Received: 8 September 2023 | Revised: 11 December 2023 | Accepted: 29 December 2023

DOI: 10.1111/tan.15345

ORIGINAL ARTICLE

HLA Immune Response Genetics WILEY

Antibody verification of HLA class I and class II eplets by human monoclonal HLA antibodies

Cynthia S. M. Kramer[1] | Suzanne Bezstarosti[1,2] | Marry E. I. Franke-van Dijk[1] | Manon Vergunst[1] | Dave L. Roelen[1] | Merve Uyar-Mercankaya[1] | Kim H. Voogt-Bakker[1] | Sebastiaan Heidt[1]

[1]Department of Immunology, Leiden University Medical Center, Leiden, The Netherlands

[2]Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands

Correspondence
Cynthia S. M. Kramer, Department of Immunology, Leiden University Medical Center, Leiden, The Netherlands.
Email: c.s.m.kramer@lumc.nl

Funding information
National Reference Center for Histocompatibility Research

In solid organ transplantation, formation of de novo donor-specific HLA antibodies is induced by mismatched eplets on donor HLA molecules. While several studies have shown a strong correlation between the number of eplet mismatches and inferior outcomes, not every eplet mismatch is immunogenic. Eplets are theoretically defined entities, necessitating formal proof that they can be recognised and bound by antibodies. This antibody verification is pivotal to ensure that clinically relevant eplets are considered in studies on molecular matching. Recombinant human HLA-specific monoclonal antibodies (mAbs) were generated from HLA-reactive B cell clones isolated from HLA immunised individuals using recombinant HLA molecules. Subsequently, the reactivity patterns of the mAbs obtained from single antigen bead assay were analysed using HLA-EMMA software to identify single or configurations of solvent accessible amino acids uniquely present on the reactive HLA alleles and were mapped to eplets. Two HLA class I and seven HLA class II-specific human mAbs were generated from four individuals. Extensive mAb reactivity analysis, led to antibody verification of three HLA-DR-specific eplets, and conversion of five eplets (one HLA-A, one HLA-B, two HLA-DR, and one HLA-DP), from provisionally verified to truly antibody-verified. Finally, one HLA-DQ-specific eplet was upgraded from level A2 to level A1 verification evidence. The generation of recombinant human HLA-specific mAbs with different specificities contributes significantly to the antibody verification of eplets and therefore is instrumental for implementation of eplet matching in the clinical setting.

KEYWORDS
antibody verification, eplet, HLA, human monoclonal antibody

1 | INTRODUCTION

In solid organ transplantation, matching for HLA results in superior graft outcome.[1] However, most recipients will receive a graft with at least one or more HLA mismatches due to the high polymorphism of HLA and scarcity of donor organs. As a consequence, those recipients are at risk of developing de novo donor-specific antibodies

(dnDSA), which is a major cause of rejection and eventually graft loss.[2] HLA-specific antibodies are induced by recognition of either a single or a configuration of polymorphic solvent accessible (SA) amino acids (AA) present on non-self HLA molecules. Eplets are theoretically defined configurations of such SA or surface-exposed polymorphic AA.[3] In recent years, studies on molecular mismatching in solid organ transplantation have shown that the development of dnDSA is indeed associated with the number of eplet mismatches between donor and recipient.[4–6]

Nonetheless, for the implementation of eplets in clinical histocompatibility assessment, it is essential to verify whether alloantibodies can actually bind to eplets, as it has been suggested that not every eplet is equally immunogenic.[7,8] The complete eplet repertoire along with their antibody verification status is listed on the HLA Eplet Registry (formerly HLA Epitope registry).[9–11] A recent evaluation by Bezstarosti et al.[12] indicated that the current antibody verification status of eplets is based on several different methods and that for numerous eplets not sufficient evidence has been provided to conclusively confirm the antibody-verification status. To this end, a classification system was proposed to indicate the level of evidence for antibody verification, with human HLA-specific monoclonal antibodies (mAbs) tested with single antigen bead (SAB) assay being classified as level A1, the highest level of evidence, and adsorption elution studies, of which elute must be tested with SAB assay, as level A2.[12] Eplets for which preliminary evidence for antibody verification is provided, but not sufficient according to the classification system, are labelled as provisionally antibody-verified (levels B-D).

Based on these definitions, only 12% of eplets are antibody-verified with sufficient quality of data (levels A1 or A2), with another 13% of eplets being provisionally verified (levels B-D).[12] To expand the list of antibody-verified eplets, we continuously strive to generate new human HLA-specific mAbs using our established recombinant technology.[13,14] With this method, several HLA-specific mAbs with distinct specificities have been generated, resulting in the antibody verification of multiple HLA-DR and HLA-DQ eplets.[13,15]

Here, we describe nine new recombinant human HLA-specific mAbs, two specific for HLA class I and seven for HLA class II. Extensive reactivity analysis resulted in the verification of three previously non-verified eplets. Furthermore, for five eplets that are considered provisionally verified, the level of evidence of antibody verification could be upgraded to A1, rendering these eplets truly antibody-verified. Finally, one eplet could be upgraded from level A2 to level A1 verification evidence.

2 | MATERIALS AND METHODS

2.1 | Subjects

From four healthy women who developed HLA-specific antibodies upon pregnancy, peripheral blood and serum samples were collected and stored in the Biobank of the Department of Immunology, LUMC with informed consent under guidelines issued by the medical ethics committee of Leiden University Medical Center (B22.099, Leiden, the Netherlands). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (Pharmacy Leiden University Medical Center, Leiden, the Netherlands) density gradient centrifugation and kept frozen in liquid nitrogen until further use. Serum samples were stored at −80°C until further use. All individuals and their respective HLA immunisers, if available, were HLA typed by next-generation sequencing (NGS) on Illuminia platform (Illumina, San Diego, CA, USA) using NGSgo kits (GenDx, Utrecht, the Netherlands) as previously described.[13] For analysis of HLA typing, IPD-IMGT/HLA database versions 3.35 and 3.47 were used.

2.2 | Generation of recombinant human HLA-specific monoclonal antibodies

For the generation of recombinant human HLA-specific mAbs, HLA-reactive memory B cells were isolated from PBMC of pregnancy immunised women as previously described.[13] B cells were enriched from PBMC by negative selection using EasySep Human B cell enrichment kit (Stem Cell Technologies, Grenoble, France) and incubated with recombinant HLA molecules (monomer, tetramer or dextramer) of interest (Table 1). For HLA monomers, enriched B cells were first incubated with biotinylated soluble HLA monomer (1 μg per 2×10^6 cells) (Pure Protein, LLC, Oklahoma City, OK, USA) for 30 min at 4°C.[15] After washing with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, Zwijndrecht, the Netherlands), cells were stained with streptavidin-PE, streptavidin-APC, mouse anti-human CD3 (Pacific blue, SP34-2), IgD (PE-Cy7, IA6-2) (all from BD Biosciences, Breda, the Netherlands) and CD27 (FITC, CLB-27/1, ThermoFisher Scientific, Waltham, MA, USA) for 30 min at 4°C in the dark. For HLA tetramers and dextramers, the enriched B cells were stained in PBS containing 1% fetal bovine serum (FBS) (Sigma-Aldrich) with 0.5 μg phycoerythrin (PE) and 0.5 μg allophycocyanin (APC)-labelled HLA-specific tetramers (ProImmune, Oxford, UK) or HLA-specific dextramers (Immudex, Copenhagen, Denmark) and mouse anti-human CD3, IgD and CD27 as described

TABLE 1 Overview of antibody producers and reagents used per mAb.

Individual	HLA antibody producer	HLA immuniser	HLA molecule	mAb
1	*A*01:01 A*02:07* *B*37:01 B*55:02* *C*03:03 C*06:02* *DRB1*04:04* *DRB4*01:03* *DQB1*03:02 DQB1*04:02* *DQA1*03:01 DQA1*03:03* *DPB1*104:01 DPB1*135:01* *DPA1*01:03 DPA1*02:02*	*A*01:01 A*68:01* *B*37:01* *C*06:02* *DRB1*04:04 DRB1*11:01* *DRB3*02:02 DRB4*01:03/01:134* *DQB1*03:01 DQB1*04:02* *DQA1*03:03 DQA1*05:05* *DPB1*04:01 DPB1*104:01* *DPA1*01:03*	*A*68:01* monomer *DRB1*03:01/ DRA1*01:01* tetramer *DPB1*04:01/ DPA1*01:03* dextramer	LB_A68_A LB_DR17_A LB_DR17_B LB_DR17_C LB_DP4_A
2	*A*01:01* *B*08:01* *C*07:01* *DRB1*03:01/03:147 DRB1*12:01/12:10 DRB3*01:01 DRB3*02:02 DQB1*02:01/02:109 DQB1*03:01 DQA1*05:01 DQA1*05:05* *DPB1*04:01* *DPA1*01:03*	Unknown	*B*15:01* monomer *DQB1*06:04/ DQA1*01:02* monomer	LB_B62_A LB_DQB0604_A
3	*A*03:01* *B*07:02 B*37:01* *C*06:02 C*07:02* *DRB1*13:01 DRB1*15:01* *DRB3*02:02 DRB5*01:01* *DQB1*06:02 DQB1*06:03* *DQA1*01:02 DQA1*01:03* *DPB1*04:01* *DPA1*01:03*	*A*03:01* *B*35:01 B*37:01* *C*03:03 C*06:02* *DRB1*04:07 DRB1*13:01* *DRB3*02:02 DRB4*01:03/01:134* *DQB1*03:01 DQB1*06:03* *DQA1*01:03 DQA1*03:03* *DPB1*04:01* *DPA1*01:03*	*DRB1*04:01/ DRA1*01:01* tetramer	LB_DR4_D
4	*A*29:02 A*30:02* *B*08:01 B*45:01* *C*06:02 C*07:01* *DRB1*09:01 DRB1*15:01* *DRB4*01:01 DRB5*01:01* *DQB1*02:02 DQB1*06:02* *DQA1*01:02 DQA1*03:03* *DPB1*04:01* *DPA1*01:03*	*A*02:01 A*30:02* *B*08:01 B*50:01* *C*06:02 C*07:01* *DRB1*07:01 DRB1*15:01* *DRB4*01:01 DRB5*01:01* *DQB1*02:02 DQB1*06:02* *DQA1*01:02 DQA1*02:01* *DPB1*04:01* *DPA1*01:03 DPA1*02:02*	*DRB1*07:01/ DRA1*01:01* tetramer	LB_DR7_G

above for 30 min at 4°C in the dark. Subsequently, $CD3^{-}CD27^{+}IgD^{-}APC^{+}PE^{+}$ cells were sorted using a FACSAria II sorter (BD Biosciences) in 96-well flat-bottom plate (Costar, Corning, NY, USA) at 1 cell per well with each well containing 1×10^5 irradiated CD40L-expressing EL4-B5 cells[16] in Iscove's modified Dulbecco's medium (IMDM, Lonza, Basel, Switzerland) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (both Gibco Invitrogen, Paisley, UK), 50 μM 2-mercaptoethanol, 0.5 μg/mL R848 (toll-like receptor 7/8 agonist, resiquimod), 20 μg/mL insulin-transferrin-sodium selenite (Sigma-Aldrich), 50 ng/mL IL-21 (Gibco), 1 ng/mL IL-1β and 0.3 ng/mL TNF-α (both from Miltenyi, Leiden, the Netherlands).[17] After 13 days of expansion, the supernatants were screened for the presence of IgG HLA-specific antibodies as described.

From HLA-antibody positive memory B cell clones mAbs were generated using recombinant technology.[14] After RNA isolation using TRIzol reagent (Thermo Fisher Scientific) and cDNA synthesis (Takara, Saint-Germain-en-Laye, France), the variable heavy (VH) and light (VL) chain encoded genes were obtained by 5′-RACE polymerase chain reaction. Then, the VH and VL fragments were cloned into pcDNA3.3 plasmids containing the corresponding human constant domain IgG1, kappa or lambda. The plasmids were co-transfected in Expi293F cells with Expi293 expression medium, ExpiFectamine, Opti-Mem (all ThermoFisher Scientific) and SV40-LT plasmid[18] to produce mAbs. All plasmids were Sanger

sequenced (Macrogen, Amsterdam, the Netherlands) to obtain the nucleotide sequence data of the variable domain to define the V(D)J gene usage using IgBLAST.[19]

2.3 | HLA-specific antibody detection

An in-house developed enzyme-linked immunosorbent assay (ELISA) was used to screen the supernatant of memory B cell clones for the presence of IgG, and to determine the IgG concentration of the produced mAbs as previously described.[20]

Either serum, supernatant of memory B cell clones, or HLA-specific recombinant mAbs were tested with Lifecodes Lifescreen Delux screening kit (Immucor Transplant Diagnostics, Stanford, CT, USA) to detect the presence of HLA-specific antibodies. Subsequently, HLA antibody specificities were determined by either Lifecodes HLA class I or HLA class II SAB assay (Immucor) and/or Labscreen single antigen HLA Class II combi (One Lambda, West Hills, CA, USA). Serum samples were treated with ethylenediaminetetraacetic acid (EDTA; end concentration 8 mM) prior to testing.

2.4 | Reactivity analysis of recombinant human HLA mAbs

Antibody reactivity analysis of the generated human HLA mAbs was performed as previously described.[13,15] The SA AA mismatches between the HLA typing of the producer and the mismatched HLA of the immuniser, which are reactive in SAB assay, or the HLA molecule used for memory B cell sorting, were determined using HLA-EMMA version 1.06.[21] HLA-EMMA version 2.0 beta was used to define eplet mismatches between the HLA makeup of antibody producer and immuniser. For SA AA mismatches, an intralocus comparison was performed, while eplet mismatches were interlocus defined for HLA class I and intralocus for HLA class II. Subsequently, the reactive and non-reactive HLA alleles in SAB analysis were compared to determine whether one or more of the SA AA were uniquely shared by the reactive HLA alleles. To establish whether SA AA were within 3.5 Å or 15 Å radius of each other and to visualise these AA positions, the following HLA structures were used in Swissviewer[22]: Protein Data Bank (PDB) 4MD4, 3PDO, 1A6A, 4HWZ, 1XR9, 4P5M, 1UVQ (downloaded from https://www.rcsb.org/). The identified crucial SA AA were mapped to eplets, for which the eplet description and antibody-verification status were extracted from the HLA Eplet Registry (http://www.EpRegistry.com.br, accessed 3 August 2023). Finally, the level of evidence classification for antibody verification as defined by Bezstarosti et al.[12] was used.

2.5 | Flow cytometric crossmatch assay

Flow cytometric crossmatch assay was performed with either HLA-typed Epstein-Barr virus transformed lymphoblastoid cell lines (EBV-LCLs) or PBMC from healthy donors based on availability. EBV-LCLs were cultured in IMDM containing 10% FBS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine and 100 U/mL penicillin with 100 μg/mL streptomycin. EBV-LCL were incubated with HLA-specific mAb (supernatant, 20 μg/mL) or PBS for 30 min at room temperature. After washing, the cells were stained with rabbit anti-human IgG F(ab')2 (FITC, Dako, Leiden, the Netherlands) at 4°C in the dark for 30 min.[13]

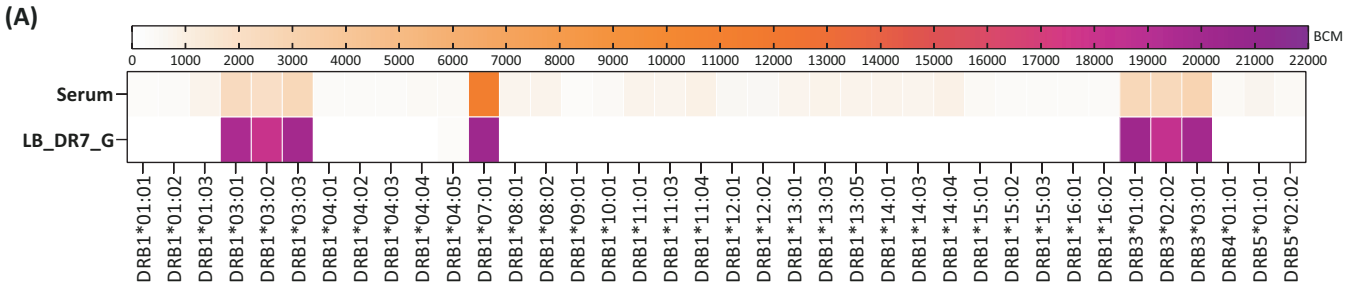
PBMC were incubated with HLA-specific mAb (supernatant, 20 μg/mL or purified, 62.5 nM) or PBS for 30 min at room temperature, and after washing, the cells were stained with mouse anti-human CD3 (Pacific blue, SP34-2), mouse anti-human CD19 (APC, HIB19, BD Bioscience), and rabbit anti-human IgG F(ab′)2 (FITC DAKO) or mouse anti-human CD19 (APC), rabbit anti-human IgG F(ab′)2 (FITC DAKO) and anti-IgM (Pacific blue, SA-DA4, Beckman Coulter, Pasadena, California, USA), or only rabbit anti-human IgG F(ab′)2 (FITC DAKO) at 4°C in the dark for 30 min. Upon washing, cells were acquired using an Accuri C6, FACSCanto (both from BD Bioscience), or Navios (Beckman Coulter) flow cytometer. Data were analysed using FlowJo V10 software (Ashland, OR, USA).

3 | RESULTS

For all nine generated human HLA mAbs, the reactivity pattern was compared to the pattern observed in serum of the antibody producer (Figures 1–9). For all HLA mAbs, the specificities detected were also present in serum albeit with different mean fluorescence intensity (MFI) values. In addition, for each mAb, binding to at least one natively expressed HLA molecule reactive in SAB was confirmed by flow cytometric crossmatch analysis, as well as absence of binding for at least one non-reactive HLA molecule (Figure S1).

3.1 | Newly antibody-verified eplets

LB_DR7_G mAb was generated from an individual who was immunised by *HLA-DRB1*07:01*. While five



(B)

				Residues on DRB1/3/4/5				
	HLA allele	BCM	F-XM	11	14	25	73	74
Immuniser / HLA tetramer	**DRB1*07:01**	**20285**	**Positive**	**G**	**K**	**Q**	**G**	**Q**
	DRB3*01:01	**20186**		**R**	**E**	**R**	**G**	**R**
	DRB3*03:01	**19893**	**Positive**	**L**	**E**	**R**	**G**	**Q**
	DRB1*03:03	**19871**		**S**	**E**	**R**	**G**	**R**
	DRB1*03:01	**19439**		**S**	**E**	**R**	**G**	**R**
	DRB3*02:02	**18176**	**Positive**	**L**	**E**	**R**	**G**	**Q**
	DRB1*03:02	**17894**		**S**	**E**	**R**	**G**	**R**
	DRB1*04:05	226		V	E	R	A	A
	DRB1*01:01	−42	Negative	L	E	R	A	A
	DRB1*04:01	−98		V	E	R	A	A
	DRB1*01:02	−78		L	E	R	A	A
	DRB1*01:03	−98		L	E	R	A	A
	DRB1*04:02	−105		V	E	R	A	A
	DRB1*04:03	−107		V	E	R	A	E
	DRB1*04:04	−110		V	E	R	A	A
	DRB1*08:01	−114		S	E	R	A	L
	DRB1*08:02	−120		S	E	R	A	L
Antibody-producer	DRB1*09:01	−122		D	E	R	A	E
	DRB1*10:01	−125		V	E	R	A	A
	DRB1*11:01	−126		S	E	R	A	A
	DRB1*11:03	−131		S	E	R	A	A
	DRB1*11:04	−132		S	E	R	A	A
	DRB1*12:01	−135		S	E	R	A	A
	DRB1*12:02	−140		S	E	R	A	A
	DRB1*13:01	−144		S	E	R	A	A
	DRB1*13:03	−154		S	E	R	A	A
	DRB1*13:05	−158		S	E	R	A	A
	DRB1*14:01	−159		S	E	R	A	E
	DRB1*14:03	−159		S	E	R	A	L
	DRB1*14:04	−165		S	E	R	A	E
Antibody-producer	DRB1*15:01	−167		P	E	R	A	A
	DRB1*15:02	−176		P	E	R	A	A
	DRB1*15:03	−176		P	E	R	A	A
	DRB1*16:01	−184		P	E	R	A	A
	DRB1*16:02	−192		P	E	R	A	A
Antibody-producer	DRB4*01:01	−205		A	E	W	A	E
Antibody-producer	DRB5*01:01	−206		D	E	R	A	A
	DRB5*02:02	−291		D	E	R	A	A

(C)

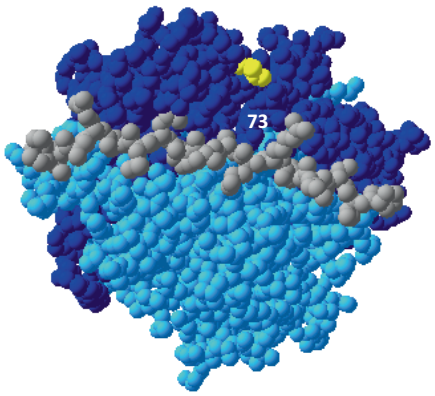
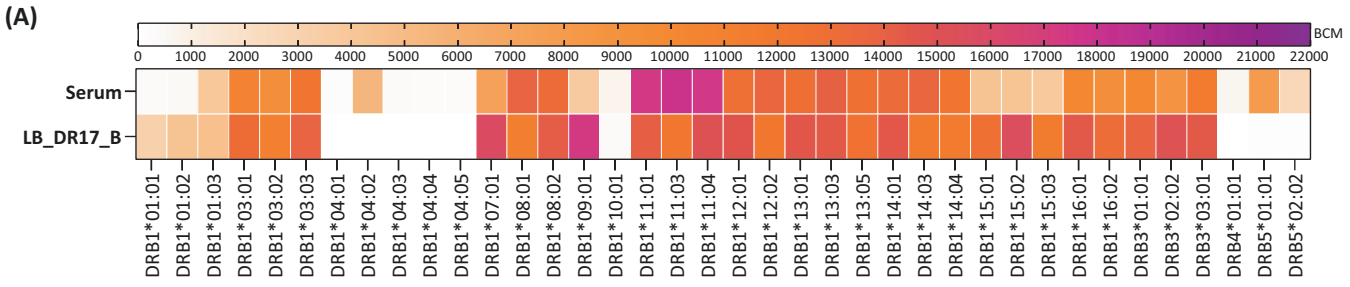


FIGURE 1 Antibody verification of eplet 73G. (A) Lifecodes HLA class II single antigen bead (SAB) data of serum from pregnancy immunised individual (lot 3012342 3012194-SA2, Immucor) and 20μg/mL LB_DR7_G mAb (lot 3008091 3008090-SA2, Immucor) (only DR beads are shown as all other loci were negative). (B) Table showing the HLA-DR molecules present in SAB, including HLA molecule of tetramer, the antibody producer and immuniser. In BCM column, the background corrected MFI values are shown and the results of the flow cytometric crossmatch can be found in F-XM. HLA highlighted in bold are considered as reactive. In the residues on DRB1/3/4/5 column the surface exposed amino acid mismatches (highlighted in light grey) between reactive HLA of the immunizer (*DRB1*07:01*) and the HLA-DR of the antibody producer (*DRB1*09:01 DRB1*15:01 DRB4*01:01 DRB5*01:0*1) are depicted, and the 73G amino acid residue uniquely shared by reactive HLA alleles is highlighted in dark grey. (C) The location of uniquely shared 73G residue (yellow) is indicated on *HLA-DRB1*01:01/DRA1*01:01* molecule (PDB: 3PDO). Light blue is the alpha chain, dark blue is the beta chain and peptide is grey. BCM, background corrected mean fluorescence intensity; F-XM, flow cytometric crossmatch.

SA AA mismatches between reactive HLA alleles of the immuniser and the HLA-DR of the antibody producer were defined by HLA-EMMA analysis, from the reactivity analysis only glycine at position 73 (73G) was deduced to be uniquely shared by the reactive HLA alleles (Figure 1). This indicated that 73G is the functional epitope of LB_DR7_G, which corresponds to a non-verified eplet, named 73G (covering AA 73G). As a result, eplet 73G can be considered antibody-verified by LB_DR7_G at the A1 level.

Multiple mAbs were generated from an individual that was immunised by *HLA-DRB1*11:01* and *HLA-DRB3*02:02*, of which three mAbs could be used for antibody verification of eplets. One of the mAbs, LB_DR17_B showed a broad reactivity pattern (Figure 2). Interestingly, of the 20 SA AA mismatches between reactive HLA of immuniser and the HLA-DR of the antibody producer only the serine on position 120 (120S) was uniquely shared by all reactive HLA alleles and absent on the non-reactive alleles of LB_DR17_B. Since 120S corresponds to the non-verified eplet 120S (covering AA 120S), these data provide A1 level evidence for antibody verification of eplet 120S.

Reactivity analysis of LB_DR17_C (Figure 3) obtained from the same individual resulted in the identification of another residue from the 20 SA AA mismatches to be uniquely shared by all the reactive HLA alleles for this particular mAb, namely histidine at position 96 (96H). Also, 96H corresponds to an eplet that has not been



(B)

				Residues on DRB1/3/4/5																	
	HLA Allele	BCM	F-XM	10	11	12	13	32	51	58	67	70	71	73	74	77	86	96	98	120	149
	DRB1*09:01	**17191**	**Positive**	**Q**	**D**	**K**	**F**	**Y**	**T**	**A**	**F**	**R**	**R**	**A**	**E**	**T**	**G**	**H**	**E**	**S**	**Q**
	DRB1*07:01	**15669**	**Positive**	**Q**	**G**	**K**	**Y**	**Y**	**T**	**A**	**I**	**D**	**R**	**G**	**Q**	**T**	**G**	**H**	**E**	**S**	**Q**
	DRB1*15:02	**15485**		**Q**	**P**	**K**	**R**	**Y**	**T**	**A**	**I**	**Q**	**A**	**A**	**A**	**T**	**G**	**Q**	**K**	**S**	**Q**
Immuniser	**DRB3*02:02**	**14911**		**L**	**L**	**K**	**S**	**H**	**R**	**A**	**L**	**Q**	**K**	**G**	**Q**	**N**	**G**	**H**	**Q**	**S**	**Q**
	DRB1*11:04	**14891**		**Y**	**S**	**T**	**S**	**Y**	**T**	**E**	**F**	**D**	**R**	**A**	**A**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB1*12:01	**14604**		**Y**	**S**	**T**	**G**	**H**	**T**	**A**	**I**	**D**	**R**	**A**	**A**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB1*13:01	**14441**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**I**	**D**	**E**	**A**	**A**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB1*14:01	**14437**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**L**	**R**	**R**	**A**	**E**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB1*16:01	**14352**		**Q**	**P**	**K**	**R**	**Y**	**T**	**A**	**F**	**D**	**R**	**A**	**A**	**T**	**G**	**Q**	**K**	**S**	**Q**
	DRB1*13:03	**14323**		**Y**	**S**	**T**	**S**	**Y**	**T**	**A**	**I**	**D**	**K**	**A**	**A**	**T**	**G**	**H**	**K**	**S**	**H**
	DRB3*03:01	**14291**		**L**	**L**	**K**	**S**	**H**	**T**	**A**	**L**	**Q**	**K**	**G**	**Q**	**N**	**V**	**H**	**Q**	**S**	**H**
	DRB1*08:02	**14171**		**Y**	**S**	**T**	**G**	**Y**	**T**	**A**	**F**	**D**	**R**	**A**	**L**	**T**	**G**	**H**	**K**	**S**	**H**
Immuniser	**DRB1*11:01**	**14017**	**Positive**	**Y**	**S**	**T**	**S**	**Y**	**T**	**E**	**F**	**D**	**R**	**A**	**A**	**T**	**G**	**H**	**K**	**S**	**H**
	DRB1*03:03	**13777**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**L**	**Q**	**K**	**G**	**R**	**N**	**V**	**H***	**K***	**S***	**H***
	DRB3*01:01	**13682**		**L**	**R**	**K**	**S**	**H**	**T**	**A**	**L**	**Q**	**K**	**G**	**R**	**N**	**G**	**H**	**Q**	**S**	**Q**
	DRB1*16:02	**12995**		**Q**	**P**	**K**	**R**	**Y**	**T**	**A**	**L**	**D**	**R**	**A**	**A**	**T**	**G**	**Q**	**K**	**S**	**Q**
HLA tetramer	**DRB1*03:01**	**12935**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**L**	**Q**	**K**	**G**	**R**	**N**	**V**	**H**	**K**	**S**	**H**
	DRB1*15:01	**12616**		**Q**	**P**	**K**	**R**	**Y**	**T**	**A**	**I**	**Q**	**A**	**A**	**A**	**T**	**V**	**Q**	**K**	**S**	**Q**
	DRB1*13:05	**12498**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**F**	**D**	**R**	**A**	**A**	**T**	**G**	**H**	**K**	**S**	**H**
	DRB1*11:03	**12023**		**Y**	**S**	**T**	**S**	**Y**	**T**	**E**	**F**	**D**	**E**	**A**	**A**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB1*12:02	**11929**		**Y**	**S**	**T**	**G**	**H**	**T**	**A**	**F**	**D**	**R**	**A**	**A**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB1*14:03	**11653**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**L**	**D**	**R**	**A**	**L**	**T**	**G**	**H**	**K**	**S**	**H**
	DRB1*14:04	**11563**		**Y**	**S**	**T**	**G**	**H**	**T**	**A**	**L**	**R**	**R**	**A**	**E**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB1*15:03	**11417**		**Q**	**P**	**K**	**R**	**Y**	**T**	**A**	**I**	**Q**	**A**	**A**	**A**	**T**	**V**	**Q**	**K**	**S**	**Q**
	DRB1*08:01	**11252**		**Y**	**S**	**T**	**G**	**Y**	**T**	**A**	**F**	**D**	**R**	**A**	**L**	**T**	**G**	**H**	**K**	**S**	**H**
	DRB1*03:02	**10984**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**L**	**Q**	**K**	**G**	**R**	**N**	**G**	**H**	**K**	**S**	**H**
	DRB1*01:03	**4542**		**Q**	**L**	**K**	**F**	**Y**	**T**	**A**	**I**	**D**	**E**	**A**	**A**	**T**	**G**	**E**	**K**	**S**	**Q**
	DRB1*01:02	**4275**		**Q**	**L**	**K**	**F**	**Y**	**T**	**A**	**L**	**Q**	**R**	**A**	**A**	**T**	**V**	**E**	**K**	**S**	**Q**
	DRB1*01:01	**3091**	**Positive**	**Q**	**L**	**K**	**F**	**Y**	**T**	**A**	**L**	**Q**	**R**	**A**	**A**	**T**	**G**	**E**	**K**	**S**	**Q**
	DRB1*10:01	330	Negative	E	V	K	F	H	T	A	L	R	R	A	A	T	G	Q	K	N	Q
	DRB5*02:02	65		Q	D	K	Y	Y	T	A	I	Q	A	A	A	T	V	E	K	N	Q
	DRB5*01:01	24		Q	D	K	Y	Y	T	A	F	D	R	A	A	T	G	E	K	N	Q
	DRB1*04:03	–40		Q	V	K	H	Y	T	A	L	Q	R	A	E	T	V	Y	E	N	Q
	DRB4*01:01	–40		Q	A	K	C	Y	T	A	L	R	R	A	E	T	V	Q	K	N	Q
	DRB1*04:01	–48	Negative	Q	V	K	H	Y	T	A	L	Q	K	A	A	T	G	Y	E	N	Q
	DRB1*04:05	–54		Q	V	K	H	Y	T	A	L	Q	R	A	A	T	G	Y	E	N	Q
	DRB1*04:02	–87		Q	V	K	H	Y	T	A	I	D	E	A	A	T	V	Y	E	N	Q
Antibody-producer	DRB1*04:04	–95		Q	V	K	H	Y	T	A	L	Q	R	A	A	T	V	Y	E	N	Q
Antibody-producer	DRB4*01:03	n/a	Negative	Q	A	K	C	Y	T	A	L	R	R	A	E	T	V	Q	K	N	Q

The sequence of DRB1*03:03 is not fully known. For the unknown residues (positions 1-5 and 95-226), the same sequence as DRB1*03:01 is assumed (C.Heylen, Immucor, personal communication, August 4, 2020).

(C)

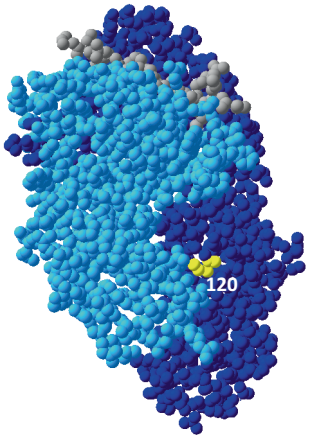


FIGURE 2 Antibody verification of eplet 120S. (A) Lifecodes HLA class II single antigen bead (SAB) data of serum from pregnancy immunised individual (lot 3007379 3007344-SA2, Immucor) and 1 μg/mL LB_DR17_B mAb (lot 3010661 3010503-SA2, Immucor) (only DR beads are shown as all other loci were negative). (B) Table showing the HLA-DR molecules present in SAB, including HLA molecule of tetramer, the antibody producer and immuniser. In BCM column, the background corrected MFI values are shown. The results of the flow cytometric crossmatch can be found in F-XM. HLA highlighted in bold are considered as reactive. In the residues on DRB1/3/4/5 column, the surface exposed amino acid mismatches (highlighted in light grey) between reactive HLA of immunizer (*DRB1*11:01* and *DRB3*02:02*) and the HLA-DR of the antibody producer (*DRB1*04:04 DRB4*01:03*) are depicted, and the 120S amino acid residue uniquely shared by reactive HLA alleles is highlighted in dark grey. (C) The location of uniquely shared 120S residue (yellow) is indicated on *HLA-DRB1*03:01/DRA1*01:01* molecule (PDB: 1A6A). Light blue is the alpha chain, dark blue is the beta chain, and peptide is grey. BCM, background corrected mean fluorescence intensity; F-XM, flow cytometric crossmatch.

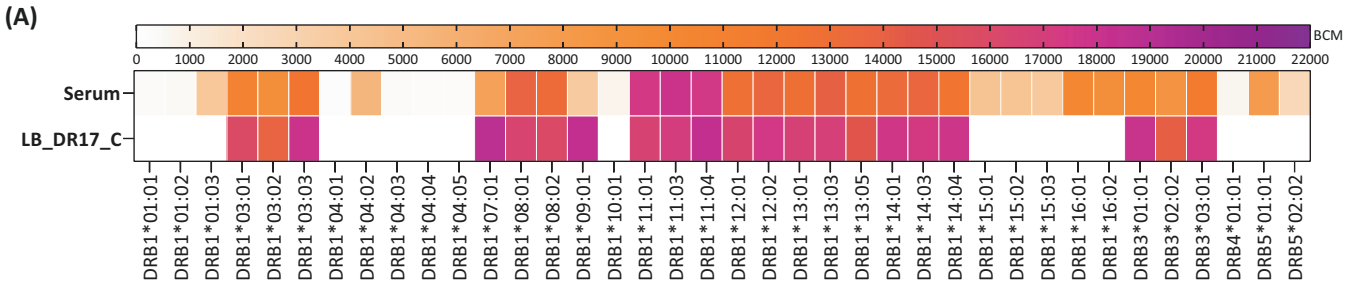
antibody-verified thus far, and as a result, the LB_DR17_C mAb verifies eplet 96H (covering AA 96H).

Overall, based on reactivity analysis of three newly generated recombinant human HLA-DR-specific mAbs, three previously non-verified eplets can now be considered antibody-verified with A1 level evidence (Table 2).

3.2 | Antibody verification of provisionally antibody-verified eplets

We have generated five mAbs that provide level A1 evidence of antibody verification for eplets that we previously considered to be provisionally verified (level B–D).[12]

From an antibody producer that was immunised by *HLA-A*68:01*, the mAb LB_A68_A was generated. While there were only three SA AA mismatches between *HLA-A*68:01* and the HLA-A alleles of the antibody producer (Figure 4), all individual SA AA mismatches were both present on reactive and non-reactive HLA alleles. Interestingly, one mismatched SA AA, 62 arginine (R), is part of the eplet 62RR (consisting of AA 62R 65R), and this eplet is unique for all reactive HLA alleles. Therefore, the configuration 62R 65R was deduced as the functional epitope of LB_A68_A. This provides sufficient evidence for provisionally verified eplet 62RR to be upgraded from



(B)

				Residues on DRB1/3/4/5																	
	HLA Allele	BCM	F-XM	10	11	12	13	32	51	58	67	70	71	73	74	77	86	96	98	120	149
	DRB1*07:01	**18708**	**Positive**	**Q**	**G**	**K**	**Y**	**Y**	**T**	**A**	**I**	**D**	**R**	**G**	**Q**	**T**	**G**	**H**	**E**	**S**	**Q**
	DRB1*09:01	**18258**	**Positive**	**Q**	**D**	**K**	**F**	**Y**	**T**	**A**	**F**	**R**	**R**	**A**	**E**	**T**	**G**	**H**	**E**	**S**	**Q**
	DRB1*11:04	**18253**		**Y**	**S**	**T**	**S**	**Y**	**T**	**E**	**F**	**D**	**R**	**A**	**A**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB3*01:01	**17901**	**Positive**	**L**	**R**	**K**	**S**	**H**	**T**	**A**	**L**	**Q**	**K**	**G**	**R**	**N**	**G**	**H**	**Q**	**S**	**Q**
	DRB1*03:03	**17741**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**L**	**Q**	**K**	**G**	**R**	**N**	**V**	**H***	**K***	**S***	**H***
	DRB1*14:04	**17622**		**Y**	**S**	**T**	**G**	**H**	**T**	**A**	**L**	**R**	**R**	**A**	**E**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB1*14:01	**17534**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**L**	**R**	**R**	**A**	**E**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB1*12:02	**17431**	**Positive**	**Y**	**S**	**T**	**G**	**H**	**T**	**A**	**F**	**D**	**R**	**A**	**A**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB3*03:01	**17294**	**Positive**	**L**	**L**	**K**	**S**	**H**	**T**	**A**	**L**	**Q**	**K**	**G**	**Q**	**N**	**V**	**H**	**Q**	**S**	**H**
	DRB1*14:03	**17233**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**L**	**D**	**R**	**A**	**L**	**T**	**G**	**H**	**K**	**S**	**H**
	DRB1*11:03	**17048**		**Y**	**S**	**T**	**S**	**Y**	**T**	**E**	**F**	**D**	**E**	**A**	**A**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB1*13:03	**16782**		**Y**	**S**	**T**	**S**	**Y**	**T**	**A**	**I**	**D**	**K**	**A**	**A**	**T**	**G**	**H**	**K**	**S**	**H**
	DRB1*13:01	**16646**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**I**	**D**	**E**	**A**	**A**	**T**	**V**	**H**	**K**	**S**	**H**
Immuniser	**DRB1*11:01**	**16504**		**Y**	**S**	**T**	**S**	**Y**	**T**	**E**	**F**	**D**	**R**	**A**	**A**	**T**	**G**	**H**	**K**	**S**	**H**
	DRB1*08:01	**16331**		**Y**	**S**	**T**	**G**	**Y**	**T**	**A**	**F**	**D**	**R**	**A**	**L**	**T**	**G**	**H**	**K**	**S**	**H**
	DRB1*12:01	**16325**		**Y**	**S**	**T**	**G**	**H**	**T**	**A**	**I**	**D**	**R**	**A**	**A**	**T**	**V**	**H**	**K**	**S**	**H**
	DRB1*08:02	**15850**		**Y**	**S**	**T**	**G**	**Y**	**T**	**A**	**F**	**D**	**R**	**A**	**L**	**T**	**G**	**H**	**K**	**S**	**H**
HLA tetramer	**DRB1*03:01**	**15776**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**L**	**Q**	**K**	**G**	**R**	**N**	**V**	**H**	**K**	**S**	**H**
	DRB1*13:05	**14564**		**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**F**	**D**	**R**	**A**	**A**	**T**	**G**	**H**	**K**	**S**	**H**
Immuniser	**DRB3*02:02**	**14025**		**L**	**L**	**K**	**S**	**H**	**R**	**A**	**L**	**Q**	**K**	**G**	**Q**	**N**	**G**	**H**	**Q**	**S**	**Q**
	DRB1*03:02	**13814**	**Positive**	**Y**	**S**	**T**	**S**	**H**	**T**	**A**	**L**	**Q**	**K**	**G**	**R**	**N**	**G**	**H**	**K**	**S**	**H**
	DRB1*01:01	−91	Negative	Q	L	K	F	Y	T	A	L	Q	R	A	A	T	G	E	K	S	Q
	DRB1*04:02	−97		Q	V	K	H	Y	T	A	I	D	E	A	A	T	V	Y	E	N	Q
	DRB1*01:02	−98		Q	L	K	F	Y	T	A	L	Q	R	A	A	T	V	E	K	S	Q
	DRB1*16:02	−103		Q	P	K	R	Y	T	A	L	D	R	A	A	T	G	Q	K	S	Q
	DRB1*04:05	−114		Q	V	K	H	Y	T	A	L	Q	R	A	A	T	G	Y	E	N	Q
	DRB1*10:01	−118		E	V	K	F	H	T	A	L	R	R	A	A	T	G	Q	K	N	Q
Antibody-producer	DRB1*04:04	−126		Q	V	K	H	Y	T	A	L	Q	R	A	A	T	V	Y	E	N	Q
	DRB1*01:03	−131		Q	L	K	F	Y	T	A	I	D	E	A	A	T	G	E	K	S	Q
	DRB5*02:02	−131		Q	D	K	Y	Y	T	A	I	Q	A	A	A	T	V	E	K	N	Q
	DRB1*04:03	−134		Q	V	K	H	Y	T	A	L	Q	R	A	E	T	V	Y	E	N	Q
	DRB1*04:01	−143	Negative	Q	V	K	H	Y	T	A	L	Q	K	A	A	T	G	Y	E	N	Q
	DRB1*15:03	−149		Q	P	K	R	Y	T	A	I	Q	A	A	A	T	V	Q	K	S	Q
	DRB1*15:02	−156		Q	P	K	R	Y	T	A	I	Q	A	A	A	T	G	Q	K	S	Q
	DRB5*01:01	−165		Q	D	K	Y	Y	T	A	F	D	R	A	A	T	G	E	K	N	Q
	DRB1*15:01	−176		Q	P	K	R	Y	T	A	I	Q	A	A	A	T	V	Q	K	S	Q
	DRB1*16:01	−176		Q	P	K	R	Y	T	A	F	D	R	A	A	T	G	Q	K	S	Q
	DRB4*01:01	−183		Q	A	K	C	Y	T	A	L	R	R	A	E	T	V	Q	K	N	Q
Antibody-producer	DRB4*01:03	n/a	Negative	Q	A	K	C	Y	T	A	L	R	R	A	E	T	V	Q	K	N	Q

The sequnece of DRB1*03:03 is not fully known. For the unknown residues (positions 1-5 and 95-226), the same sequence as DRB1*03:01 is assumed (C.Heylen, Immucor, personal communication, August 4, 2020).

(C)

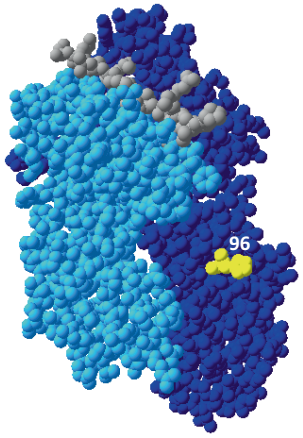


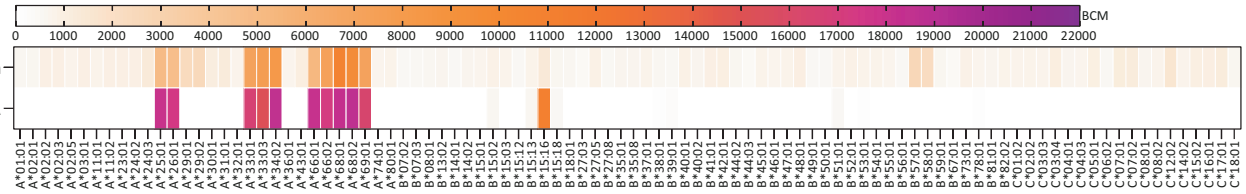
FIGURE 3 Antibody verification of eplet 96H. (A) Lifecodes HLA class II single antigen bead data of serum from pregnancy immunised individual and 20μg/mL LB_DR17_C mAb (both lot 3007379 3007344-SA2, Immucor) (only DR beads are shown as all other loci were negative). (B) Table showing the HLA-DR molecules present in SAB, including HLA molecule of tetramer, the antibody producer and immuniser. In BCM column, the background corrected MFI values are shown, and the results of the flow cytometric crossmatch can be found in F-XM. HLA highlighted in bold are considered as reactive. In the residues on DRB1/3/4/5 column, the surface exposed amino acid mismatches (highlighted in light grey) between reactive HLA of immunizer (*DRB1*11:01* and *DRB3*02:02*) and the HLA-DR of the antibody producer (*DRB1*04:04 DRB4*01:03*) are depicted, and the 96H amino acid residue uniquely shared by reactive HLA alleles is highlighted in dark grey. (C) The location of uniquely shared 96H residue (yellow) is indicated on *HLA-DRB1*03:01/DRA1*01:01* molecule (PDB: 1A6A). Light blue is the alpha chain, dark blue is the beta chain and peptide is grey. BCM, background corrected mean fluorescence intensity; F-XM, flow cytometric crossmatch.

level D (reactivity analysis with antibodies from non-human species) to level A1 formal antibody verification.

The mAb LB_B62_A was obtained from an individual with unknown immunising events. Based on the SA AA mismatches between the HLA-B alleles of the antibody producer and the *HLA-B*15:01* monomer used for sorting in combination with the reactivity pattern analysis of this mAb, 45 methionine (M) and 46 alanine (A) were identified as the functional epitope (Figure 5). The residues 45M and 46A are part of the provisionally antibody-verified eplet 44RMA (covering AA 44R 45M 46A) and indeed the configuration 44R 45M 46A is unique for the reactive HLA alleles. These data allow for the level of evidence of the eplet 44RMA to be raised from provisionally verified level B to fully verified level A1.

The mAb LB_DR4_D was produced from an individual immunised by *HLA-DRB1*04:07* (Figure 6). In the SAB assay, the LB_DR4_D showed to be reactive against HLA-DR4, -DR7, and -DR9. From the six SA AA mismatches between *HLA-DRB1*04:07* and HLA-DR of the antibody producer, which were the same SA AA mismatches between reactive *HLA-DRB1*04:03* and antibody producer, only glutamic acid (E) on position 98 was uniquely shared by the reactive HLA alleles. Since this

(A)



(B)

	HLA Allele	BCM	F-XM	Residues on HLA class I 62	65	70	97
	A*68:02	**18579**	**Positive**	**R**	**R**	**Q**	**R**
	A*34:02	**18438**		**R**	**R**	**Q**	**I**
Immuniser / HLA monomer	**A*68:01**	**18183**	**Positive**	**R**	**R**	**Q**	**M**
	A*66:01	**18084**		**R**	**R**	**Q**	**R**
	A*25:01	**17922**		**R**	**R**	**H**	**R**
	A*26:01	**17315**		**R**	**R**	**H**	**R**
	A*66:02	**17162**		**R**	**R**	**Q**	**R**
	A*33:01	**16555**	**Positive**	**R**	**R**	**H**	**M**
	A*69:01	**16254**		**R**	**R**	**Q**	**R**
	A*33:03	**15183**		**R**	**R**	**H**	**M**
	B*15:16	**10996**	**Positive**	**R**	**R**	**S**	**R**
	B*15:13	554		R	Q	N	R
	B*15:02	549	Negative	R	Q	N	R
	B*51:01	474		R	Q	N	T
	B*15:18	439	Negative	R	Q	N	R
	B*39:01	175	Negative	R	Q	N	R
	B*53:01	73		R	Q	N	R
	B*38:01	55		R	Q	N	R
	B*78:01	55		R	Q	N	T
	A*24:02	−52	Negative	E	G	H	M
	B*35:01	−54		R	Q	N	R
	A*32:01	−54	Negative	Q	R	H	M
	A*02:05	−56		G	R	H	R
	A*74:01	−57		Q	R	H	M
	A*02:03	−61		G	R	H	R
	B*15:01	−67	Negative	R	Q	N	R
Antibody-producer	C*03:03	−68	Negative	R	Q	Q	R
	B*13:02	−71	Negative	R	Q	N	T
Antibody-producer	B*37:01	−73		R	Q	N	R
	A*11:02	−75		Q	R	Q	I
	A*11:01	−78	Negative	Q	R	Q	I
	A*23:01	−81		E	G	H	M
	A*02:02	−82		G	R	H	R
Antibody-producer	C*06:02	−87	Negative	R	Q	Q	W
	B*57:01	−87		G	R	S	V
	A*43:01	−87		L	R	H	R
	A*02:01	−95	Negative	G	R	H	R
	A*80:01	−101		E	R	H	I
	A*29:02	−102		L	R	Q	M
	A*36:01	−102		Q	R	H	I
	A*29:01	−106		L	R	Q	M
	A*24:03	−112		E	G	H	M
	A*03:01	−114		Q	R	Q	I
	A*31:01	−114		Q	R	H	M
	A*30:01	−116	Negative	Q	R	Q	I
	B*27:05	−119	Negative	R	Q	K	N
Antibody-producer	A*01:01	−124	Negative	Q	R	H	I
	B*58:01	−126		G	R	S	R
	B*40:02	−158	Negative	R	Q	N	S
	B*55:01	−209	Negative	R	Q	Q	T
Antibody-producer	A*02:07	n/a		G	R	H	R
Antibody-producer	B*55:02	n/a		R	Q	Q	T
	rest HLA class I beads	≤0		R	Q	K/N/Q	N/R/S/T/W

(C)

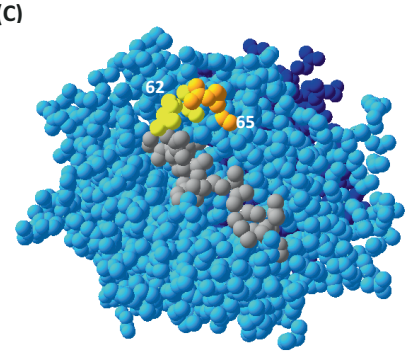
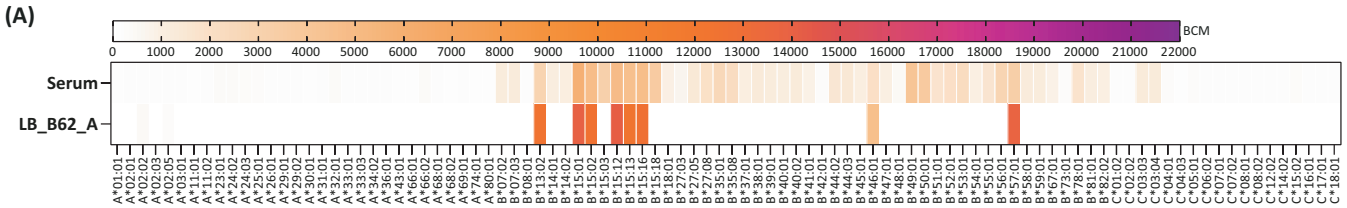


FIGURE 4 Antibody verification of eplet 62RR. (A) Lifecodes HLA class I single antigen bead data of serum from pregnancy immunised individual (lot 3012700 3012637-SA1, Immucor) and 5 μg/mL LB_A68_A mAb (lot 3010512 3010394-SA1, Immucor). (B) Table showing a selection of HLA class I molecules present in SAB, including the antibody producer, immuniser and HLA monomer. In BCM column, the background corrected MFI values are shown and the results of the flow cytometric crossmatch are shown in F-XM. HLA highlighted in bold are considered as reactive. In the residues on HLA class I column, the intralocus surface exposed amino acid mismatches (highlighted in light grey) between reactive HLA of immunizer (*A*68:01*) and HLA-A of the antibody producer (*A*01:01 A*02:07*) are depicted, and the 62R 65R amino acid residue uniquely shared by reactive HLA alleles are highlighted in dark grey. (C) Locations of amino acid 62R (yellow) and 65R (orange) are indicated on *HLA-A*68:01* structure (PDB: 4HWZ). Light blue is the alpha chain, dark blue is the beta chain and peptide is grey. BCM, background corrected mean fluorescence intensity; F-XM, flow cytometric crossmatch.

residue corresponds to the provisionally verified eplet 98E (covering AA 98E) (level B evidence), this eplet can now be considered antibody verified with A1 level evidence.

LB_DR17_A mAb was obtained from the same individual as LB_DR17_B and LB_DR17_C. However, LB_DR17_A has a slightly different reactivity pattern, as only *HLA-DRB1*11:01* of immuniser was reactive (Figure 7). Of the 11 SA AA mismatches between reactive *HLA-DRB1*11:01* of immuniser and antibody producer 10Y 11S 12T were exclusively present on the reactive HLA alleles in SAB, but the 10Y 11S 12T are located in peptide-binding groove and are therefore not considered as potential functional epitope of LB_DR17_A mAb.[12] Interestingly, the SA AA mismatches 96H and 120S in combination with residue 98 lysine (K) were shared by the reactive HLA alleles. This configuration of AA corresponds to the provisionally antibody-verified (level B) eplet 96HK (covering AA 96H 98K 120S). The reactivity analysis of LB_DR17_A confirms binding of this mAb to configuration 96H 98K 120S and thereby provides level A1 antibody verification of eplet 96HK.

From an *HLA-DPB1*04:01* immunised individual, the recombinant human HLA mAb LB_DP4_A was generated. A total of five SA AA mismatches were determined between reactive HLA alleles of the immuniser and the HLA-DP of the antibody producer of which three residues, 85G, 86 proline (P) and 87M, are all uniquely



(B)

	HLA Allele	BCM	F-XM	Residues on HLA class I 44	45	46	114	131	152	163	177	180
	B*15:12	**14010**		**R**	**M**	**A**	**D**	**S**	**E**	**L**	**E**	**Q**
HLA monomer	**B*15:01**	**13938**	**Positive**	**R**	**M**	**A**	**D**	**S**	**E**	**L**	**E**	**Q**
	B*57:01	**13798**	**Positive**	**R**	**M**	**A**	**D**	**S**	**V**	**L**	**E**	**Q**
	B*15:16	**12622**		**R**	**M**	**A**	**D**	**S**	**E**	**L**	**E**	**Q**
	B*15:02	**12491**	**Positive**	**R**	**M**	**A**	**D**	**S**	**E**	**L**	**E**	**Q**
	B*15:13	**12349**		**R**	**M**	**A**	**D**	**S**	**E**	**L**	**E**	**Q**
	B*13:02	**12311**	**Positive**	**R**	**M**	**A**	**N**	**S**	**V**	**E**	**E**	**Q**
	B*46:01	**4588**		**R**	**M**	**A**	**D**	**S**	**E**	**L**	**E**	**Q**
	A*02:02	373	Negative	R	M	E	H	R	V	T	E	Q
	A*02:05	209		R	M	E	H	R	V	T	E	Q
	A*02:03	−31		R	M	E	H	R	E	T	E	Q
	B*35:01	−33		R	T	E	D	S	V	L	E	Q
	B*40:01	−37		R	K	E	N	R	V	E	D	E
	B*39:01	−38		R	E	E	N	S	V	T	E	Q
	A*32:01	−39		R	M	E	Q	R	V	T	E	Q
	B*47:01	−39		R	K	E	H	S	V	E	E	Q
	B*15:18	−47		R	E	E	D	S	E	L	E	Q
	B*78:01	−48		R	T	E	N	S	E	L	E	Q
	B*35:08	−50		R	T	E	D	S	V	L	E	Q
	B*45:01	−51		R	K	E	N	S	V	L	E	Q
	B*37:01	−53		R	T	E	N	S	V	T	E	Q
	B*53:01	−53	Negative	R	T	E	D	S	V	L	E	Q
	B*51:01	−54		R	T	E	N	S	E	L	E	Q
	A*23:01	−56		R	M	E	H	R	V	T	E	Q
	A*68:02	−56		R	M	E	H	R	V	T	E	Q
	B*40:02	−58		R	K	E	N	R	V	E	E	Q
	B*50:01	−59		R	K	E	N	S	E	L	E	Q
	A*68:01	−61		R	M	E	R	R	V	T	E	Q
	B*14:01	−62		R	E	E	N	S	E	T	E	Q
	A*66:01	−63		R	M	E	Q	R	E	R	E	Q
	A*03:01	−67		R	M	E	R	R	E	T	E	Q
	A*33:01	−67	Negative	R	M	E	Q	R	V	T	E	Q
	B*18:01	−67		R	T	E	D	S	V	T	E	Q
	A*24:03	−69		R	M	E	H	R	V	T	E	Q
	A*26:01	−71		R	M	E	Q	R	E	R	E	Q
	A*25:01	−76		R	M	E	Q	R	E	R	E	Q
	A*74:01	−79	Negative	R	M	E	Q	R	V	T	E	Q
	B*44:03	−82		R	K	E	D	S	V	L	E	Q
	A*36:01	−83		K	M	E	R	R	A	T	E	Q
	A*29:02	−84		R	M	E	R	R	V	T	E	Q
	A*34:02	−86		R	M	E	R	R	E	T	E	Q
	A*02:01	−87		R	M	E	H	R	V	T	E	Q
	B*41:01	−87		R	K	E	N	R	V	T	D	E
Antibody-producer	B*08:01	−88		R	E	E	N	R	V	T	D	E
	A*24:02	−90		R	M	E	H	R	V	T	E	Q
	B*44:02	−91		R	K	E	D	S	V	L	E	Q
	B*49:01	−95		R	K	E	N	S	E	L	E	Q
	B*07:02	−96	Negative	R	E	E	D	R	E	E	D	E
	B*15:03	−96		R	E	E	D	S	E	L	E	Q
Antibody-producer	A*01:01	−124		K	M	E	R	R	A	R	E	Q
	B*14:02	−144	Negative	R	E	E	N	S	E	T	E	Q
Antibody-producer	C*07:01	−146		R	G	E	D	R	A	T	E	Q
	rest of HLA class I beads	≤0		R	E/G/M /G	E	D/E/H /N/Q/	R/S	A/E/R /T/V/	E/L/R/ T	D/E/ K	E/Q

(C)

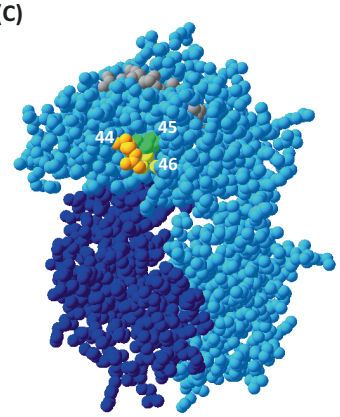


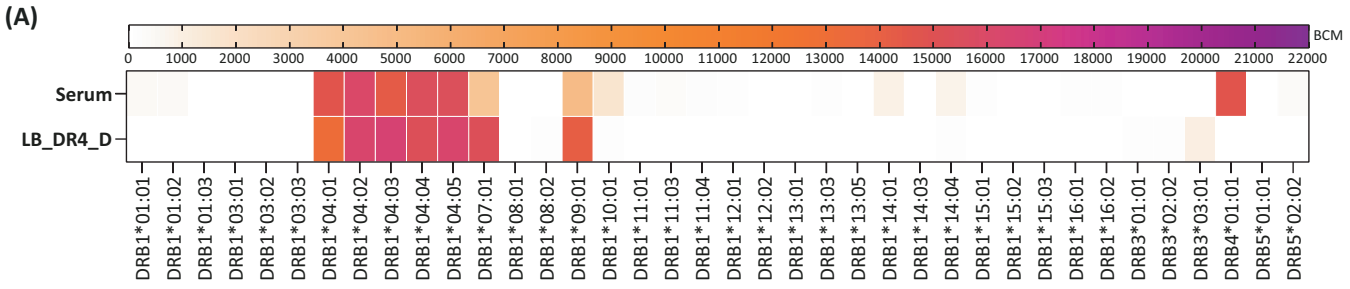
FIGURE 5 Antibody verification of eplet 44RMA. (A) Lifecodes HLA class I single antigen bead data of serum from pregnancy immunised individual (lot 3012700 3012637-SA1, Immucor) and 0.5µg/mL LB_B62_A mAb (lot 3010512 3010394-SA1, Immucor). (B) Table showing a selection of HLA class I molecules present in SAB, including the antibody producer and HLA monomer. In BCM column, the background corrected MFI values are shown and the results of the flow cytometric crossmatch are shown in F-XM. HLA highlighted in bold are considered as reactive. In the residues on HLA class I column, the intralocus surface exposed amino acid mismatches (highlighted in light grey) between HLA-B monomer (*B*15:01*), as immuniser is not known, and HLA-B of the antibody producer (*B*08:01*) are depicted, and the 44R 45M 46A amino acid residue uniquely shared by reactive HLA alleles are highlighted in dark grey. (C) Locations of amino acid 44 (orange), 45 (green) and 46 (yellow) are indicated on *HLA-B*15:01* structure (PDB: 1XR9). Light blue is the alpha chain, dark blue is the beta chain and peptide is grey. BCM, background corrected mean fluorescence intensity; F-XM, flow cytometric crossmatch.

present on the reactive HLA alleles (Figure 8). These SA AA are located within a 3.5 Å radius of each other and form a functional epitope that corresponds to eplet 85GPM (covering AA 85G 86P 87M). While in the publication of Bezstarosti et al.,[12] eplet 85GPM was already listed as antibody verified on A1 level, here we provide the underlying data for verification.

3.3 | Upgrade from level A2 to level A1 antibody verification status

Using an *HLA-DQB1*06:04/DQA1*01:02* monomer, the mAb LB_DQB0604_A was generated from an individual of whom the immunising event was unknown. From the 14 SA AA mismatches between monomer and antibody producer, none of the residues were solely shared by the reactive HLA alleles (Figure 9). Interestingly, the residues comprising eplet 45GV (covering AA 45G 46 valine (V)) were mismatched between HLA-DQB1 alleles of the antibody producer and monomer, and uniquely present on the reactive HLA alleles. While eplet 45GV is already verified by adsorption/elution study (level A2 evidence), this mAb provided additional evidence for confirmation at A1 level.

Overall, five provisionally antibody-verified eplets were formally verified to A1 level evidence (Table 3), and one A2 level antibody-verified eplet was upgraded to A1 level evidence by newly generated recombinant human HLA-specific mAbs (Table 4).



(B)

				Residues on DRB1/3/4/5					
	HLA Allele	BCM	F-XM	11	13	33	74	96	98
Immuniser	**DRB1*04:07**	**n/a**		**V**	**H**	**H**	**E**	**Y**	**E**
	DRB1*04:03	**16510**		**V**	**H**	**H**	**E**	**Y**	**E**
	DRB1*04:02	**16184**		**V**	**H**	**H**	**A**	**Y**	**E**
	DRB1*04:05	**16183**		**V**	**H**	**H**	**A**	**Y**	**E**
	DRB1*07:01	**15254**	**Positive**	**G**	**Y**	**N**	**Q**	**H**	**E**
	DRB1*04:04	**15241**		**V**	**H**	**H**	**A**	**Y**	**E**
	DRB1*09:01	**14029**	**Positive**	**D**	**F**	**N**	**E**	**H**	**E**
HLA tetramer	**DRB1*04:01**	**13065**	**Positive**	**V**	**H**	**H**	**A**	**Y**	**E**
	DRB3*03:01	972	Negative	L	S	N	Q	H	Q
Antibody-producer	DRB3*02:02	82	Negative	L	S	N	Q	H	Q
	DRB3*01:01	58	Negative	R	S	N	R	H	Q
	DRB1*08:02	14		S	G	N	L	H	K
	DRB1*14:04	2		S	G	N	E	H	K
	DRB1*10:01	1		V	F	N	A	Q	K
	DRB1*13:05	−4		S	S	N	A	H	K
	DRB1*14:01	−9	Negative	S	S	N	E	H	K
	DRB5*02:02	−10		D	Y	N	A	E	K
	DRB1*12:01	−14		S	G	N	A	H	K
Antibody-producer	DRB1*15:01	−18		P	R	N	A	Q	K
	DRB1*16:01	−19		P	R	N	A	Q	K
Antibody-producer	DRB5*01:01	−22		D	Y	N	A	E	K
	DRB1*15:02	−27		P	R	N	A	Q	K
	DRB1*01:01	−32		L	F	N	A	E	K
	DRB1*03:03	−34		S	S	N	R	H*	K*
	DRB1*08:01	−37		S	G	N	L	H	K
	DRB1*11:03	−39		S	S	N	A	H	K
	DRB1*16:02	−39		P	R	N	A	Q	K
	DRB1*13:03	−42		S	S	N	A	H	K
	DRB1*03:01	−44		S	S	N	R	H	K
	DRB1*14:03	−44		S	S	N	L	H	K
	DRB1*03:02	−45	Negative	S	S	N	R	H	K
	DRB1*11:01	−47		S	S	N	A	H	K
	DRB1*12:02	−51	Negative	S	G	N	A	H	K
	DRB1*01:02	−53		L	F	N	A	E	K
Antibody-producer	DRB1*13:01	−59		S	S	N	A	H	K
	DRB4*01:01	−63		A	C	N	E	Q	K
	DRB1*11:04	−66		S	S	N	A	H	K
	DRB1*01:03	−71		L	F	N	A	E	K
	DRB1*15:03	−91		P	R	N	A	Q	K
Immuniser	DRB4*01:03	n/a		A	C	N	E	Q	K

*The sequnece of DRB1*03:03 is not fully known. For the unknown residues (positions 1-5 and 95-226), the same sequence as DRB1*03:01 is assumed (C.Heylen, Immucor, personal communication, August 4, 2020).

(C)

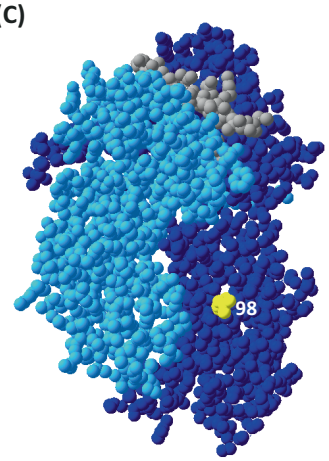
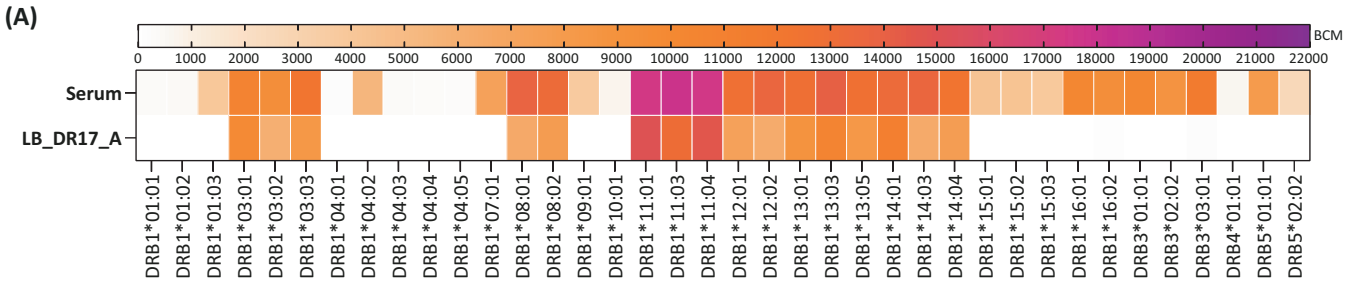


FIGURE 6 Antibody verification of eplet 98E. (A) Lifecodes HLA class II single antigen bead (SAB) data of serum from pregnancy immunised individual (lot 3009113 3009074-SA2, Immucor) and 0.5 μg/mL LB_DR4_D mAb (lot 3010661 3010503-SA2, Immucor) (only DR beads are shown as all other loci were negative). (B) Table showing the HLA-DR molecules present including HLA molecule of tetramer, the antibody producer and immuniser. In BCM column, the background corrected MFI values are shown. The results of the flow cytometric crossmatch can be found in F-XM. HLA highlighted in bold are considered as reactive. In the residues on DRB1/3/4/5 column, the surface exposed amino acid mismatches (highlighted in light grey) between reactive HLA of immunizer (*DRB1*04:07*) and the HLA-DR of the antibody producer (*DRB1*13:01 DRB1*15:01 DRB3*02:02 DRB5*01:01*) are depicted, and the 98E amino acid residue uniquely shared by reactive HLA alleles is highlighted in dark grey. (C) The location of uniquely shared 98E residue (yellow) is indicated on *HLA-DRB1*04:01/DRA1*01:01* molecule (PDB: 4MD4). Light blue is alpha chain, dark blue is beta chain and peptide is in grey. BCM, background corrected mean fluorescence intensity; F-XM, flow cytometric crossmatch.

4 | DISCUSSION

Development of dnDSA is a major complication after solid organ transplantation, as it is not only a major risk factor for graft loss, but the presence of HLA-specific antibodies also complicates finding a suitable donor for repeat transplantation.[23,24] Since eplet mismatches between donor and recipient have been shown to be associated with dnDSA formation,[25–28] the transplant field is eager to start using eplets in the clinical setting.[29] However, it is important to realise that the immunogenicity of individual eplet mismatches is not identical.[7,8,30,31] Eplets have been theoretically defined by Rene Duquesnoy based on identification of polymorphic surface exposed AA located in closed proximity of each other using common HLA AA sequences and available HLA molecule structures.[3] In order to determine clinically relevant eplets, it needs to be established which eplets can actually be recognised and bound by antibodies.

All theoretically defined eplets together with their antibody verification status are documented on the HLA Eplet Registry.[11] Previous versions of this registry contained three levels of verification: non-verified, provisionally verified, and antibody-verified. However, no



(B)

				Residues on DRB1/3/4/5												
	HLA Allele	BCM	F-XM	10	11	12	13	58	67	70	86	96	98	120	149
Immuniser	**DRB1*11:01**	**14950**		**Y**	**S**	**T**	**S**	**E**	**F**	**D**	**G**	**H**	**K**	**S**	**H**
	DRB1*11:04	**14545**		**Y**	**S**	**T**	**S**	**E**	**F**	**D**	**V**	**H**	**K**	**S**	**H**
	DRB1*11:03	**12917**		**Y**	**S**	**T**	**S**	**E**	**F**	**D**	**V**	**H**	**K**	**S**	**H**
	DRB1*14:01	**11052**		**Y**	**S**	**T**	**S**	**A**	**L**	**R**	**V**	**H**	**K**	**S**	**H**
	DRB1*13:03	**10416**		**Y**	**S**	**T**	**S**	**A**	**I**	**D**	**G**	**H**	**K**	**S**	**H**
HLA tetramer	**DRB1*03:01**	**9719**		**Y**	**S**	**T**	**S**	**A**	**L**	**Q**	**V**	**H**	**K**	**S**	**H**
	DRB1*13:01	**8817**		**Y**	**S**	**T**	**S**	**A**	**I**	**D**	**V**	**H**	**K**	**S**	**H**
	DRB1*03:03	**8296**		**Y**	**S**	**T**	**S**	**A**	**L**	**Q**	**V**	**H***	**K***	**S***	**H***
	DRB1*13:05	**8273**		**Y**	**S**	**T**	**S**	**A**	**F**	**D**	**G**	**H**	**K**	**S**	**H**
	DRB1*08:02	**7719**		**Y**	**S**	**T**	**G**	**A**	**F**	**D**	**G**	**H**	**K**	**S**	**H**
	DRB1*14:04	**7582**		**Y**	**S**	**T**	**G**	**A**	**L**	**R**	**V**	**H**	**K**	**S**	**H**
	DRB1*12:01	**7235**		**Y**	**S**	**T**	**G**	**A**	**I**	**D**	**V**	**H**	**K**	**S**	**H**
	DRB1*08:01	**6362**		**Y**	**S**	**T**	**G**	**A**	**F**	**D**	**G**	**H**	**K**	**S**	**H**
	DRB1*14:03	**6315**		**Y**	**S**	**T**	**S**	**A**	**L**	**D**	**G**	**H**	**K**	**S**	**H**
	DRB1*12:02	**6198**	**Positive**	**Y**	**S**	**T**	**G**	**A**	**F**	**D**	**V**	**H**	**K**	**S**	**H**
	DRB1*03:02	**5884**	**Positive**	**Y**	**S**	**T**	**S**	**A**	**L**	**Q**	**G**	**H**	**K**	**S**	**H**
	DRB3*03:01	109		L	L	K	S	A	L	Q	V	H	Q	S	H
	DRB1*16:02	11		Q	P	K	R	A	L	D	G	Q	K	S	Q
	DRB5*02:02	−6		Q	D	K	Y	A	I	Q	V	E	K	N	Q
	DRB1*10:01	−8		E	V	K	F	A	L	R	G	Q	K	N	Q
	DRB3*01:01	−14		L	R	K	S	A	L	Q	G	H	Q	S	Q
	DRB4*01:01	−20	Negative	Q	A	K	C	A	L	R	V	Q	K	N	Q
	DRB1*04:03	−23		Q	V	K	H	A	L	Q	V	Y	E	N	Q
	DRB1*01:01	−27	Negative	Q	L	K	F	A	L	Q	G	E	K	S	Q
	DRB1*16:01	−28		Q	P	K	R	A	F	D	G	Q	K	S	Q
	DRB1*15:01	−30		Q	P	K	R	A	I	Q	V	Q	K	S	Q
	DRB5*01:01	−35		Q	D	K	Y	A	F	D	G	E	K	N	Q
	DRB1*07:01	−38	Negative	Q	G	K	Y	A	I	D	G	H	E	S	Q
	DRB1*01:02	−42		Q	L	K	F	A	L	Q	V	E	K	S	Q
	DRB1*09:01	−42		Q	D	K	F	A	F	R	G	H	E	S	Q
	DRB1*04:05	−44		Q	V	K	H	A	L	Q	G	Y	E	N	Q
	DRB1*01:03	−56		Q	L	K	F	A	I	D	G	E	K	S	Q
	DRB1*15:02	−56		Q	P	K	R	A	I	Q	G	Q	K	S	Q
	DRB1*04:02	−62		Q	V	K	H	A	I	D	V	Y	E	N	Q
	DRB1*15:03	−66		Q	P	K	R	A	I	Q	V	Q	K	S	Q
	DRB1*04:01	−86	Negative	Q	V	K	H	A	L	Q	G	Y	E	N	Q
Immuniser	DRB3*02:02	−97		L	L	K	S	A	L	Q	G	H	Q	S	Q
Antibody-producer	DRB1*04:04	−115		Q	V	K	H	A	L	Q	V	Y	E	N	Q
Antibody-producer	DRB4*01:03	n/a	Negative	Q	A	K	C	A	L	R	V	Q	K	N	Q

*The sequnece of DRB1*03:03 is not fully known. For the unknown residues (positions 1-5 and 95-226), the same sequence as DRB1*03:01 is assumed (C.Heylen, Immucor, personal communication, August 4, 2020).

(C)

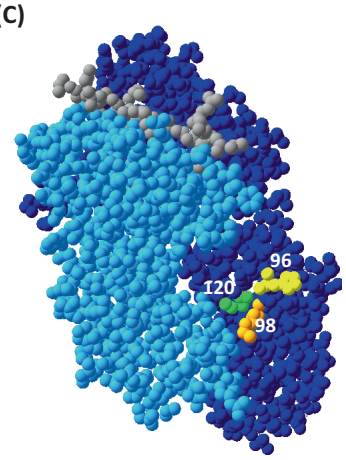
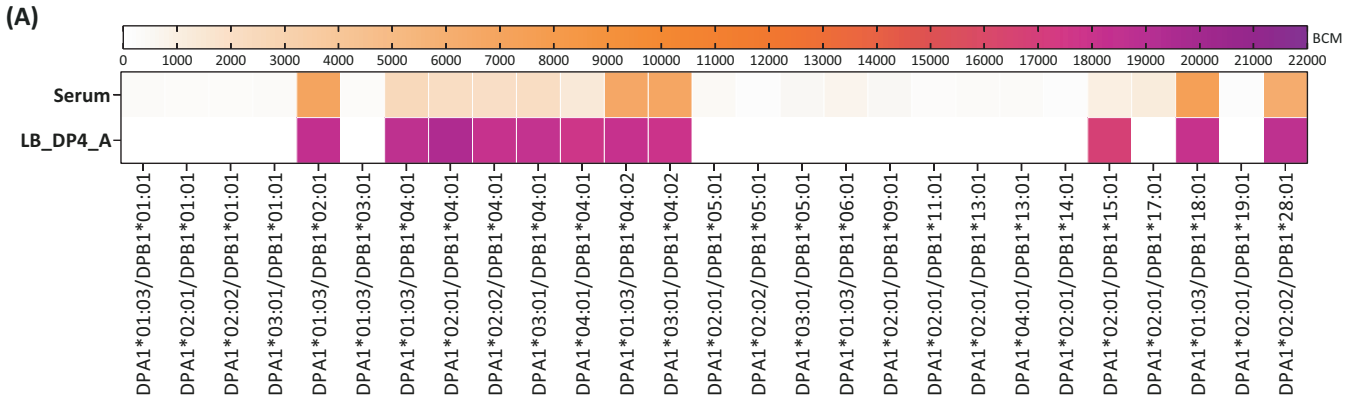


FIGURE 7 Antibody verification of eplet 96HK. (A) Lifecodes HLA class II single antigen bead (SAB) data of serum from pregnancy immunised individual (lot 3007379 3007344-SA2, Immucor) and 1 μg/mL LB_DR17_A mAb (lot 3010661 3010503-SA2, Immucor) (only DR beads are shown as all other loci were negative). (B) Table showing the HLA-DR molecules present in SAB, including HLA molecule of tetramer, the antibody producer and immuniser. In BCM column, the background corrected MFI values are shown. The results of the flow cytometric crossmatch can be found in F-XM. HLA highlighted in bold are considered as reactive. In the residues on DRB1/3/4/5 column, the surface exposed amino acid mismatches (highlighted in light grey) between reactive HLA of immunizer (*DRB1*11:01* and *DRB3*02:02*) and the HLA-DR of the antibody producer (*DRB1*04:04 DRB4*01:03*) are depicted, and the 96H 98K 120S amino acid residue uniquely shared by reactive HLA alleles are highlighted in dark grey. (C) The location of residues 96H (yellow), 98K (orange) and 120S (green) are indicated on *HLA-DRB1*03:01/DRA1*01:01* structure (PDB: 1A6A). Light blue is the alpha chain, dark blue is the beta chain and peptide is grey. BCM, background corrected mean fluorescence intensity; F-XM, flow cytometric crossmatch.

standardisation of the level of evidence for verification was used. Moreover, the provisional status was previously removed. Recently, a classification system was proposed for the level of evidence for the antibody-verified status of all eplets.[12] Based on this classification, eplets verified by human HLA-specific mAbs screened with SAB assay combined with cellular assays with high resolution HLA typed cells (level A1), or adsorption and elution studies based on SAB assay combined with cellular assays with HLA typed cells (level A2) were considered as unambiguously antibody-verified. The provisional status was reintroduced for verification using (polyclonal) patient serum tested in SAB assay and/or CDC with high-resolution HLA typed cells (level B), human mAb, adsorption and elution studies, or patient sera tested with low-resolution HLA typed cells only (level C), or reactivity analysis with antibodies from other species (level D). Using this classification system, only about 20% of all HLA class I and 7% of all HLA class II eplets are truly antibody-verified, while another 10% and 16% of HLA class I and II eplets have the provisionally verified status.

With a well-established method for the generation of recombinant human HLA-specific mAbs, we continuously expand our pool of human mAbs with various HLA specificities.[13–15] Here, the SAB reactivity patterns of nine new recombinant human HLA-specific mAbs were



(B)

	HLA allele		BCM	F-XM	Residues on DPB1 84	85	86	87	96
	DPA1*02:01	**DPB1*04:01**	**19289**	**Positive**	**G**	**G**	**P**	**M**	**R**
Immuniser / HLA dextramer	**DPA1*01:03**	**DPB1*04:01**	**18540**		**G**	**G**	**P**	**M**	**R**
	DPA1*02:02	**DPB1*28:01**	**18524**		**V**	**G**	**P**	**M**	**R**
	DPA1*03:01	**DPB1*04:01**	**18318**		**G**	**G**	**P**	**M**	**R**
	DPA1*01:03	**DPB1*02:01**	**18262**		**G**	**G**	**P**	**M**	**R**
	DPA1*01:03	**DPB1*04:02**	**18106**		**G**	**G**	**P**	**M**	**R**
	DPA1*02:02	**DPB1*04:01**	**18058**		**G**	**G**	**P**	**M**	**R**
	DPA1*01:03	**DPB1*18:01**	**18021**		**V**	**G**	**P**	**M**	**K**
	DPA1*03:01	**DPB1*04:02**	**17744**	**Positive**	**G**	**G**	**P**	**M**	**R**
	DPA1*04:01	**DPB1*04:01**	**17617**		**G**	**G**	**P**	**M**	**R**
	DPA1*02:01	**DPB1*15:01**	**16666**		**V**	**G**	**P**	**M**	**K**
	DPA1*02:01	DPB1*05:01	−84		D	E	A	V	K
	DPA1*02:01	DPB1*09:01	−92		D	E	A	V	K
	DPA1*04:01	DPB1*13:01	−99		D	E	A	V	K
	DPA1*02:01	DPB1*19:01	−103		D	E	A	V	K
	DPA1*03:01	DPB1*05:01	−108		D	E	A	V	K
	DPA1*02:01	DPB1*14:01	−109		D	E	A	V	K
	DPA1*02:01	DPB1*13:01	−117		D	E	A	V	K
	DPA1*02:01	DPB1*11:01	−118		D	E	A	V	K
	DPA1*01:03	DPB1*03:01	−123		D	E	A	V	K
	DPA1*02:01	DPB1*01:01	−125		D	E	A	V	K
	DPA1*01:03	DPB1*06:01	−134	Negative	D	E	A	V	K
	DPA1*02:02	DPB1*01:01	−139		D	E	A	V	K
	DPA1*01:03	DPB1*01:01	−144		D	E	A	V	K
	DPA1*02:02	DPB1*05:01	−150		D	E	A	V	K
	DPA1*03:01	DPB1*01:01	−161	Negative	D	E	A	V	K
	DPA1*02:01	DPB1*17:01	−187		D	E	A	V	R
Antibody producer	DPA1*01:03	DPB1*104:01	n/a		D	E	A	V	K
Antibody producer	DPA1*02:02	DPB1*135:01	n/a		D	E	A	V	K

(C)

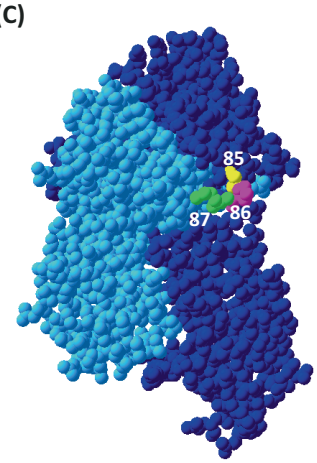
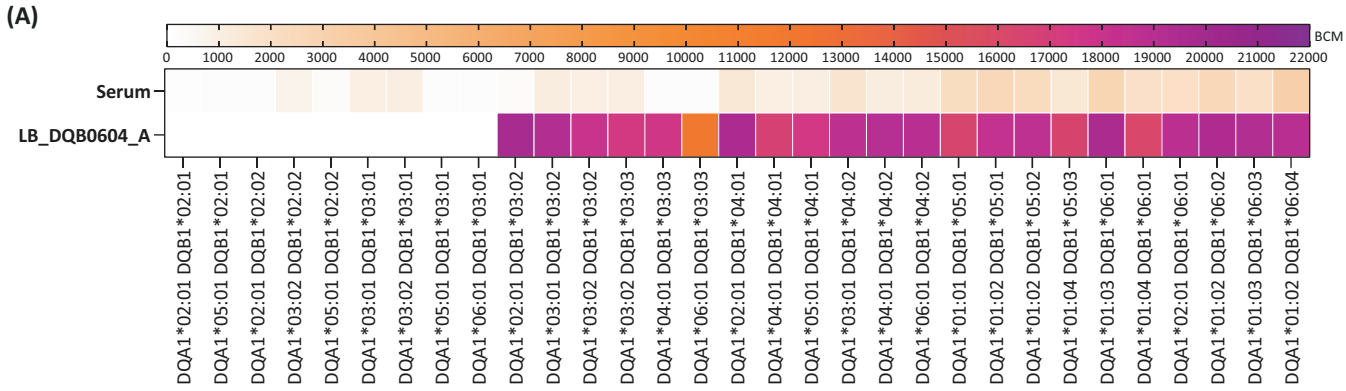


FIGURE 8 Antibody verification of eplet 85GPM. (A) Lifecodes HLA class II single antigen bead (SAB) data of serum from pregnancy immunised individual (lot 3007379 3007344-SA2, Immucor) and 20 µg/mL LB_DP4_A mAb (lot 3009113 3009074-SA2, Immucor) (only DP beads are shown as all other loci were negative). (B) Table showing the HLA-DP molecules present in SAB, including the molecules antibody producer, immuniser and HLA dextramer. In BCM column, the background corrected MFI values are shown and the results of the flow cytometric crossmatch are shown in F-XM. HLA highlighted in bold are considered as reactive. In the residues on DPB1 column, the surface exposed amino acid mismatches (highlighted in light grey) between reactive HLA of immunizer (*DPB1*04:01*) and HLA-DP of the antibody producer (*DPB1*104:01 DPB1*135:01*) are depicted, and the 85G 86P 87M amino acid residue uniquely shared by reactive HLA alleles are highlighted in dark grey. (C) Locations of amino acid 85G (yellow), 86P (pink) and 87M (green) are indicated on *HLA-DPB1*02:01/DPA1*01:03* structure (PDB: 4P5M). Light blue is the alpha chain, dark blue is the beta chain and peptide is grey. BCM, background corrected mean fluorescence intensity; F-XM, flow cytometric crossmatch.

extensively analysed considering the high-resolution HLA typing of antibody producer and immuniser (if available). For testing mAbs with SAB assay, it is essential to consider the concentration as well as the affinity of the mAb. When testing HLA-specific mAbs at high concentration in SAB assay background of non-reactive HLA can be observed, and this background disappears upon dilution. Therefore, for this analysis, the reactivity patterns of the HLA-specific mAbs in combination with binding assays to natively expressed HLA were considered rather than solely the obtained MFI values. The binding of the mAbs to natively expressed HLA molecules was confirmed by flow cytometric crossmatches and mAbs were tested for lack of binding to HLA molecules that were considered non-reactive in SAB assay. Hereby, we provided sufficient evidence for antibody verification of three previously non-verified eplets, the further verification of five currently provisionally verified eplets and one eplet was upgraded from level A2 to A1. With the majority of the eplets verified here being located on HLA class II, this work contributes to the expansion of HLA class II eplet antibody verification. This is of importance since a recent large cohort study suggested that seemingly clinically relevant eplets located on HLA class II have not yet been antibody-verified.[32]

While the antibody reactivity analysis of the nine recombinant human HLA mAbs resulted in clear identification of either a single SA AA or configuration of SA AA comprising the functional epitope, this is not always the case.[12,15] Occasionally, the reactivity patterns of



(B)

	HLA Allele		BCM	F-XM	Residues on DQB1 45	46	53	55	57	84	85	86	87	89	90	125	130	203	220	221
	DQA1*02:01	DQB1*03:02	19671		G	V	L	P	A	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*01:03	DQB1*06:01	19461		G	V	Q	R	D	E	V	A	F	G	I	G	R	I	R	Q
	DQA1*02:01	DQB1*04:01	19428		G	V	L	R	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*01:02	DQB1*06:02	19280		G	V	Q	R	D	E	V	A	F	G	I	G	R	V	R	Q
	DQA1*03:01	DQB1*03:02	19176		G	V	L	P	A	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*01:03	DQB1*06:03	19094		G	V	Q	R	D	E	V	A	F	G	I	G	R	V	R	Q
	DQA1*04:01	DQB1*04:02	19005	Positive	G	V	L	R	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*06:01	DQB1*04:02	18879		G	V	L	R	D	Q	L	E	L	T	T	A	R	I	H	H
HLA monomer	DQA1*01:02	DQB1*06:04	18806		G	V	Q	R	V	E	V	G	Y	G	I	G	Q	V	R	Q
	DQA1*02:01	DQB1*06:01	18757		G	V	Q	R	D	E	V	A	F	G	I	G	R	I	R	Q
	DQA1*03:01	DQB1*04:02	18698		G	V	L	R	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*01:02	DQB1*05:02	18579		G	V	Q	R	S	E	V	A	Y	G	I	S	R	V	R	Q
	DQA1*01:02	DQB1*05:01	18314		G	V	Q	R	V	E	V	A	Y	G	I	S	R	V	R	Q
	DQA1*03:02	DQB1*03:02	17927		G	V	L	P	A	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*04:01	DQB1*03:03	17481		G	V	L	P	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*05:01	DQB1*04:01	17358		G	V	L	R	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*03:02	DQB1*03:03	17201	Positive*	G	V	L	P	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*04:01	DQB1*04:01	16579		G	V	L	R	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*01:04	DQB1*05:03	16307	Positive	G	V	Q	R	D	E	V	A	Y	G	I	S	R	V	R	Q
	DQA1*01:01	DQB1*05:01	16242		G	V	Q	R	V	E	V	A	Y	G	I	S	R	V	R	Q
	DQA1*01:04	DQB1*06:01	16074		G	V	Q	R	D	E	V	A	F	G	I	G	R	I	R	Q
	DQA1*06:01	DQB1*03:03	11843		G	V	L	P	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*03:02	DQB1*02:02	−87		G	E	L	L	A	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*03:02	DQB1*03:01	−97		E	V	L	P	D	Q	L	E	L	T	T	A	R	I	H	H
Antibody producer	DQA1*05:01	DQB1*02:01	−98		G	E	L	L	A	Q	L	E	L	T	T	A	R	I	H	H
Antibody producer	DQA1*05:01	DQB1*03:01	−105		E	V	L	P	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*05:05	DQB1*03:01	n/a	Negative	E	V	L	P	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*03:01	DQB1*03:01	−117		E	V	L	P	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*05:01	DQB1*02:02	−123		G	E	L	L	A	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*06:01	DQB1*03:01	−129		E	V	L	P	D	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*02:01	DQB1*02:01	−136		G	E	L	L	A	Q	L	E	L	T	T	A	R	I	H	H
	DQA1*02:01	DQB1*02:02	−140		G	E	L	L	A	Q	L	E	L	T	T	A	R	I	H	H

* HLA typing of cells was DQB1*03:03 DQA1*03

(C)

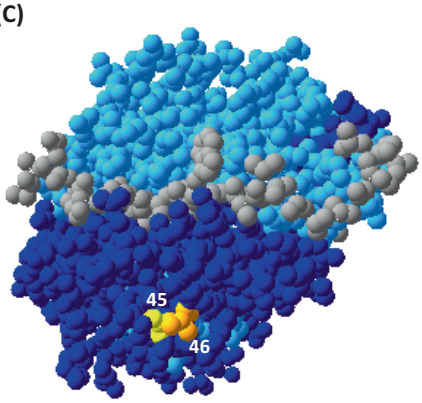


FIGURE 9 Antibody verification of eplet 45GV. (A) Lifecodes HLA class II single antigen bead (SAB) data (lot 3008523 3008518-SA2, Immucor) of serum from pregnancy immunised individual and 5 μg/mL LB_DQB0604_A mAb (lot 3012342 3012194-SA2, Immucor) (only DQ beads are shown as all other loci were negative). (B) Table showing the HLA-DQB1 molecules present in SAB, including HLA molecule of monomers, and the antibody producer. In BCM column, the background corrected MFI values are shown. The results of the flow cytometric crossmatch can be found in F-XM. HLA highlighted in bold are considered as reactive. In the residues on DQB1 column, the surface exposed amino acid mismatches (highlighted in light grey) between HLA monomer (*DQB1*06:04*) and HLA-DQ of the antibody producer (*DQB1*02:01/02:109 DQB1*03:01*) are depicted, and the 45G 46V amino acid residues uniquely shared by reactive HLA alleles are highlighted in dark grey. (C) The location of residues 45G (yellow) and 46V (orange) are indicated on HLA-*DQB1*06:02/DQA1*01:02* structure (PDB: 1UVQ). Light blue is the alpha chain, dark blue is the beta chain and peptide is grey. BCM, background corrected mean fluorescence intensity; F-XM, flow cytometric crossmatch.

human HLA mAbs are highly complex with multiple potential SA AA outside a 3.5 Å radius could potentially form the functional epitope. We have recently shown that these complicated reactivity patterns can be resolved by site-directed mutagenesis of HLA molecules to determine the crucial SA AA for binding of a mAbs to its target HLA.[33] Such strategy will be of interest for a number of HLA class II mAbs for which we have not yet been able to identify the functional epitope.

The increasing number of newly generated human HLA-specific mAbs with unique specificities together with mutation studies (when necessary), as well as adsorption elution studies will contribute significantly to the antibody verification endeavour. However, to accomplish standardisation of eplet verification, it is of vital importance that both the classification system and re-evaluation of the antibody-verified status of eplets by Bezstarosti et al.[12] are implemented in the HLA Eplet Registry. In addition, newly antibody-verified eplets, such as described here, must be adopted regularly in a versioned HLA Eplet Registry to ensure an accurate and up-to-date antibody verification status of the eplets. This is essential since the HLA Eplet Registry is the main source of information for both researchers and clinicians for eplet information. As a result, such a uniform database of eplets with corresponding antibody verification could be consistently used in eplet studies, such as the definition of the most immunogenic eplet mismatches or

TABLE 2 New antibody-verified eplets.

Human mAb	Reactive HLA alleles	Eplet	Polymorphic residues	Level of evidence
LB_DR7_G	*DRB1*03:01 DRB1*03:02 DRB1*03:03 DRB1*07:01 DRB3*01:01 DRB3*02:02 DRB3*03:01*	73G	73G	A1
LB_DR17_B	*DRB1*01:01 DRB1*01:02 DRB1*01:03 DRB1*03:01 DRB1*03:02 DRB1*03:03 DRB1*07:01 DRB1*08:01 DRB1*08:02 DRB1*09:01 DRB1*11:01 DRB1*11:03 DRB1*11:04 DRB1*12:01 DRB1*12:02 DRB1*13:01 DRB1*13:03 DRB1*13:05 DRB1*14:01 DRB1*14:03 DRB1*14:04 DRB1*15:01 DRB1*15:02 DRB1*15:03 DRB1*16:01 DRB1*16:02 DRB3*01:01 DRB3*02:02 DRB3*03:01*	120S	120S	A1
LB_DR17_C	*DRB1*03:01 DRB1*03:02 DRB1*03:03 DRB1*07:01 DRB1*08:01 DRB1*08:02 DRB1*09:01 DRB1*11:01 DRB1*11:03 DRB1*11:04 DRB1*12:01 DRB1*12:02 DRB1*13:01 DRB1*13:03 DRB1*13:05 DRB1*14:01 DRB1*14:03 DRB1*14:04 DRB3*01:01 DRB3*02:02 DRB3*03:01*	96H	96H	A1

TABLE 3 Upgrade of provisionally verified eplets.

Human mAb	Reactive HLA alleles	Eplet	Polymorphic residues	Current level of evidence[12]	New level of evidence
LB_A68_A	*A*25:01 A*26:01 A*33:01 A*33:03 A*34:02 A*66:01 A*66:02 A*68:01 A*68:02 A*69:01 B*15:16*	62RR	62R 65R	D	A1
LB_B62_A	*B*13:02 B*15:01 B*15:02 B*15:12 B*15:13 B*15:16 B*46:01 B*57:01*	44RMA	44R 45M 46A	B	A1
LB_DR4_D	*DRB1*04:01 DRB1*04:02 DRB1*04:03 DRB1*04:04 DRB1*04:05 DRB1*07:01 DRB1*09:01*	98E	98E	B	A1
LB_DR17_A	*DRB1*03:01 DRB1*03:02 DRB1*03:03 DRB1*08:01 DRB1*08:02 DRB1*11:01 DRB1*11:03 DRB1*11:04 DRB1*12:01 DRB1*12:02 DRB1*13:01 DRB1*13:03 DRB1*13:05 DRB1*14:01 DRB1*14:03 DRB1*14:04*	96HK	96H 98K 120S	B	A1
LB_DP4_A	*DPB1*02:01 DPB1*04:01 DPB1*04:02 DPB1*15:01 DPB1*18:01 DPB1*28:01*	85GPM	85G 86P 87M	B	A1

TABLE 4 Upgrade of eplet from A2 to A1 level.

Human mAb	Reactive HLA alleles	Eplet	Polymorphic residues	Current level of evidence[12]	New level of evidence
LB_DQB0604_A	*DQB1*03:02 DQB1*03:03 DQB1*04:01 DQB1*04:02 DQB1*05:01 DQB1*05:02 DQB1*05:03 DQB1*06:01 DQB1*06:02 DQB1*06:03 DQB1*06:04*	45GV	45G 46V	A2	A1

the effect of eplet mismatch levels on transplant outcomes.

ACKNOWLEDGMENTS

The authors thank the Flow Cytometry Core Facility of the Leiden University Medical Center and the HLA Typing and Screening Laboratory Leiden, the Netherlands, for technical assistance.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INFORMED CONSENT

Informed consent was obtained under guidelines issued by the medical ethics committee of Leiden University Medical Center (B22.099, Leiden, the Netherlands).

ORCID

Cynthia S. M. Kramer https://orcid.org/0000-0003-1350-2336
Suzanne Bezstarosti https://orcid.org/0000-0002-0315-115X
Sebastiaan Heidt https://orcid.org/0000-0002-6700-188X

REFERENCES

1. Heidt S, Haasnoot GW, van Rood JJ, Witvliet MD, Claas FHJ. Kidney allocation based on proven acceptable antigens results in superior graft survival in highly sensitized patients. *Kidney Int*. 2018;93(2):491-500. doi:10.1016/j.kint.2017.07.018
2. Lachmann N, Terasaki PI, Budde K, et al. Anti-human leukocyte antigen and donor-specific antibodies detected by luminex posttransplant serve as biomarkers for chronic rejection of renal allografts. *Transplantation*. 2009;87(10):1505-1513. doi:10.1097/TP.0b013e3181a44206
3. Duquesnoy RJ, Askar M. HLAMatchmaker: a molecularly based algorithm for histocompatibility determination. V. Eplet matching for HLA-DR, HLA-DQ, and HLA-DP. *Hum Immunol*. 2007;68(1):12-25. doi:10.1016/j.humimm.2006.10.003
4. Kosmoliaptsis V, Mallon DH, Chen Y, Bolton EM, Bradley JA, Taylor CJ. Alloantibody responses after renal transplant failure can be better predicted by donor-recipient HLA amino acid sequence and physicochemical disparities than conventional HLA matching. *Am J Transplant*. 2016;16(7):2139-2147. doi:10.1111/ajt.13707
5. Snanoudj R, Kamar N, Cassuto E, et al. Epitope load identifies kidney transplant recipients at risk of allosensitization following minimization of immunosuppression. *Kidney Int*. 2019;95(6):1471-1485. doi:10.1016/j.kint.2018.12.029
6. Wiebe C, Pochinco D, Blydt-Hansen TD, et al. Class II HLA epitope matching-a strategy to minimize de novo donor-specific antibody development and improve outcomes. *Am J Transplant*. 2013;13(12):3114-3122. doi:10.1111/ajt.12478
7. Kramer CSM, Israeli M, Mulder A, et al. The long and winding road towards epitope matching in clinical transplantation. *Transpl Int*. 2019;32(1):16-24. doi:10.1111/tri.13362
8. Kramer C, Heidt S, Claas FHJ. Towards the identification of the relative immunogenicity of individual HLA antibody epitopes. *Hum Immunol*. 2019;80(4):218-220. doi:10.1016/j.humimm.2019.02.002
9. Duquesnoy RJ, Marrari M, Mulder A, Sousa LCDD, da Silva AS, do Monte SJH First report on the antibody verification of HLA-ABC epitopes recorded in the website-based HLA epitope registry. *Tissue Antigens*. 2014;83(6):391–400. 10.1111/tan.12341
10. Duquesnoy RJ, Marrari M, Tambur AR, et al. First report on the antibody verification of HLA-DR, HLA-DQ and HLA-DP epitopes recorded in the HLA epitope registry. *Hum Immunol*. 2014;75(11):1097-1103. doi:10.1016/j.humimm.2014.09.012
11. Duquesnoy RJ, Marrari M, da M Sousa LCD, et al. 16th IHIW: a website for antibody-defined HLA epitope registry. *Int J Immunogenet*. 2013;40(1):54-59. doi:10.1111/iji.12017
12. Bezstarosti S, Bakker KH, Kramer CSM, et al. A comprehensive evaluation of the antibody-verified status of eplets listed in the HLA epitope registry. *Front Immunol*. 2022;12:800946. doi:10.3389/fimmu.2021.800946
13. Kramer CSM, Franke-van Dijk MEI, Bakker KH, et al. Generation and reactivity analysis of human recombinant monoclonal antibodies directed against epitopes on HLA-DR. *Am J Transplant*. 2020;20(12):3341-3353. doi:10.1111/ajt.15950
14. Kramer CSM, Franke-van Dijk MEI, Priddey AJ, et al. Recombinant human monoclonal HLA antibodies of different IgG subclasses recognising the same epitope: excellent tools to study differential effects of donor-specific antibodies. *HLA*. 2019;94(5):415-424. doi:10.1111/tan.13664
15. Bezstarosti S, Kramer CSM, Franke-van Dijk MEI, et al. HLA-DQ-specific recombinant human monoclonal antibodies allow for in-depth analysis of HLA-DQ epitopes. *Front Immunol*. 2022;12(5533):761893. doi:10.3389/fimmu.2021.761893
16. Wen L, Hanvanich M, Werner-Favre C, Brouwers N, Perrin LH, Zubler RH. Limiting dilution assay for human B cells based on their activation by mutant EL4 thymoma cells: total and anti-malaria responder B cell frequencies. *Eur J Immunol*. 1987;17(6):887-892. doi:10.1002/eji.1830170624
17. Lighaam LC, Vermeulen E, Bleker T, et al. Phenotypic differences between IgG4+ and IgG1+ B cells point to distinct regulation of the IgG4 response. *J Allergy Clin Immunol*. 2014;133(1):267-270. doi:10.1016/j.jaci.2013.07.044
18. Vink T, Oudshoorn-Dickmann M, Roza M, Reitsma JJ, de Jong RN. A simple, robust and highly efficient transient expression system for producing antibodies. *Methods (San Diego, Calif)*. 2014;65(1):5-10. doi:10.1016/j.ymeth.2013.07.018
19. Ye J, Ma N, Madden TL, Ostell JM. IgBLAST: an immunoglobulin variable domain sequence analysis tool. *Nucleic Acids Res*. 2013;41(Web Server issue):W34-W40. doi:10.1093/nar/gkt382

20. Heidt S, Roelen DL, Eijsink C, et al. Calcineurin inhibitors affect B cell antibody responses indirectly by interfering with T cell help. *Clin Exp Immunol*. 2010;159(2):199-207. doi:10.1111/j.1365-2249.2009.04051.x
21. Kramer CSM, Koster J, Haasnoot GW, Roelen DL, Claas FHJ, Heidt S. HLA-EMMA: a user-friendly tool to analyse HLA class I and class II compatibility on the amino acid level. *HLA*. 2020;96(1):43-51. doi:10.1111/tan.13883
22. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*. 1997;18(15):2714-2723. doi:10.1002/elps.1150181505
23. Everly MJ, Rebellato LM, Haisch CE, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. *Transplantation*. 2013;95(3):410-417. doi:10.1097/TP.0b013e31827d62e3
24. Kosmoliaptsis V, Gjorgjimajkoska O, Sharples LD, et al. Impact of donor mismatches at individual HLA-A, -B, -C, -DR, and -DQ loci on the development of HLA-specific antibodies in patients listed for repeat renal transplantation. *Kidney Int*. 2014;86(5):1039-1048. doi:10.1038/ki.2014.106
25. Ladowski JM, Mullins H, Romine M, et al. Eplet mismatch scores and de novo donor-specific antibody development in simultaneous pancreas-kidney transplantation. *Hum Immunol*. 2020;82:139-146. doi:10.1016/j.humimm.2020.12.009
26. Senev A, Coemans M, Lerut E, et al. Eplet mismatch load and de novo occurrence of donor-specific anti-HLA antibodies, rejection, and graft failure after kidney transplantation: an observational cohort study. *J Am Soc Nephrol*. 2020;31:2193-2204. doi:10.1681/asn.2020010019
27. Tafulo S, Malheiro J, Santos S, et al. HLA class II eplet mismatch load improves prediction of dnDSA development after living donor kidney transplantation. *Int J Immunogenet*. 2020;48:1-7. doi:10.1111/iji.12519
28. Wiebe C, Kosmoliaptsis V, Pochinco D, et al. HLA-DR/DQ molecular mismatch: a prognostic biomarker for primary alloimmunity. *Am J Transplant*. 2019;19(6):1708-1719. doi:10.1111/ajt.15177
29. Bryan CF, Chadha V, Warady BA. Donor selection in pediatric kidney transplantation using DR and DQ eplet mismatching: a new histocompatibility paradigm. *Pediatr Transplant*. 2016;20(7):926-930. doi:10.1111/petr.12762
30. Honger G, Niemann M, Schawalder L, et al. Toward defining the immunogenicity of HLA epitopes: impact of HLA class I eplets on antibody formation during pregnancy. *HLA*. 2020;96(5):589-600. doi:10.1111/tan.14054
31. Schawalder L, Honger G, Kleiser M, et al. Development of an immunogenicity score for HLA-DQ eplets: a conceptual study. *HLA*. 2021;97(1):30–43. 10.1111/tan.14110
32. Sapir-Pichhadze R, Zhang X, Ferradji A, et al. Epitopes as characterized by antibody-verified eplet mismatches determine risk of kidney transplant loss. *Kidney Int*. 2020;97(4):778-785. doi:10.1016/j.kint.2019.10.028
33. Meng T, Bezstarosti S, Singh U, et al. Site-directed mutagenesis of HLA molecules reveals the functional epitope of a human HLA-A1/A36-specific monoclonal antibody. *HLA*. 2023;101(2):138-142. doi:10.1111/tan.14895

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kramer CSM, Bezstarosti S, Franke-van Dijk MEI, et al. Antibody verification of HLA class I and class II eplets by human monoclonal HLA antibodies. *HLA*. 2024;103(1):e15345. doi:10.1111/tan.15345