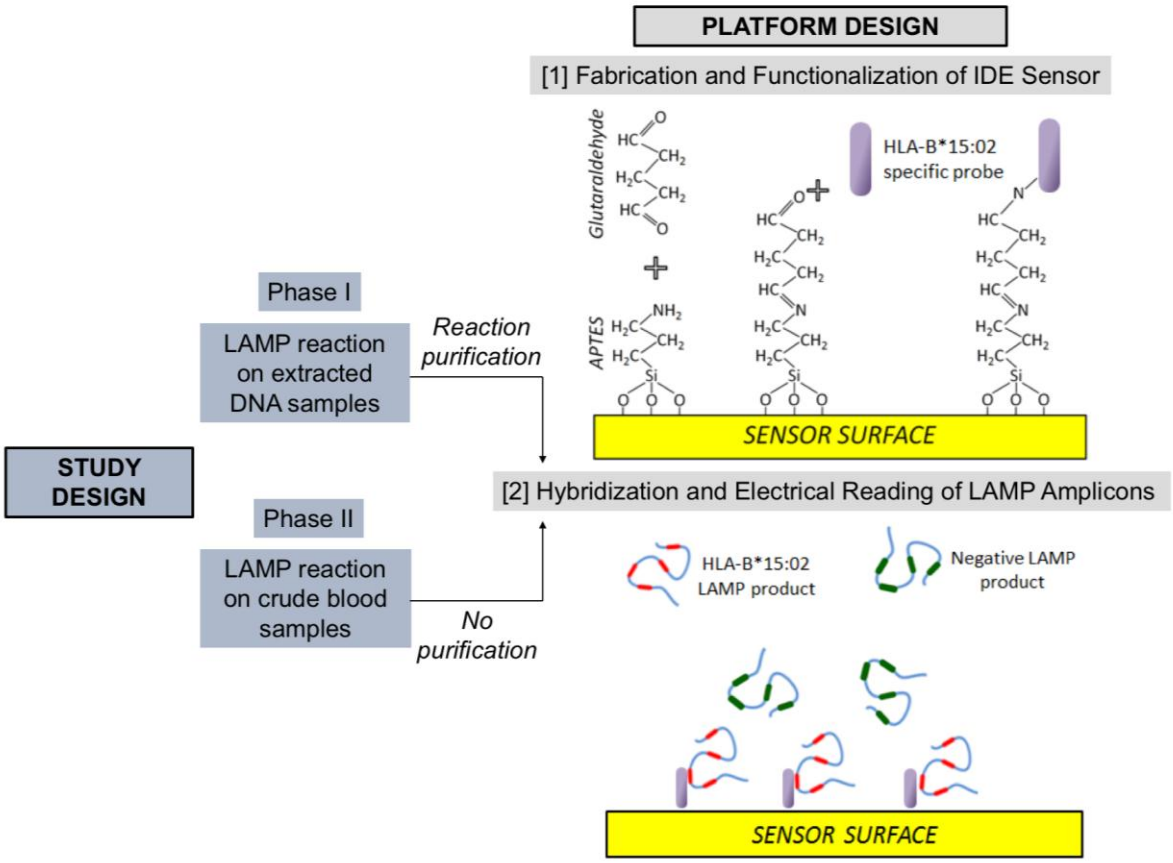


Fig. 1. Schematic representation of the LAMP-IDE platform and study design. The platform integrates primary LAMP-based amplification of the HLA-B gene with secondary solid-phase hybridization of the HLA-B*15:02 LAMP amplicon on capture DNA probes immobilized on an IDE sensor configuration. The study was divided into two phases: phase I involved optimization and proof-of-concept of the LAMP-IDE sensor on purified DNA samples while phase II evaluated its sensitivity and specificity on crude blood samples.

This study was divided into two phases (Fig. 1). Phase I involved optimization and proof-of-concept experiments using purified human genomic DNA samples. Each sample underwent LAMP reaction followed by reaction purification and sequencing to confirm presence of the gene region of interest. Amplicons were subsequently incubated on the pre-functionalized IDE sensors for hybridization at a known concentration. The optimal sensor geometry and applied frequency for the IDE platform were determined. Signal characterization was performed on a panel of purified LAMP amplicons that were either positive or negative for HLA-B*15:02. In phase II, the protocol developed in phase I was evaluated for its sensitivity and specificity in detecting HLA-B*15:02 using a panel of crude blood samples.

2. Materials and Methods

2.1 Samples

Phase I. LAMP reactions were performed using human genomic DNA samples of known HLA-B genotypes obtained from the International Histocompatibility Working Group (IHWG) DNA Bank. Amplicons were purified (refer to Section 2.4) to remove contaminants. In the hybridization step, LAMP amplicons used were either entirely complementary to the probe, or non-complementary by 2 bases. A sequence of complementary oligonucleotide was also used as a positive control. Sequences of the probe and positive control oligonucleotide are provided in Fig. 2A.

Phase II. Venous blood samples collected in EDTA tubes from 27 patients with epilepsy were used to evaluate the protocol developed in phase I. The study was approved by local IRBs and all subjects provided written informed consent. The HLA-B genotype of each sample was pre-determined by sequence based typing (BGI Hong Kong, Hong Kong). Three groups of blood samples were tested based on their known HLA-B alleles: (1) positive samples heterozygous or homozygous for HLA-B*15:02 (n=14) expected to produce LAMP amplicons complementary to the probe on the sensors, (2) negative samples with either one or two HLA-B alleles expected to produce amplicons non-complementary to the probe by 2 bases (n=5), and (3) negative samples with HLA-B alleles that would either not amplify or will only produce primer repeats non-complementary to the probe by more than 2 bases (n=8), chosen at random. Each sample was subjected to one LAMP reaction, and the reaction products were divided for hybridization on 3 sensors.

2.2 LAMP-IDE assay design

LAMP primers were designed to produce amplicons that contained a section of HLA-B gene exon 2 suitable for subsequent hybridization with an oligonucleotide probe specific for the HLA-B*15:02 allele (Fig. 2A and B). The primer set included forward and backward outer primers (F3 and B3), and forward and backward inner primers (FIP and BIP). The primers were designed by modifying outputs from the primer design software (http://primerexplorer.jp/e/). Suggested primers were compared with sequences of HLA-B*15:02 against other HLA alleles to provide the first step differentiation. The optimal primer design was predicted to cross-react with 5.67% of known HLA-B alleles with amplicons that differed from those of HLA-B*15:02 by 2 bases.

To achieve a higher level of specificity to meet clinical standards, an 11-mer oligonucleotide probe was designed to target the region between the FIP (F1c) and BIP (B1c) of the HLA-B LAMP amplicon for the secondary hybridization step on IDE sensors (Fig 2B). The primer and probe combination is expected to differentiate HLA-B*15:02 from 99.31% of all known HLA-B alleles (Supplementary Table S1).

(A) LAMP-IDE Platform Primers and Oligonucleotides

LAMP Reaction Primers			
Primer Name	Modification	Target Genome Position	Primer Sequence (5' to 3')
F3	-	Exon 2 (287-303)	CGACACCCAGTTCGTGA
FIP (F1c/TTTT/F2)	5' Biotin	Exon 2 (353-369/TTTT/306-321)	GGCCCCTCCTGCTCTATC-TTTT-TTCGACAGCGACGCCG
BIP (B1c/TTTT/B2)	-	Exon 2 – Intron 2 (398-419/TTTT/464-478)	CCAAGACCAACACACAGACT-TTTT-TCACTCACCGGCCTCG
B3	-	Intron 2 (494-509)	GGGAGTCGTGACCTGC
Surface Functionalized Probes and Positive Control			
Oligonucleotide Name	Modification	Target Genome Position	Oligonucleotide Sequence (5' to 3')
Probe	5' Amine	Exon 2 (384-394)	TGTGTGTTCCG
Positive Control	5' Biotin	Reverse Complementary to Probe	CGGAACACACA

(B) HLA-B*15:02 LAMP Primer Target Regions

F3 F2
284 5'- GGACGACACCCAGTTCGTGAGGTTCGACAGCGACGCCGCGAGTCCGAGGATGGCGCCCCGG

F1c Probe Target Region B1c
345 5'- GCGCCATGGATAGAGCAGGAGGGGCCGGAGTATTGGGACCGGAACACACAGATCTCCAAGA

B1c B2
406 5'- CCAACACACAGACTTACCGAGAGAGCCTGCGGAACCTGCGCGGCTACTACAACCAGAGCGAG

B2 B3
468 5'- GCCGGTGAGTGACCCCGGCCCGGGGCGCAGGTCACGACTCCCCATCCCCCACGTACGGCCC

(C) LAMP Product Visualization on Gel Electrophoresis and Chromatogram Confirmation

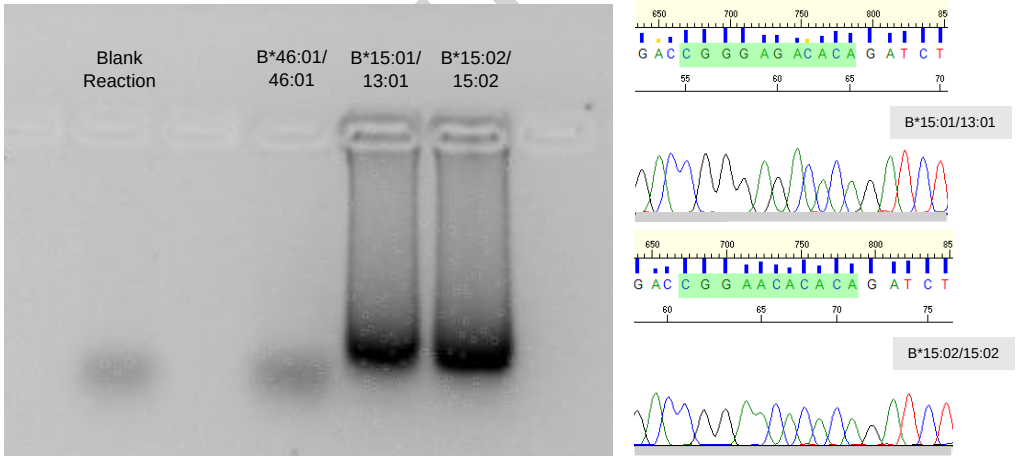


Fig. 2. LAMP-IDE assay. (A) LAMP reaction primer and oligonucleotide sequences (capture probe and positive control) used in the IDE detection step. F3 = forward outer primer, B3 = backward outer primer, FIP = forward inner primer, BIP = backward inner primer (B) HLA-B*15:02 exon 2 sequence with corresponding LAMP primer target regions (grey highlights) and probe hybridization target region (green highlight) with polymorphic sites in bold. (C) Example of LAMP reaction on gel electrophoresis (left) with sequencing confirmation of amplicons on chromatograms (right). On gel, the amplified reactions can be visualized as a 'smear' composed of a heterogeneous mix of amplicons in various sizes and lengths. On

chromatogram, the HLA-B*15:02/15:02 LAMP amplicon shows full complementarity with the probe sequence, as shown in the probe target region (green highlight) between nucleotides 650-800. In comparison, the HLA-B*13:01/15:01 amplicon shows non-complementary bases (vertical red highlights) with both alleles in the sample being non-complementary towards the probe sequence by 2 bases.

2.3 LAMP reaction conditions

Each LAMP reaction was performed in a mix of 1× Isothermal Amplification Buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50mM KCl, 2mM $MgSO_4$, 0.1% Tween® 20 at pH 8.8 (New England Biolabs, Ipswich, Massachusetts), 1.4 mmol/L dNTP (New England Biolabs, Ipswich, Massachusetts), 1.3 mol/L betaine (Sigma Aldrich, St Louis, Missouri), 1.6 μmol biotin-labelled FIP primer, 1.6 μmol BIP primer, 0.4 μmol F3 and B3 primers, 0.24 units of Bst 2.0 *WarmStart* DNA Polymerase (New England Biolabs, Ipswich, Massachusetts). All primers used were of high-performance liquid chromatography purified grade (Life Technologies Australia, Mulgrave, Victoria).

A template volume of 4 μL was added into the mix. In phase I, the template consisted of human genomic DNA at 10 ng/μL. In phase II, 2 μL of crude blood was diluted 1:5 in nuclease-free water and heated for 5 minutes at 90°C. Following heat treatment, 4 μL of diluted blood was used as template for each LAMP reaction. Nuclease-free water was used as template for the blank negative control. Each reaction was adjusted to a final volume of 25 μL using nuclease-free water. LAMP was performed at 63°C for 60 minutes using a thermal cycler (Bio-Rad, Hercules, California). Following amplification, the LAMP products were visualized via electrophoresis using a 1.3% agarose gel (Scientifix, Cheltenham, Victoria) with Gel Red dye (Biotium, Hayward, California).

2.4 Purification and sequence confirmation of LAMP amplicons

This step was performed in phase I to confirm that the LAMP amplicons contained the DNA region of interest. LAMP amplicons were purified using the Wizard® PCR clean up kit (Promega, Fitchburg, Wisconsin) and quantified using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Scoresby, Victoria). Amplicons were then sequenced using a sequencing primer (Life Technologies Australia, Mulgrave, Victoria) via an ABI 3730 XL genetic analyzer located at the Centre for Translational Pathology, University of Melbourne and. Sequencing chromatogram results were aligned with HLA-B*15:02 sequence using ClustalW (Thompson et al. 1994). Fig. 2C shows an example of gel electrophoresis of LAMP products with corresponding sequencing confirmation. Positive samples were amplified while

no product was detected on blank control (water template) and negative HLA-B*46:01/46:01 sample. As expected, cross-reactions may occur, with the HLA-B*15:01/13:01 LAMP reaction shown as an example.

2.5 IDE fabrication

Details of the fabrication process are provided in Supplementary Fig. S2. In brief, sensors were fabricated at the Melbourne Centre for Nanofabrication (Clayton, Victoria, Australia) using UV-lithography on photoresist coated BOROFLOAT® borosilicate glass wafer containing 80% silicon dioxide (SiO_2) in its naïve form. Each sensor comprised of gold electrodes (100 nm), with finger length of 1 mm and finger width that matched the selected gap width (Fig. 3A). For optimization in phase I, 3 different finger/gap widths (6 µm, 8 µm, and 10 µm) were fabricated and tested. Following fabrication, each sensor was subject to additional SiO_2 deposition (25 nm thickness) through e-beam evaporation to increase surface coverage of the SiO_2 layer and to limit the region of polarization through passivation.

2.6 IDE functionalization and probe immobilization

Photoresist was removed using a wash of acetone, ethanol, and H_2O. Sensors were then plasma treated (PE-25 Plasma Etch, Carson City, Nevada) with argon (75%) and oxygen (25%) for 5 minutes at 100 W power, 0.30 Torr pressure, and 30 sccm flow rate to remove organic contaminants which can cause failure of silane growth upon surface functionalization (Gupta et al. 2013). The importance of plasma cleaning prior to functionalization of DNA sensors has been elucidated in previous studies (Wang et al. 2017b) as a major factor in achieving uniform covalent binding of DNA probes. We also found this to be a major contributor for effective probe immobilization (Supplementary Fig. S3).

The treated sensors were functionalized according to our established protocol (Abeyrathne et al. 2016a; Abeyrathne et al. 2016b; Soraya et al. 2017). Sensors were dipped into 2% APTES (Sigma Aldrich, St Louis, Missouri) solution for 1.5 hours followed by 5-minute wash in 100% ethanol for 3 times. Sensors were then immediately placed in a 2.5% glutaraldehyde solution (Sigma Aldrich, St Louis, Missouri) for 3 hours and washed for 5 minutes 3 times in Milli-Q® water and dried under N_2 gas. APTES and glutaraldehyde solutions were filtered using Minisart® 0.2 µm pore size filters (Sigma Aldrich, St Louis, Missouri) to remove impurity. Following functionalization, 1.5 mm diameter wells fabricated from acrylic sheets (Plastic Center Australia, Cheltenham, Victoria) were adhered over the

sensors using medical grade pressure sensitive adhesive (PSA) tape (ARcare 90445; Adhesive Research, Glen Rock, Pennsylvania). The wells serve as incubation reservoirs.

The amine-terminated oligonucleotide probe (Life Technologies Australia, Mulgrave, Victoria) was suspended in 4 µL nuclease-free H_2O at a concentration of 10 µM (Lai et al. 2012; Wang et al. 2017b) and placed within each well in a humid chamber at 4°C for 15-16 hours. Following incubation, probe solution was removed and sensors were blocked with 1% ethanolamine (Sigma Aldrich, St Louis, Missouri) for 30 minutes to quench residual amine groups on the sensor surface. Blocking solution was washed 3 times in Milli-Q® water and dried. Sensors were immediately used for detection of LAMP amplicons.

2.7 Hybridization of LAMP amplicons on IDE sensors

In phase I, purified LAMP amplicons and positive control oligonucleotides were suspended in 1× SSC (150 mM sodium chloride, 15 mmol/L sodium citrate) hybridization buffer at 40 ng/µL. In phase II, hybridization was performed using untreated LAMP amplicons from crude blood samples suspended in the original reaction mix. The blood samples were divided into 3 experimental batches.

In both phases, LAMP amplicons were mixed and placed in the sample reservoir (which contained the sensors) at room temperature. The reservoir was sealed with a glass cap to prevent evaporation. After 20 minutes the LAMP amplicons were aspirated and the sensors were subjected to wash using Milli-Q® water.

2.8 Electrical measurements

The electrical measurement setup (Fig. 3B) has been previously described (Abeyrathne et al. 2016a; Abeyrathne et al. 2016b; Soraya et al. 2017). The sensor was connected in series to a 1 kΩ reference resistor (R_{ref}). A function generator provided the input sinusoidal AC excitatory signal (V_{in}) at 20 mV peak-to-peak voltage (V_{pp}). Three different frequencies (1 kHz, 10 kHz, and 100 kHz) were applied during phase I to determine the most optimal frequency. A lock-in amplifier (SR830; Standford Research, Sunnyvale, California) recorded the amplitude of the output voltage (V_{out}) and phase across the reference resistor. The impedance magnitude (Z) of the sensor can then be calculated with the following formula

$$V_{out}/V_{in} = R_{ref}/(Z + R_{ref}), \quad (1)$$

where V_{out} is the output voltage amplitude, V_{in} is the input voltage, R_{ref} is the resistance of the reference resistor, Z is the impedance of the sensor. Moreover, impedance properties can be described by the following formula

$$Z = R/(1 + j\omega RC), \tag{2}$$

in which R is the resistance of the sensor, C is the capacitance of the sensor and ω is the frequency in rad/s.

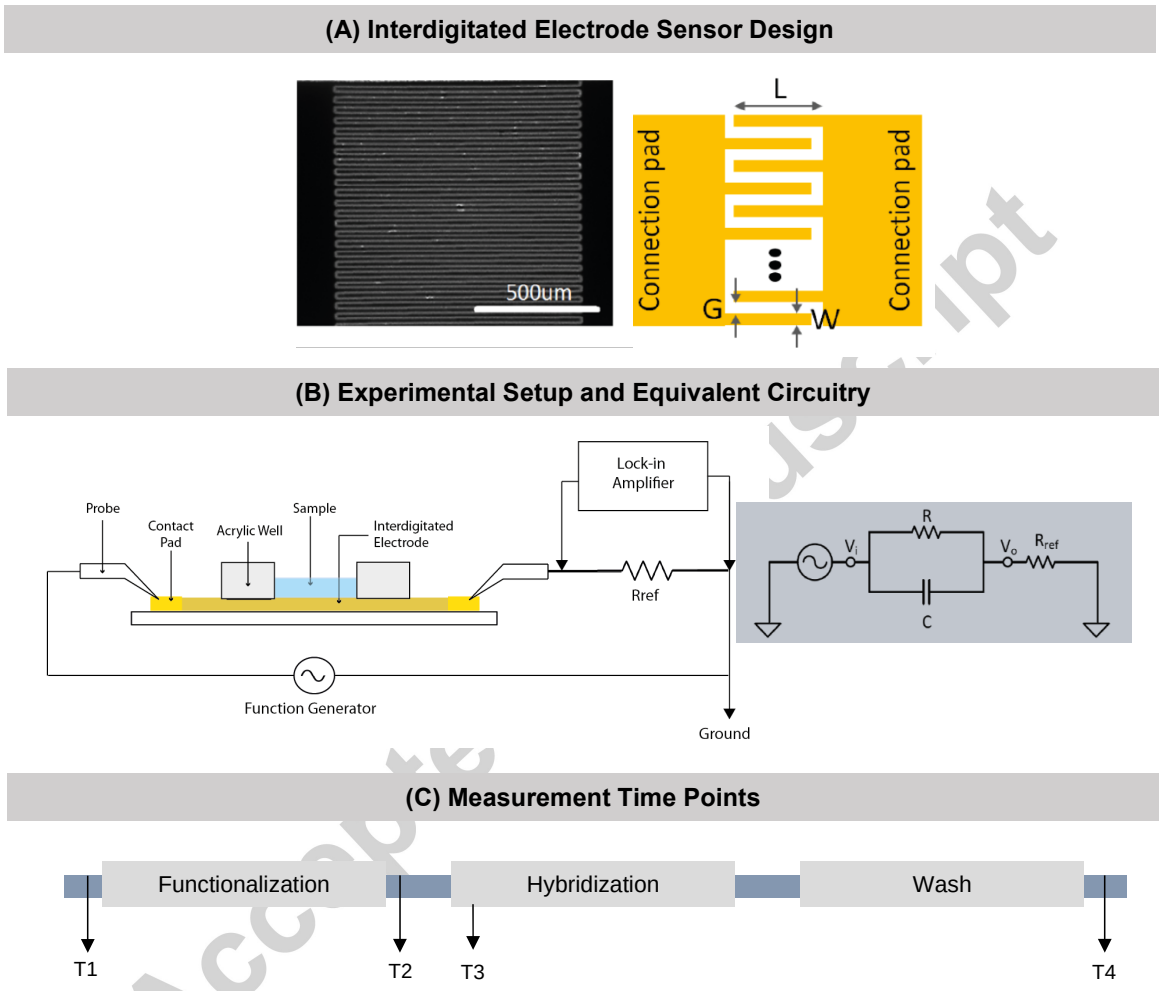


Fig. 3. Design of the IDE sensor and measurement setup. (A) Optical microscope image of IDE sensor with accompanying schematic representation showing gold electrode array with width (W) = 8 µm, length (L) = 1mm, and gap (G) = 8 µm. (B) Cross sectional view of the experimental setup along with equivalent circuitry. The sensors can be represented by a resistor (R) in parallel with a capacitor (C) connected in series with a reference resistor (R_{ref}). (C) Time point of measurements on IDE sensors. T1 = measurements before functionalization, T2 = measurements taken after functionalization and blocking, T3 = measurements taken immediately after placing samples on the sensors, and T4 = measurements taken after 20 minutes of hybridization and wash. Measurements were performed in water (T1, T2, T4) or hybridization buffer/LAMP reaction buffer (T3).

All electrical measurements (Fig. 3C) were performed in wet state. After functionalization and blocking, baseline value (T2) was taken in Milli-Q® water. Sensors that did not meet quality control were discarded (see Supplementary Fig. S4 for details on the quality control process). The T3 measurement reflected the impedance properties of the sample and the buffer, and was utilized in phase I to determine the SSC concentration required to match the impedance properties of a LAMP reaction. After the sample had been incubated on the sensor for 20 minutes, it was washed and the T4 measurement was made in Milli-Q® water.

2.9 Statistical analysis

We used MATLAB® to convert V_{out} and phase data into Z. The magnitude and percentage of change in impedance (ΔZ, $\%\Delta Z$) between points T2 and T4 will be described throughout the results section, unless otherwise stated. GraphPad Prism 7.0 software was used to perform statistical analysis and to generate plots. P-values ≤ 0.05 were considered statistically significant.

2.9.1 Phase I

Optimization of gap size was performed using $\Delta_{T3\text{-}T2}$ values obtained from the sensors. This represents the change in impedance immediately after placing the samples on the functionalized sensors. The optimal gap size was chosen based on the largest impedance magnitude shift and least variation. Optimization of frequency was performed by comparing the effect of three applied frequencies (described in Section 2.8) towards $\%\Delta Z$ following incubation of the complementary oligonucleotides, complementary LAMP amplicons, and non-complementary LAMP amplicons. Normality of $\%\Delta Z$ was determined using Shapiro-Wilk normality test. The frequency providing the largest differentiation between complementary and non-complementary targets was then determined with one-way ANOVA followed by post-hoc Tukey's multiple comparisons test.

Following optimization, the proof-of-concept step involved a panel of various HLA-B LAMP amplicons to confirm the platform performance using the optimized parameters. Selectivity was determined based on the differences in $\%\Delta Z$ between complementary and non-complementary LAMP amplicons. Comparisons were made using one-way ANOVA and post-hoc Tukey's multiple comparisons test. The dynamic range of the LAMP-IDE platform was assessed based on the average yield of DNA amplicons quantified on NanoDrop™ spectrophotometer. Dilution of LAMP amplicons were performed within the range of amplicon concentration, and the $\%\Delta Z$ obtained from the hybridization of these concentrations

were fitted into a standard curve and linear equation.

2.9.2 Phase II

In phase II, amplicons from each LAMP reaction were hybridized onto 3-4 sensors to derive the average %Δ*Z*. Average %Δ*Z* values were compared between the three groups of blood samples with different HLA-B genotypes using one-way ANOVA followed by post-hoc Tukey's multiple comparisons. A receiver-operating characteristic (ROC) curve was generated to determine the optimal cutoff of impedance change. The optimal cutoff point of %Δ*Z* was defined as the closest point towards a false positive rate of 0 (100% specificity) and a sensitivity of 100% (0, 1 on the curve) as calculated using the Youden's index (Youden 1950).

3. Results and Discussion

3.1 Phase I

Optimization of sensor geometry. The IDE sensor geometry plays an important role in signal detection. In this application, where detection was targeted towards high concentrations of amplified DNA with amplicons up to 500 base-pairs in size, sensor gap size of micrometer scale was deemed sufficient. Results of the three tested gap sizes are shown in Fig. 4A. Using the positive control oligonucleotides, $\%\Delta Z$ was not significantly different between the different gap sizes ($p=0.677$, one-way ANOVA). Nonetheless, the largest gap size tested (10 μm) trended to result in the smallest impedance change compared to the smaller gap sizes. This finding is in line with previous work demonstrating increasing sensitivity with decreasing gap size for the detection of various molecular targets (Alexander et al. 2010; Kim 2008; Radke 2004). IDE sensors with the 10 μm gap size also showed greater variation in impedance change, which may be related to the larger variations in the position of the amplicons on the sensor surface. Previous work performed on bacteria (Couniot et al. 2013) has shown that the spatial position of the target on the sensor greatly affects the changes in sensor capacitance and conductance. In this case, the smaller gap sizes reduced the likelihood of varying spatial positions. Based on these findings, the 8 μm gap size was chosen for the subsequent LAMP detection due to the numerically highest magnitude, least variation in impedance shift, and less occurrences of short circuit sensors during fabrication.

Optimization of applied frequency. A range of frequencies (1, 10, and 100 kHz) were applied to the sensors for measurement. At all three frequencies, there was greater change in impedance with the positive complementary targets, particularly the positive oligonucleotide controls, compared to the non-complementary negative samples (Fig. 4B). The difference in $\%\Delta Z$ between the complementary HLA-B*15:02/15:02 LAMP amplicons and the non-complementary HLA-B*15:01/46:01 LAMP amplicon appeared to be greatest at 100 kHz. This was consistent with our previous work that employed IDE sensors for bacteria detection using the same measurement setup in which the largest difference in output voltage between targets was also observed at 100 kHz (Abeyrathne et al. 2016b). This suggests that hybridization-associated impedance changes are also dominated by change of the bulk medium conductivity within the well-known resistive range (Abeyrathne et al. 2016b; Ma et

al. 2013; Yang et al. 2004) for sensor detection between 10-100 kHz. Based on this finding 100 kHz was chosen for the subsequent experiments, although it might be useful to explore even higher frequencies in future experiments.

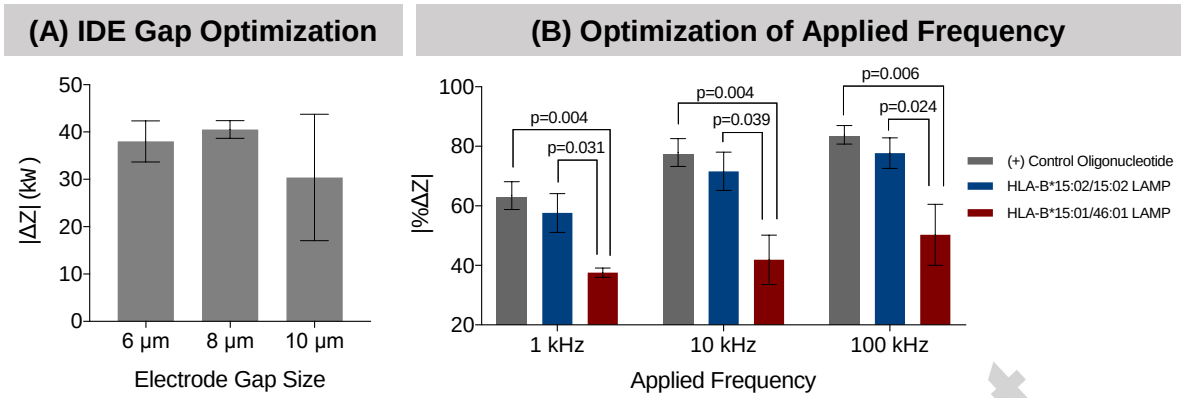
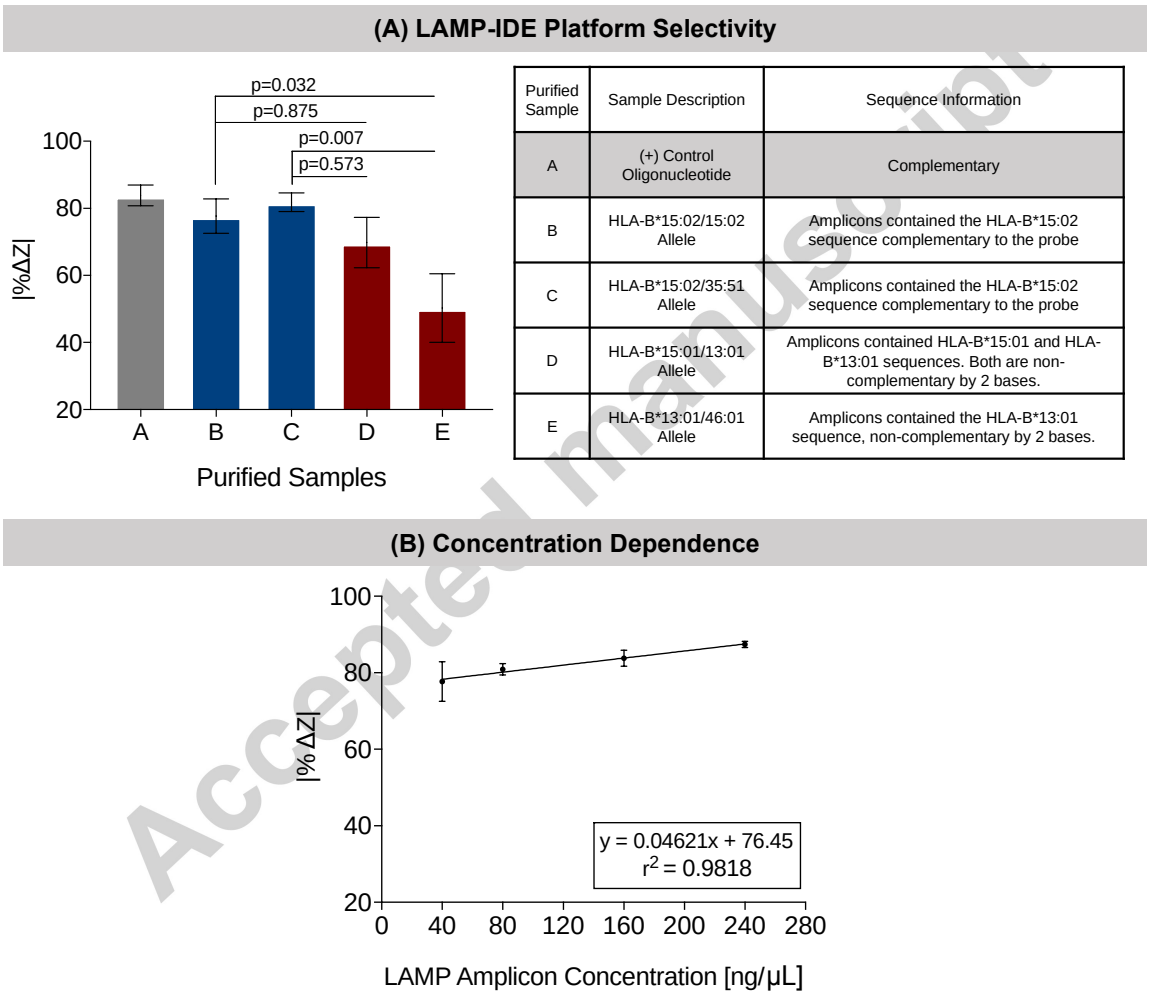


Fig. 4 Optimization of the LAMP-IDE platform. (A) Optimization of IDE sensor gap size using the positive control oligonucleotide suspended in the hybridization buffer. Applied frequency = 100 kHz. Values represented are mean ± standard error of the mean (SEM). n=3 sensors per group. (B) Optimization of applied frequency for differentiation of positive and negative LAMP amplicons. Sequencing of LAMP products confirmed that the positive LAMP sample (blue bar) contained HLA-B*15:02 LAMP amplicons fully complementary to the probe, while the negative LAMP sample (red bar) contained HLA-B*15:01 LAMP amplicons non-complementary to the probe by 2 bases. Sensors used were those with the optimized 8 µm gap. Values are mean ± SEM. n ≥ 5 sensors per group. P-values calculated using the post-hoc Tukey's multiple comparisons test.

Selectivity of LAMP-IDE Platform. Using the optimized gap size and frequency, the LAMP-IDE platform selectivity was evaluated on additional purified HLA-B LAMP amplicons. The results (Fig. 5A) confirmed the pattern of change between complementary and non-complementary LAMP amplicons, in which %Δ*Z* was greater in the heterozygous and homozygous HLA-B*15:02 LAMP amplicons (samples B and C) compared to the negative LAMP amplicons (samples D and E), although the difference with the HLA-B*15:01/13:01 (sample D) sample was not statistically significant. This may suggest cross-reactivity of the probe with the negative LAMP amplicons. Of note both alleles (HLA-B*15:01 and B*13:01) in sample D contained regions of only 2-base mismatch to the probe. This may explain the higher chance of cross-reactivity in sample D relative to sample E.

Relationship between %Δ*Z* and LAMP amplicon concentration. Because the platform aimed to detect amplified DNA, it was important to assess its performance across the typical amplicon concentrations produced by the LAMP reaction. Based on NanoDrop™ nucleic

acid quantification, the average amplicon concentration yield of the LAMP assay was 195.8 ± 64.4 ng/µL (Supplementary Fig. S5). Purified HLA-B*15:02 LAMP amplicons were diluted at four concentrations to cover this range. As seen in Fig. 5B, the results demonstrated concentration dependent, linear increase in %Δ*Z* with the increasing concentrations of complementary LAMP amplicon target. Each twofold increase in LAMP amplicon concentration resulted in an average of 3-4% change in impedance magnitude. No signs of the hook-effect or probe saturation was observed at the higher end of the concentration range, as the %Δ*Z* continued to display an increasing pattern. This concentration dependent response suggests that the LAMP-IDE platform is likely to be useful in real-world settings.



Purified Sample	Sample Description	Sequence Information
A	(+) Control Oligonucleotide	Complementary
B	HLA-B*15:02/15:02 Allele	Amplicons contained the HLA-B*15:02 sequence complementary to the probe
C	HLA-B*15:02/35:51 Allele	Amplicons contained the HLA-B*15:02 sequence complementary to the probe
D	HLA-B*15:01/13:01 Allele	Amplicons contained HLA-B*15:01 and HLA-B*13:01 sequences. Both are non-complementary by 2 bases.
E	HLA-B*13:01/46:01 Allele	Amplicons contained the HLA-B*13:01 sequence, non-complementary by 2 bases.

Fig. 5. LAMP-IDE proof of concept. (A) Selectivity of the LAMP-IDE platform assessed by various HLA-B LAMP amplicons. Values are mean ± SEM of each sample's normalized %Δ*Z*. n ≥ 5 sensors per group. Samples A, B and C were complementary to probe, while samples D and E were non-complementary by 2 bases. P-values calculated with post-hoc Tukey's multiple comparisons test. (B) Linear relationship between %Δ*Z* and complementary LAMP amplicon. Values are mean ± SEM. n ≥ 4 sensors per group.

Since the objective of the IDE detection step was to hybridize amplified DNA, it was not pertinent to optimize the IDE lower limit of detection. That approach would be relevant to the detection of unamplified DNA, where a very low limit of detection is imperative. Several studies on both macroscale and nanoscale IDE sensors have shown impressive limits of detection down to the femtomolar (Lai et al. 2012) to attomolar (Wang et al. 2017b) range through detection of hybridization-induced capacitance changes. These studies have demonstrated the sensitivity of IDE sensors on synthetic single stranded DNA in a non-crude hybridization environment, which are not directly applicable to the detection of gene targets in real-world settings. Sensors aiming to hybridize amplified DNA have also shown that very low detection limit is possible at femtomolar level (Safavieh et al. 2012). However, these studies have used an additional DNA extraction step and a post-amplification labelling step in their approaches, hence adding to the turnaround time and test-cost.

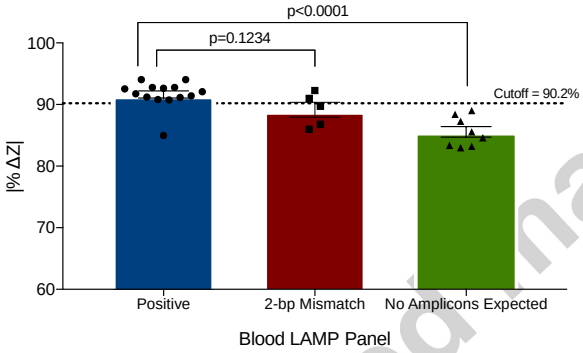
3.2 Phase II

The LAMP-IDE platform developed and optimized in phase I was evaluated in phase II using crude blood samples. When using LAMP alone, smears were observed on gel electrophoresis for all positive HLA-B*15:02 samples but also in 7 of 13 negative samples (Fig. 6A), indicating non-specific amplification. Use of the LAMP reaction alone to target the HLA-B*15:02 allele yielded sensitivity of 100% but specificity of only 46.2%. Upon incorporation of the IDE hybridization step, some false positives were excluded. Group comparison showed that $\%\Delta Z$ was significantly higher in the HLA-B*15:02 samples compared to the samples in which amplicons are not expected ($N_{1\text{-}8}$, $p<0.0001$) (Fig. 6B). In samples $N_{1\text{-}8}$, because the primers did not anneal to the primer target regions, the products visualized on gel electrophoresis typically contained primer repeats, which were non-complementary to the probe by > 2 bases. However, the difference between the HLA-B*15:02 samples and the samples that were non-complementary by 2 bases only ($C_{1\text{-}5}$) did not reach statistical significance ($p=0.1234$). This finding indicates that the selectivity of the platform is influenced by the number of non-complementary bases present in the LAMP amplicons derived from crude blood samples.

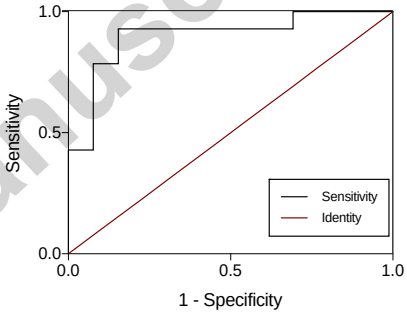
(A) Amplification and Hybridization of HLA-B Alleles

Sample	Blood Sample HLA-B Type		Amplicon Match to Probe	Visualization on Gel Electrophoresis	Mean (SD) of |% ΔZ|
P1	B*15:02	B*44:03	Complementary	(+)	92.07 (1.89)
P2	B*15:02	B*46:01	Complementary	(+)	92.8 (0.3029)
P3	B*15:02	B*15:02	Complementary	(+)	94.06 (0.1864)
P4	B*15:02	B*51:01	Complementary	(+)	91.41 (2.463)
P5	B*15:02	B*38:02	Complementary	(+)	92.64 (0.1801)
P6	B*15:02	B*40:01	Complementary	(+)	91.14 (1.322)
P7	B*15:02	B*35:05	Complementary	(+)	92.55 (0.5787)
P8	B*15:02	B*40:01	Complementary	(+)	91.74 (1.822)
P9	B*15:02	B*35:51	Complementary	(+)	91.23 (2.454)
P10	B*15:02	B*46:01	Complementary	(+)	90.81 (1.866)
P11	B*15:02	B*58:01	Complementary	(+)	94.05 (0.9876)
P12	B*15:02	B*40:01	Complementary	(+)	92.78 (2.201)
P13	B*15:02	B*40:01	Complementary	(+)	90.76 (1.809)
P14	B*15:02	B*38:02	Complementary	(+)	84.99 (6.181)
C1	B*13:01#	B*15:01#	2 bp non-complementary	(+)	89.7 (2.215)
C2	B*13:01#	B*46:01	2 bp non-complementary	(+)	90.99 (3.497)
C3	B*15:01#	B*38:02	2 bp non-complementary	(+)	85.99 (2.194)
C4	B*13:25#	B*07:05	2 bp non-complementary	(+)	86.77 (2.466)
C5	B*13:01#	B*54:01	2 bp non-complementary	(+)	92.27(1.911)
N1	B*35:146	B*51:11	No amplicons expected	(-)	84.61 (3.842)
N2	B*46:01	B*46:01	No amplicons expected	(-)	89.01 (3.429)
N3	B*40:01	B*48:03	No amplicons expected	(-)	85.57 (6.189)
N4	B*27:04	B*58:01	No amplicons expected	(+)	87.28 (5.755)
N5	B*46:01	B*56:01	No amplicons expected	(-)	83.23 (3.623)
N6	B*39:51	B*56:01	No amplicons expected	(-)	88.43 (0)
N7	B*35:51	B*40:01	No amplicons expected	(+)	82.97 (4.298)
N8	B*07:05	B*40:01	No amplicons expected	(-)	83.39 (7.213)

(B) Complementarity and Impedance Change



(C) ROC Curve Analysis



(D) Implementation of Cutoff Value

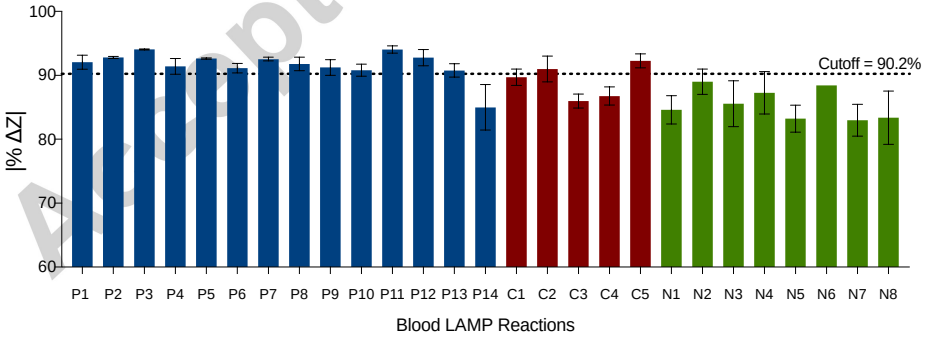


Fig. 6. Phase II validation results. (A) List of blood samples used in phase II validation with corresponding HLA-B genotypes, probe complementarity to amplicons, gel electrophoresis result in which (+) indicates presence of smear and (-) indicates no smear, and the mean ± SD of %Δ*Z* obtained from IDE hybridization. Samples were grouped according to complementarity of amplicons, with HLA-B*15:02 positives $P_{1\text{-}14}$ being fully complementary (highlighted in blue), $C_{1\text{-}5}$ non-complementary by 2 bases (red) in which the primer-amplified alleles are indicated with #, and $N_{1\text{-}8}$ not expected to produce amplicons. Each sample was hybridized on ≥ 3 IDE sensors to obtain the mean %Δ*Z*, except sample N6 that did not meet

quality control standards. (B) Effect of complementarity towards sensor impedance change following hybridization of blood LAMP amplicons. Values are group mean ± SEM. P-values were calculated from post-hoc Tukey's multiple comparison analysis. Dotted line indicates the optimal cutoff. (C) Receiver operating characteristic (ROC) curves of the IDE platform for detection of HLA-B*15:02 blood LAMP amplicons. Calculations were based on mean %Δ*Z* of sensor readings of HLA-B*15:02 blood samples (n=14) and negative HLA-B*15:02 blood samples (n=13). (D) Normalized %Δ*Z* of IDE sensors for individual samples. Values are mean ± SEM. Dotted line indicates the optimal cutoff.

Sensitivity and specificity of the LAMP-IDE platform was assessed using the ROC curve (Fig. 6C) generated based on the mean %Δ*Z* values of the individual samples. Using the optimal %Δ*Z* cutoff of 90.2% (refer to Section 2.9.2), the platform yielded a sensitivity of 92.9% and specificity of 84.6% for the detection of the HLA-B*15:02 allele. Although the sensitivity of the platform decreased slightly when incorporating the IDE hybridization step in comparison to performing LAMP alone, the specificity was substantially improved, demonstrating the advantage of the LAMP-IDE platform for detecting highly polymorphic gene regions such as the HLA-B.

As indicated in Fig. 6D, at this cutoff value, the LAMP-IDE platform resulted in one false negative (P_{14}), and two false positives (C_2 and C_5). In this application, where a positive HLA-B*15:02 result implies increased risk of carbamazepine-induced SJS/TEN, false negative results should be avoided. Closer examination of the %Δ*Z* values showed that the false negative sample had standard deviation of 6.1%, which was considerably higher than other HLA-B*15:02 samples. Future studies should therefore focus on reducing these false negative results via improving consistency of the sensors.

Both false positive samples (C_2 and C_5) contained the HLA-B*13:01 allele, the amplicons of which were non-complementary to the probe by 2 bases. HLA-B*13:01 is frequently found in Asian populations (e.g. 15.4% in Hong-Kong Chinese, 16.8% in Singaporean Chinese, and 45.5 % in Taiwan Bunun populations) (González-Galarza et al. 2014). This imperfect hybridization selectivity should be addressed in future work. One strategy may involve platform integration with recent developments in microfluidics that can potentially allow more specific hybridization in a shorter amount of time through bypassing the diffusion limited processes (Henry and O'Sullivan 2012), and hence avoiding the high background signal resulting from prolonged static incubation. Through applications of various sample mixing and mobilizing strategies such as electrophoretic manipulation (Cheng et al. 2010) or centrifugal force (Li et al. 2009) to increase the mass transfer rate, DNA hybridization can

subsequently be speeded up to several minutes whilst maintaining sensitivity. Other strategies to improve the stringency of hybridization can be through increase of hybridization temperature and modification of the wash protocol.

Our study has limitations. Multiple sensors were used to determine the average change for each sample, increasing the complexity of the test. Purification of crude blood samples might improve performance, although this would need to be balanced against added test time and cost. Although the blood samples used in this study provided proof-of-concept, a larger sample size would be needed for clinical validation. It would also be important to test the platform in more diverse HLA genotypes and across various ethnic populations to assess its generalizability.

The LAMP-IDE platform has a number of potential advantages over the conventional HLA typing methods. It requires a considerably smaller volume of blood, and no DNA extraction step is required. Unlike other reported methods of on-chip LAMP hybridization (Nakamura et al. 2007; Safavieh et al. 2012), our electrical detection format does not require post-amplification labeling or staining of the DNA. The turnaround time of the LAMP-IDE platform was substantially faster over conventional HLA-B*15:02 typing methods. This will not only facilitate timely clinical decision-making but also reduce the cost of HLA screening by avoiding the need for additional clinic visits. Importantly, through customizing the LAMP primers and DNA probe on the sensor surface, the platform can be adapted to detect other genetic variants relevant for disease diagnosis. Finally, the platform is expected to offer a much cheaper alternative to conventional testing, thereby having the potential to increase uptake of conducting such tests in healthcare settings where costs and a lack of specialist equipment are currently prohibitive. Although other DNA amplification approaches have been reported (Jaruthamsophon et al. 2016; Virakul et al. 2012; Wang et al. 2017a), they still require specialized equipment and time-consuming DNA extraction step, which add to test cost and turnaround time when used in the field. In comparison, our proposed approach of combining LAMP and IDE needs minimal sample preparation and can be fabricated at low cost through UV lithography, rendering it amendable for future miniaturization and integration into a point-of-care device.

4. Conclusion

We have developed a LAMP-IDE sensor platform for HLA-B*15:02 genotyping. The findings provided proof-of-concept that the platform can selectively identify the HLA-B*15:02 allele from other HLA-B alleles. The total turnaround time is 1 hour and 20 minutes using crude blood samples (60-minute LAMP reaction and 20-minute hybridization on IDE sensors). The magnitude of impedance change increased linearly with the amplicon concentrations typically produced by LAMP reactions. Using the optimal cutoff value, the platform had a sensitivity of 92.9% and specificity of 84.6%. The amplicon hybridization step enabled more specific gene detection compared to using the LAMP reaction alone.

Based on these characteristics, the LAMP-IDE platform has the potential to be more time-, sample- and cost-efficient compared to conventional methods and is amenable for further development into a POC device. Further, the platform may be adapted for the detection of other pharmacogenetic markers, gene based pathogens and other nucleic acid detecting applications, when prompt genetic information is required for clinical decision.

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Highlights

- Novel approach to HLA-B*15:02 genotyping to prevent severe adverse drug reactions
- Low-cost platform utilizes LAMP combined with amplicon hybridization on IDE sensor
- Turnaround time 1 hour and 20 minutes, faster than current methods (days-weeks)
- Sensitivity and specificity is 92.86% and 84.62% for typing on crude blood samples
- LAMP-IDE platform potentially adaptable for rapid typing of other genetic variants