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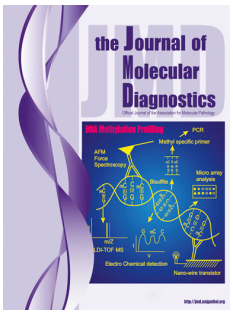

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Rapid, Loop-Mediated Isothermal Amplification Detection of Coeliac Disease Risk Alleles

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Abstract

Human leukocyte antigen (HLA) genotyping has become a useful investigation in the diagnostic work-up of coeliac disease (CD), with utility in risk stratification and screening. However, broad application of this technology has been hindered by the cost and time burden of conventional laboratory-based assays. We have developed and validated CD–loop-mediated isothermal amplification (CD-LAMP), a LAMP assay, which enables rapid identification of the signature CD risk genotypes, HLA-DQ2.5, HLA-DQ8, HLA-DQ2.2, and HLA-DQA1*05. Sample-to-answer is achieved in approximately 65 minutes without DNA purification, thermal cycling, or specialized analytical equipment. CD-LAMP genotyping of samples was 100% concordant with accredited pathology genotyping on a panel of 40 blood and 20 saliva samples. In a panel of 100 purified DNA samples, genotyping of the high risk DQ2.5 genotype was 100% concordant with accredited pathology genotyping, with slightly reduced sensitivity for the DQ8 genotype (97.1%), and reduced specificity for the DQ8 (93.9%) and DQ2.2 genotypes (95.1%). CD-LAMP results are easily visualized, instrument free, through the addition of a DNA intercalating dye following amplification. Combined with point-of-care antibody testing, CD-LAMP may enable immediate, confident CD diagnosis at a low cost in the clinical setting.

Introduction

Coeliac Disease (CD) is a chronic, immune-mediated enteropathy of the small intestine resulting from ingestion of gluten, the alcohol insoluble protein component of wheat and related grains[1]. CD is a common disease, with a prevalence of 0.5% to 2% in Europe[2], North America[3], Australia[4], and the Middle East[5]. Symptoms of CD include diarrhoea, nausea and vomiting, fatigue, abdominal pain, weight loss, and iron-deficiency anaemia[6]. Long term health effects of undiagnosed CD include reproductive problems[7], excess mortality [8], and the development of other autoimmune disorders[9], sepsis[10], and malignancy[11]. Given the broad and non-specific clinical presentation of CD, it is estimated that approximately 80% of patients are undiagnosed [3, 4]. Adhering to a strict, permanent, gluten-free diet (GFD) will result in a reversal of intestinal damage and normalization of symptoms in most CD patients[12].

Although measurement of sensitive CD-specific antibody markers (transglutaminase-IgA and deamidated gliadin peptide-IgG) are used to screen for CD, the current diagnostic gold standard is the demonstration of characteristic small intestinal inflammation and damage (villous atrophy) while on a gluten-containing diet[13]. Intestinal biopsies are obtained by upper gastrointestinal endoscopy, typically under intravenous sedation. Endoscopy is resource intensive, invasive, and inconvenient for patients. Accurate diagnosis is crucially dependent on adequate sampling of the intestine and expert histologic interpretation. CD serology is generally used to stratify which patient may benefit from a confirmatory endoscopy; however, false positive serology can lead to unnecessary testing. Hence, better risk stratification strategies are needed to reduce unnecessary endoscopies and improve the cost-effectiveness of CD investigation[4].

One such strategy involves human leukocyte antigen (HLA) genotyping based on the exceptionally strong association of CD with specific HLA genotypes[14]. Three major susceptibility alleles, HLA-DQA1*05, HLA-DQB1*02, and HLA-DQB1*03:02[14] are associated with CD, with greater than 99% of individuals with CD expressing one or a combination of these risk alleles as part of the HLA-DQ2.5

(DQA1*05, DQB1*02), DQ2.2 (DQA1*02, DQB1*02), DQA1*05 (without DQB1*02, also known as DQ7), or DQ8 (DQB1*03:02) genotypes[15] (**Table 1).** Although HLA genotyping has limited positive predictive value for CD due to the high population frequency of at-risk susceptibility genotypes (30% to 50%)[16, 17], it is clinically useful given its exceptionally strong negative predictive value, allowing a negative result to exclude a diagnosis of CD[14]. This makes HLA genotyping particularly useful to exclude patients who have self-treated with a GFD and are unable or unwilling to undertake an oral gluten challenge, when investigations for CD are equivocal, in patients with presumed CD not responding to a GFD, or when stratifying high-risk individuals, such as family members of individuals with CD, to guide their work-up[13, 14, 18]. Furthermore, combining HLA testing with CD-specific serology provides strong predictive value for CD, and enables false positive serological results to be excluded, providing a cost-effective and potentially useful "one-step" diagnostic approach[4]. Indeed, owing to its utility in the work-up of CD and community health concerns over gluten intake, HLA genotyping has become one of the most commonly performed genetic tests in Australia[14, 19].

Despite its value, the widespread application of HLA genotyping for CD diagnosis is hampered by technical and economic barriers. This is because diagnostic HLA-DQ genotyping is conventionally performed in specialized laboratories using high resolution PCR–based sequence-specific priming (SSP), sequenced base typing (SBT), or micro bead hybridization approaches[20]. Although highly accurate, these technologies are time consuming and expensive, limiting utility in low resource environments or for high-throughput applications such as population screening or research[17]. To address these limitations, more efficient and cost-effective alternatives have been proposed utilizing HLA-tagging single nucleotide polymorphisms[17], real-time PCR[21], and multiplex ligation-dependent probe amplification[22]. A common feature of these approaches is a reliance on PCR-based technologies in combination with sensitive fluorescence-based detection of amplified products. This

restricts their application to laboratory settings where costly DNA purification, thermal-cycling, and fluorescence detection equipment are available.

As an alternative technology, we have previously demonstrated the use of loop-mediated isothermal amplification (LAMP) to overcome the barriers in detecting specific HLA alleles associated with drug hypersensitivity[23, 24]. LAMP is a rapid, isothermal nucleic acid amplification technique utilizing a strand displacement *Bst* DNA polymerase and four to six sequence-specific primers[22, 25]. LAMP reactions typically require under 60 minutes of incubation and function reliably across a broad temperature range (2 to 4 °C), eliminating the need for high accuracy thermal-cycling equipment[26]. Furthermore LAMP assays are more tolerant than PCR-based technologies to inhibitors in clinical samples and may be performed directly on minimally processed blood[23] or saliva[27]. Following incubation, amplification status can be rapidly determined, instrument free, by employing a fluorescent metal indicator[28], dsDNA intercalating dye[29], pH-sensitive dye[30], or lateral flow dipstick[31]. LAMP assays have been developed for hundreds of applications[32], primarily the detection of pathogenic microorganisms[33, 34], but have also been designed for screening of pharmacogenetic risk alleles[23, 35]

Here, we have developed and validated CD-LAMP, an assay capable of identifying and stratifying patient risk for CD based on the presence of the risk alleles HLA-DQB1*02, HLA-DQB1*03:02, and HLA-DQA1*05. Sample-to-answer is achieved within 65 minutes. This novel assay has immediate application for rapid sample analysis in research settings, such as for the pre-screening of samples prior to high resolution genotyping or high throughput screening of large sample cohorts. CD-LAMP functions on purified DNA and minimally processed blood and saliva samples making it an excellent candidate as the foundation technology of a point-of-care (POC) platform for low cost patient diagnostics and population screening purposes.

Materials and Methods

CD-LAMP overall approach

Effective HLA genotyping for CD risk genotypes can be achieved through the identification of the HLA-DQA1*05 allele group, the HLA-DQB1*02 allele group, the HLA-DQA1*03 allele group, and HLA-DQB1*03:02 alleles. Due to the strong linkage disequilibrium between HLA-DQB1*03:02 alleles and HLA-DQA1*03 alleles (D'=1.00), detection of HLA-DQB1*03:02 is sufficient for attribution of the HLA-DQ8 risk type[36]. Similarly, the detection of an HLA-DQB1*02 allele in the absence of an HLA-DQA1*05 allele is sufficient for attribution of the HLA-DQ2.2 risk type due to strong linkage disequilibrium between the HLA-DQB1*02:02 and HLA-DQA1*02:01 alleles (D' = 0.99)[36]. A strategy was designed comprised of three LAMP reactions targeting HLA-DQA1*05, HLA-DQB1*02, and HLA-DQB1*03:02 alleles. The pattern of positive or negative results is informative of risk genotype and relative risk (**Table 2)** and a negative result to all tests can effectively exclude a CD diagnosis (NPV = 99.6%)[15]. An overview of the CD-LAMP process used in this study is presented in **Figure 1A.**

Primer design

Risk allele–specific target regions of the HLA-DQA1 and HLA-DQB1 loci were identified using the IPD-IMGT/HLA database[37] with focus on variation in alleles listed in the Common and Well-Documented (CWD) alleles catalog[38]. Alignments were constructed using the IMGT/HLA sequence alignment tool **(Supplemental Figures S1, S2, and S3)**. We also confirmed that the target region was unique to the target gene through sequence alignment with known paralogs HLA-DQA2 and HLA-DQB2 **(Supplemental Figure S4)**. Primers were designed targeting these regions with the Primer Explorer V4 software (Eiken Chemical Co., Ltd., Japan; http://primerexplorer.jp/e). LAMP primer sets for these reactions are shown in **Table 3.**

Samples and HLA genotype data

Purified DNA samples for initial validation were obtained from the International Histocompatibility Working Group (IHWG) cell and DNA bank. DNA samples used for validation experiments were provided by the Australian Red Cross Blood Service following purification from patient blood samples. Blood samples used for initial validation experiments were also provided by the Australian Red Cross Blood Service. Blood and saliva samples used for validation experiments were collected from participants in the Gluten Immunity in Coeliac Disease study at the Royal Melbourne Hospital/Walter and Eliza Hall Institute. The use of patient samples in this study is approved by the Health Sciences Human Ethics Sub-Committee of the University of Melbourne (project ID: 1443204). The HLA genotypes in all samples used for validation of the CD-LAMP assay were determined by NATA accredited pathology laboratories using high resolution PCR-reverse SSO genotyping (LABType™ or Gen-Probe™) and blinded prior to CD-LAMP genotyping.

Processing of samples

Concentration of purified DNA samples was quantified using a NanoDrop 2000c Spectrophotometer (Thermo Scientific) and normalised to 10ng/µL in ultrapure water. Minimally processed blood samples were prepared by mixing 4uL of whole blood collected in an EDTA coated tube with a lysis buffer solution (25mM NaOH, 200µM EDTA) in a 1:49 ratio and incubating at 98 °C for 5 minutes. Minimally processed saliva samples were prepared by mixing 4uL of saliva collected with an OG-500 collection kit (DNA genotek) with ultrapure water in a 1:49 ratio and incubating at 98 °C for 5 minutes.

CD-LAMP reaction conditions

The reaction mix for each of the three CD-LAMP reactions is detailed in **Table 4.** For each reaction 11µL of reaction mix was added to 4µL of DNA or processed blood or saliva to make up a total reaction volume of 15 µL. This reaction mix was incubated for 60 minutes at 63 °C in a thermal-cycler, then incubated at 85 °C for 5 minutes to terminate the reaction for storage of samples or

immediately visualized by electrophoresis on a 2% agarose gel and via fluorescence as described below.

CD-LAMP product visualization

Five microliter of 100X GelGreen Nucleic Acid Stain (Biotium) was added to 5uL of reaction product and fluorescence was measured with a Spectramax M3 microplate reader, visualized using a UV transilluminator, and visualized by eye by illuminating samples with a blue light emitting diode (LED). GelGreen was chosen over stronger intercalation dyes due to its long term thermostability and low cost[39].

Statistical analysis

Statistical analyses of sensitivity and specificity values were performed using MedCalc for Windows, version 15.0 (MedCalc Software, Ostend, Belgium).

Results

Optimization of CD-LAMP

Performance of CD-LAMP reactions for their respective target alleles was first optimized and assessed on a panel of human genomic DNA samples from typed B-cell lines encompassing the relevant sequence region **(Supplemental Figure S5, Supplemental Table S1)**. After optimization HLA-DQA1*05 and HLA-DQB1*02 reactions amplified in the presence of all available alleles from their respective target allele groups. No amplification was observed following incubation with alleles from any other HLA-DQ allele group. Following optimization, the HLA-DQB1*03:02 reaction only amplified in the presence of the HLA-DQB1*03:02 allele and did not amplify in the presence of other HLA-DQB1*03 group alleles or alleles from other HLA-DQB1 allele groups. All reactions functioned reliably with incubation for 60 minute at 63°C.

LAMP protocols were then further optimized to accommodate sample processing on a panel of blood samples **(Supplemental Figure S6, Supplemental Table S2)**. Again, after optimization all reactions only amplified in the presence of their respective targets.

Validation of the CD-LAMP assay on previously typed patient samples

The optimized CD-LAMP protocols were validated using 100 DNA samples (purified from blood samples), 40 blood samples, and 20 saliva samples that had been previously genotyped for clinical purposes by an accredited genetic testing laboratory. Reaction status for each of the three reactions was analyzed by gel electrophoresis. Representative genotyping results for 10 blood samples are shown in **Figure 1B.**

Using purified DNA samples, CD-LAMP identified 100% of HLA-DQA1*05 (37/37), 100% of HLA-DQB1*02 (41/41), and 97% of HLA-DQB1*03:02 (33/34) positive samples. No false positives were observed for the HLA-DQA1*05 reaction (0/63) and four false positives were observed for both the HLA-DQB1*02 (4/59) and HLA-DQB1*03:02 reactions (4/66). All HLA-DQB1*03:02 false positive samples were HLA-DQB1*03:03 positives, suggesting cross reactivity with this allele in this sample type **(Table 5, Supplemental Table S2)**.

Using minimally processed blood samples CD-LAMP correctly identified 100% of HLA-DQA1*05 (30/30), HLA-DQB1*02 (24/24), and HLA-DQB1*03:02 (6/6) positive samples. Using minimally processed saliva samples CD-LAMP correctly identified 100% of HLA-DQA1*05 (15/15), HLA-DQB1*02 (14/14), and HLA-DQB1*03:02 (5/5) positive samples. In both sample types, no false positives were observed and results were 100% concordant with PCR-SSO genotyping. **(Table 5, and Supplemental Table S3 and S4)**.

Across all sample types as stratified by risk genotype, CD-LAMP identified 100% (63/63) of DQ2.5, 97.7% (44/45) of DQ8, 100% (25/25) of DQ2.2, and 100% (22/22) of DQA1*05 genotypes.

Rapid detection of CD-LAMP status with GelGreen DNA stain

Addition of GelGreen stain to the CD-LAMP product allowed for rapid distinction of positive and negative reaction status without gel electrophoresis. Across all reactions and sample types fluorescence readings in positive samples were at least 15 times higher than negative samples, allowing a threshold value of 1000 AFU for positive reaction status to be established **(Figure 2A)**. Utilizing this fluorescence shift, risk genotype status could readily be determined using a fluorimeter, UV transilluminator, or by eye under blue LED illumination **(Figure 2B)**.

Discussion

Based on the strong association of CD with specific HLA alleles, the clinical role for HLA genotyping to assist in the diagnosis and management of CD is now firmly established[14]. Indeed, practice guidelines are beginning to incorporate HLA testing to streamline the diagnostic work-up for CD[18]. In addition to excluding CD in clinical scenarios when the diagnosis is in doubt, HLA genotyping can stratify CD risk and determine if further investigations for CD are required. This may be particularly useful in screening high-risk individuals such as first-degree family members of CD patients. A negative HLA genotyping result means that endoscopy can generally be avoided in the investigation of CD. This may be a particularly cost-effective strategy when combined with CD serology to improve testing accuracy and prevent unnecessary endoscopies[4].

Despite this broad clinical utility, application of HLA genotyping has been hindered by the expense and long turn-around-time of conventional genotyping technologies[40]. Although more efficient alternatives have been developed, these remain reliant on DNA purification, expensive laboratory equipment including thermal-cyclers and sensitive fluorimeters, and have not been widely adopted. The clinical benefits of HLA genotyping would be substantially enhanced if testing could return a positive result sooner and the test was more affordable.

To address the need for more cost and time effective HLA genotyping for CD, CD-LAMP, a rapid assay capable of identifying the HLA-DQ2.5, HLA-DQ8, HLA-DQ2.2, and HLA-DQA1*05 risk genotypes, not

only using purified DNA samples but directly on minimally processed human blood and saliva samples, was developed and validated. Sample-to-answer using blood or saliva samples may be achieved within 65 minutes. In contrast to existing alternatives, CD-LAMP is performed without thermal-cycling and the result may be easily visualized by eye using a DNA intercalating dye and a blue LED. Due to these minimal processing and equipment requirements of this assay, CD-LAMP may be a more accessible alternative to conventional testing approaches.

CD-LAMP genotyping had 100% concordance with gold-standard PCR-SSO genotyping on all tested blood and saliva samples. However, reduced sensitivity for the DQ8 genotype (97.1%), and reduced specificity for the DQ8 (93.9%) and DQ2.2 genotypes (95.1%) was observed in the large purified DNA panel. This may be due to the optimization of the CD-LAMP assay on minimally processed blood samples, and further optimization for function on DNA may improve sensitivity and specificity with this sample type. All HLA-DQB1*03:02 false positive DNA samples were HLA-DQB1*03:03 positive, suggesting cross reactivity of the CD-LAMP assay with this allele. Although this cross-reactivity was not observed in the few blood or saliva samples with this allele, genotyping of additional DQB1*03:03 blood and saliva samples, as well as those with rare HLA-DQA1*05 and HLA-DQB1*02 variants and a broader set of non-European non-susceptibility genotypes, is warranted to confirm perfect concordance. Using the reduced sensitivity and specificity observed in the purified DNA panel, CD-LAMP would be expected to identify over 99% of CD patients in European and Australian Caucasian populations **(Supplemental Table S5)**, while eliminating approximately 90% of the individuals negative for high risk CD susceptibility genotypes.

A potential limitation of the CD-LAMP assay is the inability to distinguish highest risk DQ2.5/DQ2.5 and DQ2.5/DQ2.2 genotypes from high risk DQ2.5/DQX or DQ2.2/DQA1*05 genotypes. Higher risk genotypes result from homozygosity at the DQB1*02 allele and are associated with a higher risk of CD and possibly a more severe clinical phenotype, an effect attributed to more effective antigen presentation of gluten (gene dose effect). Currently, knowledge of gene-dose does not impact

patient care. Although this inability to account for gene dose reduces the predictive capacity of the CD-LAMP assay for identifying individuals at highest risk, it does not compromise the negative predictive power of testing. Where homozygosity information is deemed necessary CD-LAMP may act as a method of pre-screening of samples prior to DNA purification and higher resolution genotyping, reducing the overall cost of testing.

POC testing provides a simple, convenient, and attractive approach to patient care by avoiding the wait times for laboratory-based testing, and minimizing the discomfort associated with traditional venesection. Clinical management decisions can be made on the same day, improving clinical care and maximizing the use of health care resources. Several POC antibody testing devices for CD screening have been developed, utilizing lateral flow immunochromatography to provide a colorimetric readout in the presence of circulating CD-specific IgA and IgG antibodies[41-43]. POC antibody testing has been shown to reduce the time to biopsy when compared with laboratory-based antibody testing[42], an important factor as delay in diagnosis has been associated with poorer health–related quality of life[44]. Although POC antibody tests are considered an attractive technology by practitioners, they have received some criticism due to subjective results and inferior accuracy when compared with laboratory-based testing (http://www.coeliac.org.nz/_literature_149201/PoCT_CAus_Medical_Advisory_Committee_Position_Statement_2014) [45]. Furthermore, these test are reliant on gluten consumption which patients on a GFD are often unwilling or unable to resume.[14]

To date, no POC genetic testing devices have been developed to assist with CD diagnosis. LAMP assays have been highlighted as an excellent candidate for POC genetic testing devices[46], and devices capable of performing LAMP reactions POC are under development[27, 47]. In the future, CD-LAMP may be adaptable for use in immediate POC HLA genotyping utilizing such a device. Combined with POC antibody testing, CD-LAMP paves the way for immediate, confident CD diagnosis or exclusion at a low cost in the clinical setting.

In conclusion, we have developed and validated CD-LAMP as a new approach to HLA genotyping for CD research and diagnostics. CD-LAMP is rapid and can be performed without the use of specialized laboratory equipment on minimally processed blood and saliva samples. CD-LAMP has immediate utility in low-resource or high-throughput settings where existing genotyping technologies are laborious and prohibitively expensive. In the future, CD-LAMP may be combined with a serological approach to enable a highly accurate, time and cost efficient approach to CD evaluation in the clinic.

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Figure legends

Figure 1: The CD-LAMP Assay. A: Overview of the CD-LAMP Process. Saliva or blood samples are minimally processed through dilution in a lysis buffer or water and heat-treated prior to incubation with the CD-LAMP reaction mixtures. Purified DNA is directly added prior to incubation. Results are then determined by gel electrophoresis or direct addition of a nucleic acid stain. **B:** Representative genotyping of 10 blood samples using CD-LAMP. Results are visualized by gel electrophoresis (image inverted). A positive reaction is visible as a large DNA smear. All 10 genotyping results match accredited pathology results.

Figure 2: Rapid interpretation of CD-LAMP results using GelGreen DNA intercalating dye.
A: Following addition of GelGreen, sample fluorescence of positive CD-LAMP reactions are a minimum of 15X higher than negative reactions for all sample types. Consistency of negative LAMP fluorescence allows for a negative threshold value of 1000 AFU (**dotted line**) to set (n = 3 for all categories). **B:** Following addition of GelGreen, risk allele status and genotype attributed risk can easily be determined by eye under blue LED illumination or using UV trans-illumination (image inverted).

Table 1: CD risk genotypes at the HLA-DQA1 and HLA-DQB1 loci.

Genotype	HLA-DQA1	HLA-DQB1	CD Carrier rate[15]	Population Carrier rate[17]	CD Risk[14]
DQ2.5	05	02	88.0%	17.6-36.1%	High
DQ8	03	03:02	5.9%	8.9-17.3%	Moderate/Low
DQ2.2	02:01	02:02	4.1%		Low
DQA1*05	05	03	1.6%	55.0-68.0%	Very Low
Other	XX	XX	0.4%		Lowest

Table 2: Result Matrix for the CD-LAMP assay.

HLA-DQA1*05	HLA-DQB1*02	HLA-DQB1*03:02	Result	Relative Risk[14]
+	+	+	DQ2.5/DQ8	High
+	+	-	DQ2.5	
-	+	+	DQ8/DQ2.2	
+	-	+	DQ8/DQA1*05	Moderate/Low
-	-	+	DQ8	
-	+	-	DQ2.2	Low
+	-	-	DQA1*05	Very Low
-	-	-	Non-Risk	Lowest

Table 3: CD-LAMP primers.

HLA-DQA1*05 Reaction	
A1*05-F3	5’-AGAAAGAAAAGAGGGAAGGAA-3’
A1*05-B3	5’-ACAAACCCCACTGTCCAT-3’
A1*05-FIP	5’-AAGACAGAGAAGACTAAGAGAGACCTTTTAAAAGGAAGGAAGGAAGCAC-3’
A1*05-BIP	5’-TGTCATCCATCTATTTCCACCTCTTTTTAGAAGCAGGGAGTCAGAG-3’
A1*05-LF	5’-AAATGATTAATAATTAATCT-3’
A1*05-LB	5’-TCCTTTCTCCCTCTTCCCTTT-3’
HLA-DQB1*02 Reaction	
B1*02-F3	5’-TGGGCCGCACTGACTG-3’
B1*02-B3	5’-AGTACTCGGCGGCAGG-3’
B1*02-FIP	5’-GTCCCGTTGGTGAAGTAGCACATTTTGTGATTCCTCGCAGAGGATT-3’
B1*02-BIP	5’-GTGCGTCTTGTGAGCAGAAGCATTTTTCACCGCCCGGAACTC-3’
B1*02-LF	5’-TGCCCTTAAACTGGTACACGA-3’
B1*02-LB	5’-CGCTTCGACAGCGACGT-3’
HLA-DQB1*03:02 Reaction	
B1*03:02-F3	5’-GCATGTGCTACTTCACCAA-3’
B1*03:02-B3	5’-GTGCGGAGCTCCAACTG-3’
B1*03:02-FIP	5’-ATACACCCCCACGTCGCTGTCTTTTGGAGCGCGTGCGTCTT-3’
B1*03:02-BIP	5’-CCGCCGAGTACTGGAACAGCCATTTTTGTCTGCACACCGTGTCC-3’
B1*03:02-LF	5’-TCTCGGTTATAGATGTATCTGGTCA-3’
B1*03:02-LB	5’-AAGTCCTGGAGAGGACCCGG-3’

Table 4: Composition of the reaction mixtures for the three CD-LAMP reactions.

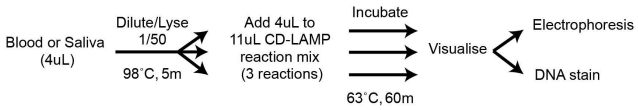
Reagent	Supplier	HLA-DQA1*05 Concentration	HLA-DQB1*02 Concentration	HLA-DQB1*03:02 Concentration
Betaine	Sigma	1.11M	1.02M	1.39M
dNTP mix	NEB	1.1mM	1.02mM	0.93mM
Isothermal Amplification Buffer	NEB	1.55X	1.43X	1.30X
Bst 2.0 Warmstart DNA polymerase	NEB	0.71U	0.98U	0.59U
$MgS0_4$	NEB	4.45mM	4.09mM	3.73mM
FIP primer	IDT	19.6µM	18µM	32.6µM
BIP primer	IDT	19.6µM	18µM	32.6µM
F3 primer	IDT	4.9µM	4.5µM	8.15µM
B3 primer	IDT	4.9µM	4.5µM	8.15µM
LF primer	IDT	4.9µM	4.5µM	8.15µM
LB primer	IDT	4.9µM	4.5µM	8.15µM

Table 5: Sensitivity and specificity of CD-LAMP genotyping for CD risk alleles and genotypes in DNA, blood, and saliva samples when compared with PCR-SSO genotyping results.

HLA-DQA1*05 LAMP			**95% Confidence Interval**
Sensitivity	DNA	100% (37/37)	90.51% to 100.00%
	Blood	100% (30/30)	88.43% to 100.00%
	Saliva	100% (15/15)	78.20% to 100.00%
	Combined	100% (82/82)	95.60% to 100.00%
Specificity	DNA	100% (63/63)	94.31% to 100.00%
	Blood	100% (10/10)	69.15% to 100.00%
	Saliva	100% (5/5)	47.82% to 100.00%
	Combined	100%(78/78)	95.38% to 100.00%
HLA-DQB1*02 LAMP			
Sensitivity	DNA	100% (41/41)	91.40% to 100.00%
	Blood	100% (24/24)	85.75% to 100.00%
	Saliva	100% (14/14)	76.84% to 100.00%
	Combined	100%(79/79)	95.44% to 100.00%
Specificity	DNA	93.2% (55/59)	83.54% to 98.12%
	Blood	100% (16/16)	79.41% to 100.00%
	Saliva	100% (6/6)	54.07% to 100.00%
	Combined	95.1% (77/81)	87.84% to 98.64%
HLA-DQB1*03:02 LAMP			
Sensitivity	DNA	97.1% (33/34)	84.67% to 99.93%
	Blood	100% (6/6)	54.07% to 100.00%
	Saliva	100% (5/5)	47.82% to 100.00%
	Combined	97.7%(44/45)	88.23% to 99.94%
Specificity	DNA	93.9% (62/66)	85.20% to 98.32%
	Blood	100% (34/34)	89.72% to 100.00%
	Saliva	100% (15/15)	78.20% to 100.00%
	Combined	96.5% (111/115)	91.33% to 99.04%
Risk Genotype Sensitivity			
DQ2.5	DNA	100% (25/25)	86.28% to 100.00%
	Blood	100% (25/25)	86.28% to 100.00%
	Saliva	100% (13/13)	75.29% to 100.00%
	Combined	100% (63/63)	94.31% to 100.00%
DQ8	DNA	97.1% (33/34)	84.67% to 99.93%
	Blood	100% (6/6)	54.07% to 100.00%
	Saliva	100% (5/5)	47.82% to 100.00%
	Combined	97.7% (44/45)	88.23% to 99.94%
DQ2.2	DNA	100% (20/20)	83.16% to 100.00%
	Blood	100% (4/4)	39.76% to 100.00%
	Saliva	100% (1/1)	N/A
	Combined	100% (25/25)	86.28% to 100.00%
DQA1*05	DNA	100% (12/12)	73.54% to 100.00%
	Blood	100% (8/8)	63.06% to 100.00%
	Saliva	100% (2/2)	N/A
	Combined	100% (22/22)	84.56% to 100.00%

95% confidence intervals are "exact" Clopper-Pearson confidence intervals.

A



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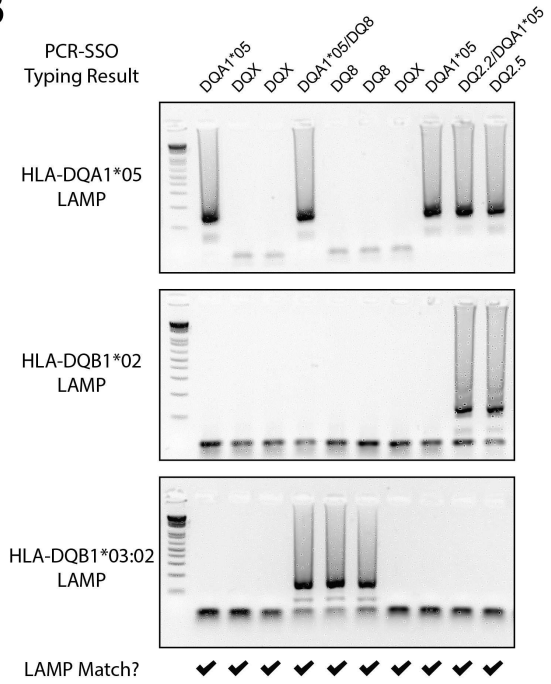

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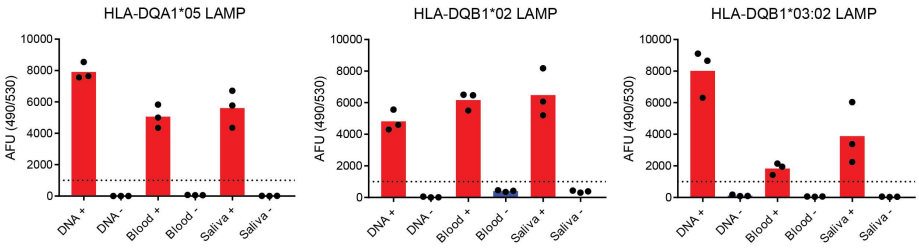

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