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A High-Throughput Population Screening System for the Estimation of Genetic Risk for Type 1 Diabetes: An Application for the TEDDY (The Environmental Determinants of Diabetes in the Young) Study

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ABSTRACT

Background: In the TEDDY (The Environmental Determinants of Diabetes in the Young) study patient eligibility is based on the presence of some selected type 1 diabetes risk-associated human leukocyte antigen DR-DQ genotypes. A practical screening strategy was needed with efficient exclusion of ineligible patients at an early stage. Also, a simple, low-cost, and fast screening system was essential for the primary step of the risk assessment including thousands of samples.

Methods: A homogeneous genotyping system utilizing an asymmetric polymerase chain reaction (PCR) and subsequent hybridization of allele-specific probes was designed to be used as the first screening step. This assay was combined with methods further elucidating the genetic risk of type 1 diabetes to screen for high-risk individuals.

Results: The homogeneous assay platform allows the typing of hundreds of samples within one working day. The costs of the assay are minimal, and the reduction in hands-on time provides considerable improvements compared to the heterogeneous genotyping methods comprising separate PCR and hybridization steps. The primary selection criteria used in the first step proved to be efficient since the numbers of samples typed in subsequent stages were markedly reduced.

Conclusions: The presented assay system provides a practical approach to the rapid screening of thousands of samples at low cost, a general starting point for large-scale screening studies.

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INTRODUCTION

TYPE 1 DIABETES (T1D) is a complex disease where environmental factors play a role in the development of the disease in subjects carrying genetic disease susceptibility. An extensive international effort, the TEDDY (The Environmental Determinants of Diabetes in the Young) study was recently launched with the aim to explore the contribution of various environmental factors to disease development.[1] In this project newborn infants with human leukocyte antigen (HLA)-conferred susceptibility to T1D are identified in an initial screening step, and those carrying defined risk haplotypes conferring increased risk are then monitored for the emergence of autoimmunity and observed for various environmental exposures for up to 15 years. Our laboratory is performing the genetic screening for the study cohort recruitment in Finland and Germany, and for this purpose we have developed the methods and the model for the multistage genetic risk definition described here.

T1D susceptibility is defined by a number of genes with a major contribution of the HLA complex.[2,3] The primary disease determinant is the HLA-DQ molecule encoded by the *DQA1* and *DQB1* genes, but certain variants of the neighboring *DRB1* genes also influence genetic susceptibility to T1D when they occur together with particular DQ alleles.[4] Consequently, HLA-encoded disease risk is best defined by the DRB1-DQA1-DQB1 haplotype combinations.[5,6] The absolute disease risk associated with different HLA Class II genotypes ranges from 5–8% to less than 0.01%.[7] Eligibility for follow-up in the TEDDY study is defined by the presence of certain high-risk associated genotypes, which are differently defined for newborn infants recruited from the general population and for those with first-degree relatives (FDRs) affected by TID[1] (Table 1).

A simple, efficient, and reliable screening system is required whenever embarking on a project involving the typing of thousands of samples. Here we describe a screening system designed to identify those individuals with a high genetic risk for developing T1D and eligible for the TEDDY study. An efficient and cheap primary screening step based on a homogeneous typing method uses whole blood dried on a sample collection card as a sample material. The whole assay can be performed in 4 h with less than 1 h of hands-on time and produces numerical results easily interpreted and incorporated into databases. The equipment needed for this assay includes only a thermal

TABLE 1. T1D HIGH-RISK GENOTYPES AND NUMBERS OF ELIGIBLE INFANTS IDENTIFIED IN THE SAMPLE COHORT

	Finland	*Germany*
General population		
Number of screened newborns	9,608	1,341
(a) DR4-DQA1*0301-DQB1*0302[a]/DR3-DQA1*0501-DQB1*0201	183	25
(b) DR4-DQA1*0301-DQB1*0302[a]/DR4-DQA1*0301-DQB1*0302[a]	94	6
(c) DR4-DQA1*0301-DQB1*0302[a]/DR8-DQA1*0401-DQB1*0402	184	3
(d) DR3-DQA1*0501-DQB1*0201/DR3-DQA1*0501-DQB1*0201	87	23
FDRs		
Number of screened newborns	137	55
(a) DR4-DQA1*0301-DQB1*0302[a]/DR3-DQA1*0501-DQB1*0201	10	1
(b) DR4-DQA1*0301-DQB1*0302[a]/DR4-DQA1*0301-DQB1*0302	6	2
(c) DR4-DQA1*0301-DQB1*0302[a]/DR8-DQA1*0401-DQB1*0402	10	3
(d) DR3-DQA1*0501-DQB1*0201/DR3-DQA1*0501-DQB1*0201	4	2
(e) DR4-DQA1*0301-DQB1*0302[a]/DR4-DQA1*0301-DQB1*0201	0	0
(f) DR4-DQA1*0301-DQB1*0302[a]/DR1[b]-DQA1*0101-DQB1*0501	7	1
(g) DR4-DQA1*0301-DQB1*0302[a]/DR13-DQA1*0102-DQB*0604	3	2
(h) DR4-DQA1*0301-DQB1*0302/DR4-DQA1*0301-DQB1*0304	0	0
(i) DR4-DQA1*0301-DQB1*0302[a]/DR9-DQA1*0301-DQB1*0303	4	0
(j) DR3-DQA1*0501-DQB1*0201/DR9-DQA1*0301-DQB1*0303	1	0

[a]Acceptable alleles in this haplotype include both DQB1*0302 and *0304.
[b]In this DQB1*0501 haplotype, DR10 must be excluded. Only DR1 is eligible.

cycler and a fluorescence counter, instruments that are common in many laboratories; thus adoption of the system by new laboratories is straightforward and rapid.

The steps following the homogeneous, primary screening either are based on a well-known DELFIA® method (PerkinElmer Life and Analytic Sciences Wallac, Turku, Finland) or they are simple polymerase chain reaction (PCR) assays based on allele-specific amplification. Depending on the results of each of the typing steps a sample may require different kinds of additional typing as described in Figures 1 and 2.

PATIENTS AND METHODS

The general population-based birth cohort sample from Finland comprised consecutive newborn infants ($n = 9{,}608$) from three cities, Turku, Tampere, and Oulu, collected during the period from November 1, 2005 until October 30, 2006. The FDR cohort comprised samples from siblings of children with T1D ($n = 137$).

The general population-based birth cohort sample from Germany included 1,341 consecutive newborn infants from Munich (study period, September 13, 2005–October 20, 2006). The

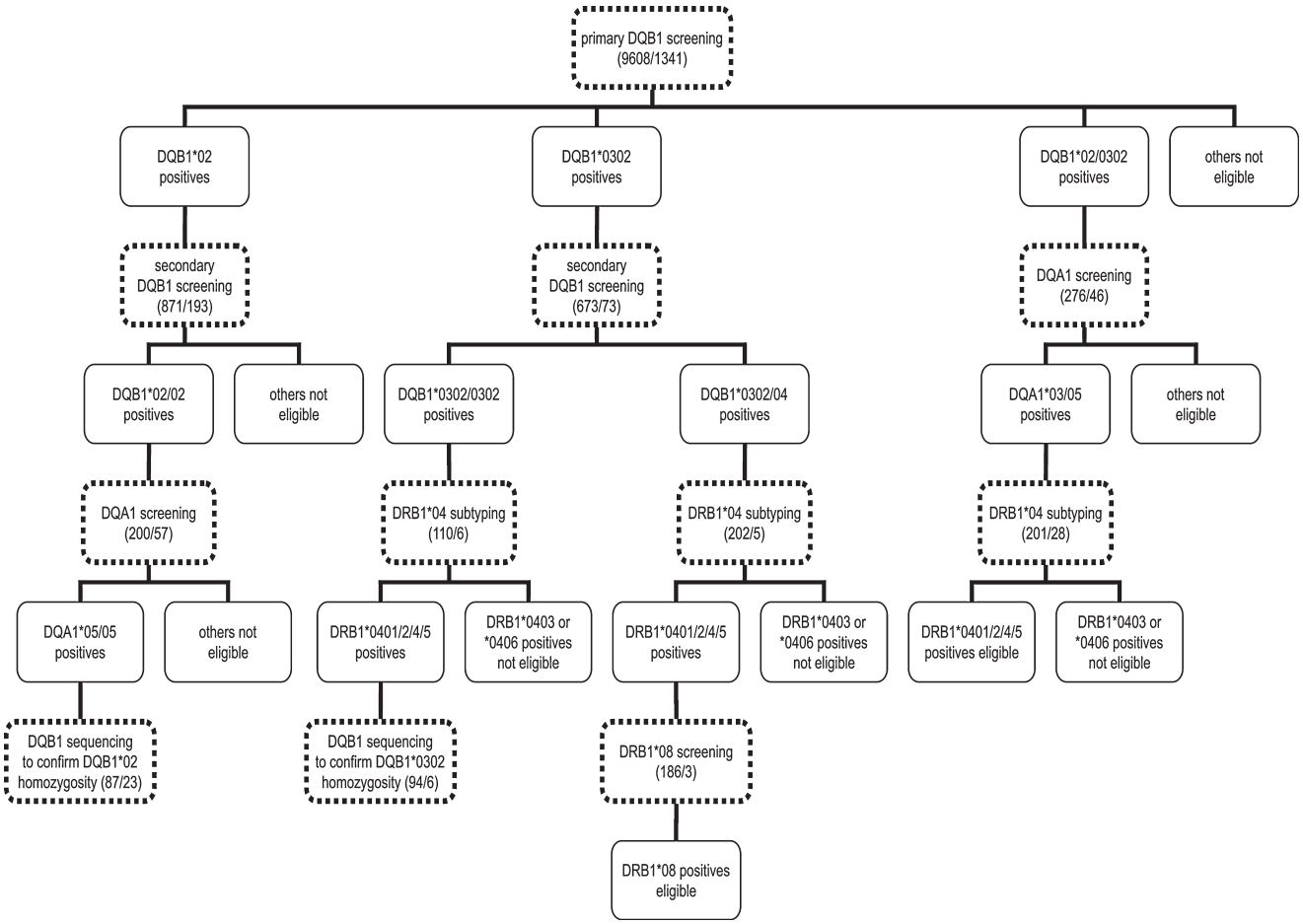

FIG. 1. Flowchart of the genotyping steps and genetic risk assessment in the general population in the TEDDY Study screening program. The assay steps are marked in boxes with dashed lines, and the results of the sample genotypes continuing in the screening program are in boxes with solid line outline. Three major risk groups are defined in the primary homogeneous assay: (1) samples positive only for DQB1*02, (2) samples positive only for DQB1*0302, and (3) samples positive for both DQB1*02 and DQB1*0302. The other genotypes are not eligible for the TEDDY study. Based on the screening results the samples are further genotyped as shown in the chart with the secondary DQB1 assay, the DQA1 screening, the DRB1*04 subtyping, and the additional DRB1 subtyping assays. A confirmatory sequencing is done of the homogeneous DQB1*02/02 and DQB1*0302/0302 genotypes to assure that no rare DQB1 alleles were left undetected. The numbers of samples typed with each assay are given in parentheses at each assay step for the Finnish and German samples, respectively.

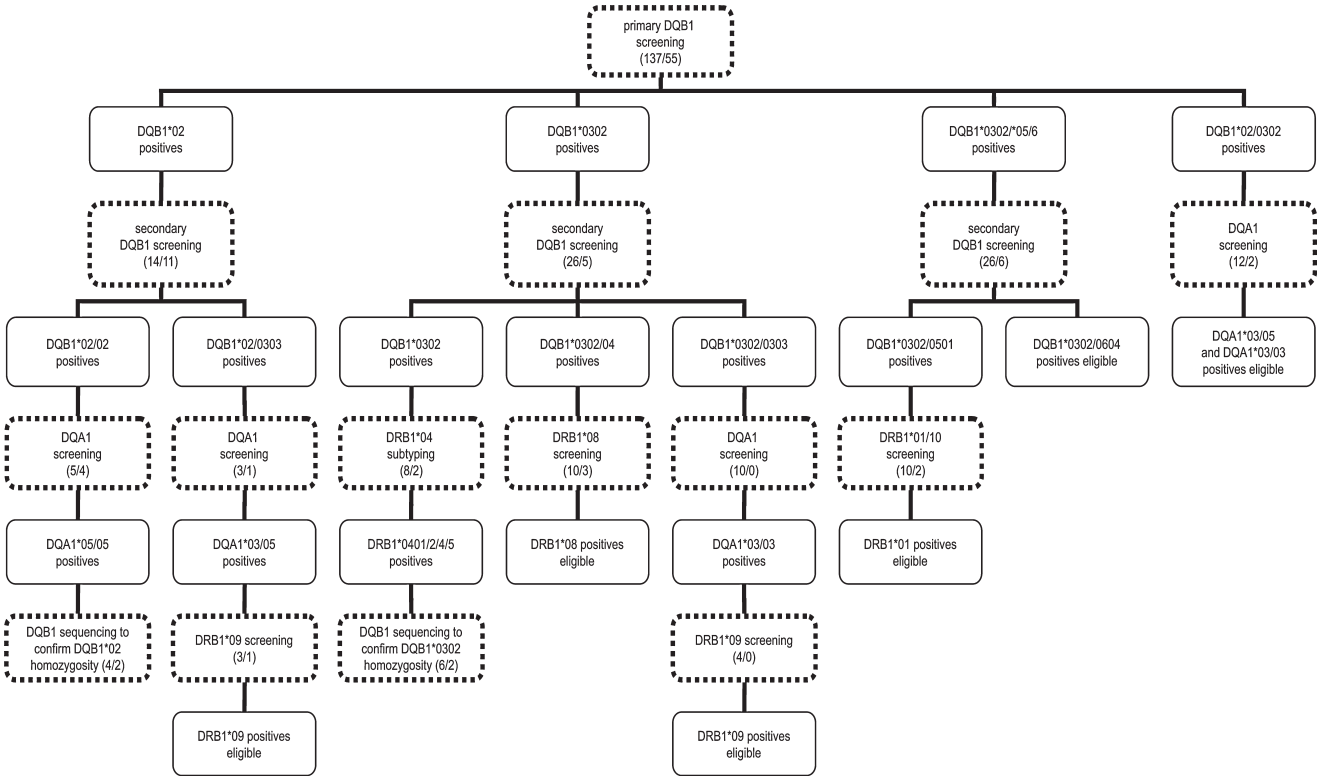

FIG. 2. Flowchart of the genotyping steps and genetic risk assessment in the FDRs in the TEDDY Study screening program. The assay steps are marked in boxes with dashed lines, and the results of the sample genotypes continuing in the screening program are in boxes with solid line outline. The samples not eligible for the study are left unmarked for clarity. Four major groups continuing in the screening program are defined in the primary homogeneous assay: (1) samples positive only for DQB1*02, (2) samples positive only for DQB1*0302, (3) samples positive for both DQB1*0302 and DQB1*05/6, and (4) samples positive for both DQB1*02 and DQB1*0302. The other genotypes are not eligible for the TEDDY study. The numbers of samples typed with each assay are given in parentheses at each assay step for the Finnish and German samples, respectively.

FDRs were recruited through the BabyDiab network in Germany ($n = 55$).[8]

Blood collected from participating newborn infants was dried on sample collection card and analyzed as described later in the homogeneous screening system for stage 1 risk assessment. Control samples with various studied alleles are used in all assays to assure correct assignment of genotypes. As an external quality control program the Centers for Disease Control and Prevention (Atlanta, GA) have provided three times Annual TEDDY Genetic Screening Proficiency Surveys consisting each of 55 samples, and these have been correctly identified at least at the demanded level of 98%.

Homogeneous HLA-DQB1 screening system for stage 1 genetic risk assessment

A homogeneous genotyping system was developed to be used in the first step of the screening. The method is based on an asymmetrical PCR and a subsequent hybridization of allele-specific probes.[9,10] It is applied in this screening program to identify the samples positive for the DQB1*02 and DQB1*0302 alleles as well as all those with DQB1*05 and DQB1*06 allele groups.

The oligonucleotides used in this study are described in Table 2. Amplifications were performed on ThermoFast® 96 plates (ABgene, Epsom, UK) and sealed with Microseal 'A' Sealing film (catalog number MSA5001, Bio-Rad, Hercules, CA). The 25-μL reaction mixture contained 1.0 U of 7T1 *Taq* polymerase (HyTest Ltd., Turku), 0.8× HyTest PCR buffer (HyTest Ltd.), 4.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphates (Fermentas Inc., Helsingborg, Sweden), 1.5 mol/L betaine (Acros Organics, Geel, Belgium), 1% dimethyl sulfoxide (Merck, Whitehouse Station, NJ), 0.6

TABLE 2. OLIGONUCLEOTIDES USED IN THIS STUDY

Assay	*Oligonucleotide*	*Sequence from 5′ to 3′ end*	*Label/position*
Primary DQB1 screening (homogeneous)	DQB1-HG 5′ primer	GGGCATGTGCTACTTCACCAACG	
	DQB1-HG 3′ primer	CCTTCTGGCTGTTCCAGTACT	
	*02 probe	AAGAGATCGTG	Tb/5′
	*02 quencher	CGCACGATCTCT	Dabcyl/3′
	*0302 probe	CCGCCTGCC	Eu/5′
	*0302 quencher	GGCAGGCGG	Dabcyl/3′
	*05/6 probe	GGGCGGCCT	Tb/5′
	*05/6 quencher	AGGCCGCCC	Dabcyl/3′
	Control probe	CGCTTCGACAG	Eu/5′
	Control quencher	CTGTCGAAGCG	Dabcyl/3′
Secondary DQB1 screening	DQB1-HG 5′ primer	GCATGTGCTACTTCACCAACG	
	DQB1-HG 3′ primer	CCTTCTGGCTGTTCCAGTACT	Biotin/5′
	*0301 probe	ACGTGGAGGTGTAC	Sm
	*0301/3 probe	CCGCCTGACGC	Tb
	*04 probe	AACGGGACCGAGC	Eu
	*04/5 probe	TGCGGGGTGTGAC	Sm
	*0501 probe	ACCGGGCAGTGAC	Sm
	*0502 probe	CCGCCTGACGC	Tb
	*0601 probe	AGGAGGACGTGC	Eu
	*0602/3 probe	GTACCGCGCGGT	Eu
	*0603/4 probe	TTGTAACCAGACACA	Sm
	Control probe	CTTCGACAGCGACG	Tb
DQA1 screening	DQA1 5′ primer	GGTAGCAGCGGTAGAGTTG	
	DQA1 3′ primer	TATGGTCTAAACTTGTACCAGT	Biotin/5′
	*0201 probe	CACAGCAACTTCCAGAC	Tb
	*03 probe	TCATGGCTGTACTG	Eu
	*05 probe	TAATCAGACTGTTCA	Sm
DRB1*04 screening		See Nejentsev et al.[11]	
DRB1*08 typing	DRB1*08 5′ primer	AGTACTCTACGGGTGAGTGTT	
	DRB1*08 3′ primer	CTGCAGTAGGTGTCCACCAG	
	Control 5′ primer (DQB1 5′ primer)	GCATGTGCTACTTCACCAACG	
	Control 3′ primer (DQB1 3′ primer)	CCTTCTGGCTGTTCCAGTACT	
DRB1*01/*10 typing	DRB1*01 5′ primer	TTGTGGCAGCTTAAGTTTGAAT	
	DRB1*10 5′ primer	CGGTTGCTGGAAAGACGCG	
	DRB1*01/*10 3′ primer	CTGCACTGTGAAGCTCTCAC	
DRB1*09/*13 typing	DRB1*09 5′ primer	GTTTCTTGAAGCAGGATAAGTTT	
	DRB1*09 3′ primer	CCCGTAGTTGTGTCTGCACAC	
	DRB1*13 5′ primer	GTTCCTGGACAGATACTTCC	
	DRB1*13 3′ primer	TCCACCGCGGCCCGCTC	

Underlined bases are locked nucleic acid.

μmol/L DQB1 5′ primer, and 0.15 μmol/L DQB1 3′ primer.

Two probe-quencher pairs were used in each reaction, and the concentrations were as follows for the *02, *0302, *05/6, and control probes and quenchers, respectively: 0.6 nmol/L and 5.3 nmol/L; 3.3 nmol/L and 16.6 nmol/L; 1.7 nmol/L and 13.3 nmol/L; and 3.3 nmol/L and 33.2 nmol/L. The *02 and *0302 probes and quenchers were combined in one reaction, and similarly the *05/6 and control probe-quencher pairs were combined in another reaction. The *05/6 probe recognizes all DQB1*05 and DQB1*06 alleles.

The sample material was EDTA-treated whole blood dried on a sample collection card. The DNA was detached from the sample card into solution, and a small aliquot of this was used as template in the actual PCR. In detail, EDTA-treated blood was applied to FTA® Classic Card (Whatman International Ltd., Maidstone, UK) sample collection cards, and 3-mm discs were cut from the dried cards into the wells of a ThermoFast 96 PCR plate. Fifty mi-

TABLE 3. PCR CONDITIONS USED IN THIS STUDY

	Screening			*Typing*		
	Secondary DQB1	*DQA1*	*DRB1*04*	*DRB1*08*	*DRB1*01/*10*	*DRB1*09/*13*
Reaction						
Volume	80 μL (110 μL)[a]	80 μL	60 μL	20 μL	20 μL	20 μL
Buffer	1 × HyTest PCR buffer	1 × HyTest PCR buffer	1 × HyTest PCR buffer	1 × HyTest PCR buffer	1 × HyTest PCR buffer	1 × HyTest PCR buffer
$MgCl_2$	3.0 mmol/L	5.0 mmol/L	3.0 mmol/L	4.0 mmol/L	4.0 mmol/L	4.0 mmol/L
dNTPs	0.2 mmol/L	0.2 mmol/L	0.2 mmol/L	0.2 mmol/L	0.2 mmol/L	0.2 mmol/L
Betaine	1.5 mmol/L	1.5 mmol/L	1.5 mmol/L	1.5 mmol/L	1.5 mmol/L	1.5 mmol/L
BSA	—	0.1 g/L	—	—	—	—
Polymerase	HyTest 7T1 0.015 U/μL	HyTest 7T1 0.019 U/μL	HyTest 7T1 0.033 U/μL	HyTest 7T1 0.025 U/μL	HyTest 7T1 0.025 U/μL	HyTest 7T1 0.025 U/μL
Primers	0.2 μmol/L	0.2 μmol/L	0.5 μmol/L	DRB1*08 primers 0.3 μmol/L; control primers 0.2 μmol/L	0.3 μmol/L	0.3 μmol/L
Thermal cycling	1) 95°C for 6 min 2) 59.5°C for 4 min 3) 0.5°C/s to 73° C 4) 73°C for 2 min 5) 95°C for 1 min 6) 59.5°C for 1 min 30 sec 7) 0.5°C/s to 73°C 8) 73°C for 2 min Repeat steps 5–8 34 times 9) 73°C for 5 min	1) 96°C for 3 min 2) 55°C for 2 min 3) 73°C for 2 min 4) 96°C for 45 s 5) 55°C for 45 s 6) 73°C for 1 min Repeat steps 4–6 39 times 7) 73°C for 2 min	1) 96°C for 4 min 2) 96°C for 20 s 3) 51°C for 30 s 4) 72°C for 30 s Repeat steps 2–4 40 times 5) 72°C for 2 min	1) 96°C for 1 min 2) 96°C for 25 s 3) 63°C or 45 s 4) 72°C for 45 s Repeat steps 2–4 4 times 5) 96°C for 25 s 6) 58°C for 50 s 7) 72°C for 45 s Repeat steps 5–7 30 times 8) 96°C for 45 s 9) 48°C for 1 min 10) 72°C for 2 min Repeat steps 8–10 4 times	1) 96°C for 1 min 2) 96°C for 25 s 3) 63°C or 45 s 4) 72°C for 45 s Repeat steps 2–4 4 times 5) 96°C for 25 s 6) 58°C for 50 s 7) 72°C for 45 s Repeat steps 5–7 30 times 8) 96°C for 45 s 9) 48°C for 1 min 10) 72°C for 2 min Repeat steps 8–10 4 times	1) 96°C for 1 min 2) 96°C for 25 s 3) 60°C or 45 s 4) 72°C for 45 s Repeat steps 2–4 4 times 5) 96°C for 25 s 6) 58°C for 50 s 7) 72°C for 45 s Repeat steps 5–7 30 times 8) 96°C for 45 s 9) 48°C for 1 min 10) 72°C for 2 min Repeat steps 8–10 3 times

BSA, bovine serum albumin; dNTPs, deoxynucleotide triphosphates.
[a]One hundred ten microliters used in typing the DQB1*0302- and *05/6-positive FDRs.

croliters of H_2O was added to each well, sealed with a Microseal 'A' Sealing film, and incubated at 100°C for 10 min. One-microliter samples were used as templates in the homogeneous PCR assay as well as in the later PCR steps of the screening program. The thermal cycling program was performed as described previously.[10]

Alternatively, Whatman ProteinSaver™ 903 cards (Whatman International Ltd.) could be used as sample material. With these sample cards a modification was required to the pretreatment protocol: before boiling in water the discs were first washed with NaOH by adding 50 μL of 10 mmol/L NaOH to the discs. The plates were briefly centrifuged, and the liquid was removed. Then 50 μL of water was added to each well, and the plate was sealed and incubated at 100°C for 10 min.

The samples that based on the results of the primary screening were classified as DQB1*02/x ($x \neq$ DQB1*05/6), DQB1*0302/x, or DQB1*02/DQB1*0302 were further analyzed with the DELFIA-based assays (Fig. 1). Among the FDR samples also those that gave a positive result with both *0302 and *05/6 probes were included in further analyses (Fig. 2).

Additional assays for stage 2–3 genetic risk assessment

Secondary DQB1 typing. A modified DELFIA assay was used to type the samples for the presence of the DQB1 alleles *0301, *0303, and *04. In brief, the sequence of interest was amplified according to the instructions previously described[12] and summarized in Table 3. The hybridization was performed using allele-specific probes for the DQB1*0301, *0303, and *04 alleles and done according to the manufacturer's instructions with minor modifications as indicated in Table 4.

For the extended secondary screening for the DQB1 alleles *0501, *0502, *0503, *0601, *0602, *0603, and *0604 applied to the FDR samples positive for the *0302 and *05/6 probes in the primary screening, only the number of probes in the hybridization was increased; other steps were similar to those described for the screening of DQB1 alleles *0301, *0303, and *04. The additional probes

TABLE 4. SECONDARY DQB1 SCREENING: HYBRIDIZATION FOR THE DQB1 ALLELES *0301, *0303, AND *04

Step number	*Procedure*
1	Add 8 μL of PCR product and 50 μL of Assay Buffer per well into a streptavidin-coated 96-well plate
2	Shake slowly for 30 min at room temperature, with the plate covered with sealer
3	Wash the plates four times with a room temperature wash solution
4	Add 150 μL of 50 m*M* NaOH per well
5	Shake slowly for 5 min at room temperature
6	Wash the plates four times with a room temperature wash solution
7	Add the probes in a 100- μL volume per well of Assay Buffer containing in final concentrations 1 *M* NaCl and 0.1% Tween: *0301 probe with samarium label 1.0 ng per well, *0301/3 probe with terbium label 1.5 ng per well, *04 probe with europium label 0.5 ng per well
8	Incubate at 37°C for 2 h without shaking, with the plate covered with sealer
9	Wash six times with washing solution heated to 46°C
10	Add 100 μL of DELFIA Enhancement Solution per well
11	Shake slowly for 25 min at room temperature
12	Measure the europium and samarium fluorescence
13	Add 25 μL of DELFIA Enhancer per well
14	Shake slowly for 5 min at room temperature
15	Measure the terbium fluorescence

TABLE 5. SECONDARY DQB1 SCREENING FOR FDRs: ADDITIONAL HYBRIDIZATION PROBES FOR DQB1 ALLELES *04/5, *0501, *0502, *0601, *0602/3, AND *0603/4

Probe	*Concentration (ng/well)*	*Alleles recognized by the probe*
*04/05-samarium	2.0	*04, *05
*0501-samarium	8.2	*0501
*0502-terbium	2.0	*0502, *0504
*0601-europium	1.0	*0601
*0602/3-europium	1.0	*0602, *0603
*0603/4-samarium	2.0	*0603, *0604

and their concentrations in the hybridization are described in Table 5.

*DQA1 screening and DRB1*04 subtyping.* DQA1 screening[13] for the DQA1 alleles *0201, *03, and *05 is in principle done as for the secondary DQB1 screening. The conditions for the amplification of the area of interest are described in Table 3, and the hybridization protocol is summarized in Table 6. The conditions for the PCR for DRB1*04 subtyping are described in Table 3, and the hybridization was done as described earlier by Nejentsev et al.[11]

DRB1 assays based on allele-specific amplification. The DRB1*08, DRB1*01/10, and DRB1 *09/13 assays were based on allele-specific PCR,[14] and the PCR conditions are summarized in Table 3. In these assays the PCR products were analyzed by agarose gel electrophoresis, and the interpretation of the results was based on the different sizes of the products originating from different DRB1 alleles in the DRB1*01/10 and DRB1*09/13 assays and on the presence or lack of a PCR product in the DRB1*08 subtyping.

RESULTS

Homogeneous screening system for stage 1 genetic risk assessment

Figure 3 shows the results of 114 samples in the primary homogeneous DQB1 screening assay. The signal-to-background ratios are calculated for each probe, and the cutoff values for all probes were set as shown in Figure 3 at 1.50 for the DQB1*02 and control probes (Fig. 3A and D) and at 1.30 for the DQB1*0302 and DQB1*05/6 probes (Fig. 3B and C). Samples that gave signal-to-background ratios higher than the cutoff were considered positive for the allele tested. As shown by the control probe all samples were amplified successfully. A sample showing an elevated signal-to-background ratio with the DQB1*05/6 probe, marked with an arrow in Figure 3C, but remaining under the cutoff value was reanalyzed and shown to be negative.

TABLE 6. HYBRIDIZATION STEPS IN THE DQA1 SCREENING

Step number	*Procedure*
1	Add 10 μL of PCR product and 50 μL of Assay Buffer per well into a streptavidin-coated 96-well plate
2–6	Same as in secondary DQB1 screening hybridization (see Table 3)
7	Add the probes in a 100-μL volume per well of Assay Buffer containing in final concentrations 1 *M* NaCl and 0.1% Tween: *0201 probe with terbium label 1.0 ng per well, *03 probe with europium label 0.5 ng per well, *05 probe with smarium label 5.0 ng per well
8–15	Same as in secondary DQB1 screening hybridization (see Table 3)

Only the deviations from the protocol used for the DQB1 secondary screening assay (Table 3) are shown.

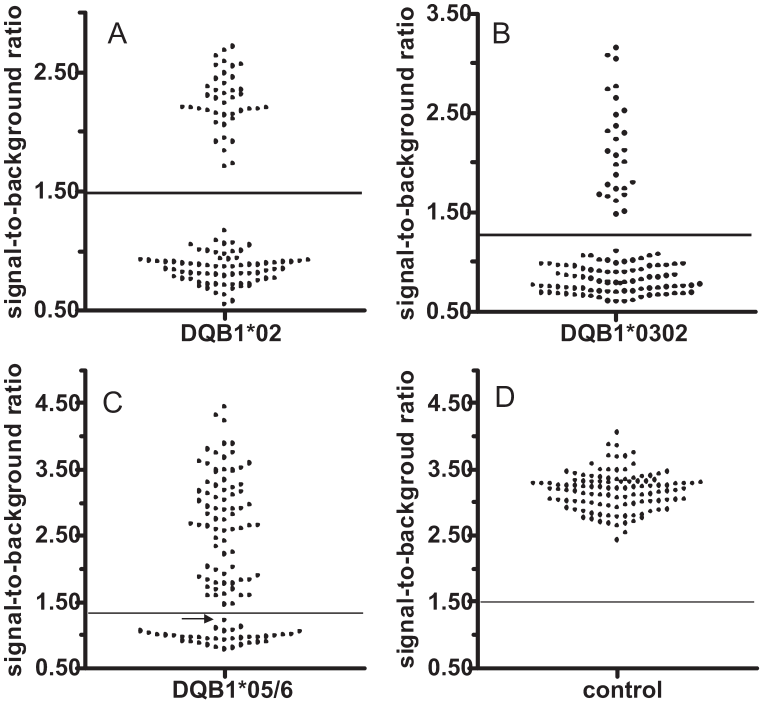

FIG. 3. Results of the preliminary homogeneous screening assay for the DQB1 alleles *02, *0302, and *05/6. (**A–D**) The signal-to-background ratios of the *02 probe, the *0302 probe, the *05/6 probe, and the control probe, respectively. The cutoff value for each probe is marked with a black horizontal line. The sample marked with an arrow in (C) was retyped and found to be negative for the DQB1 alleles *05 and *06.

Flow of the typing procedure

All samples were first analyzed using the homogeneous typing with three probes specific for HLA-DQB1*02-, DQB1*0302-, and DQB1*05/6-positive samples. The samples that were positive for either DQB1*02 or DQB1*0302 but did not give a positive result with the DQB1*05/6 probe in the primary screening were further tested for the presence of DQB1*0301, DQB1*0303, and DQB1*04 in the secondary DQB1 screening using the DELFIA method. Samples positive for DQB1*0301 and DQB1*0303 were excluded, and those with DQB1*0302/DQB1*04 and assumed homozygosity for DQB1*02 or DQB1*0302 were further studied.

DQA1 typing was performed for the DQB1*02/DQB1*0302 and DQB1*02 homozygous samples. If the DQB1*02/0302-positive samples gave a DQA1 genotype of *03/05, they were further studied for the DRB1*04 subtype.

The assumed DQB1*0302 homozygotes and samples with the DQB1*0302/DQB1*04 genotype were also typed for the DRB1*04 subtypes. The DQB1*0302/DQB1*04-positive samples were further tested for the presence of DRB1*08 if the DRB1*04 subtyping result was neither *0403 or *0406. The selection of the individuals as eligible for the TEDDY study was done as indicated in Figure 1.

More genotypes were eligible for the FDRs, and thus the procedure for their typing was more complicated. In addition to the samples that in homogeneous screening gave a positive signal with the DQB1*02 probe and/or DQB1*0302 probe without DQB1*05/6, also samples positive for both the DQB1*0302 and DQB1*05/6 probes were selected for more specific typing (Fig. 2).

After the secondary DQB1 typing also DQB1*02/DQB1*0303 heterozygotes were tested for the DQA1 alleles as well as those with DQB1*0302/DQB1*0303. DQB1*02/DQB1*0303 heterozygotes positive for DQA1*03 and DQA1*05 and those with the DQB1*0302/DQB1*0303 genotype with only DQA1*03 and none of the other tested DQA1 alleles were finally typed for the presence of DRB1*09 alleles with the DRB1*09/13 assay.

The DQB1*0302-positive samples that were also positive for DQB1*05/6 in the primary DQB1 screening were further tested for the DQB1 alleles *0501, *0502, *0503, *0601, *0602, *0603, and *0604. Of these the *0302/DQB1*0604-positives were eligible for the study without additional typing. The *0302/DQB1*0501

samples were screened for the presence of the DRB1*01 and DRB1*10 alleles. All FDR samples were classified into noneligible or eligible for the TEDDY study according to the guidelines given in Figure 2.

After all screening steps those samples that were found to be homozygous for either DQB1*02 or DQB1*0302 and eligible for the TEDDY study were sequenced for the DQB1 gene to confirm homozygosity and that no rare DQB1 alleles were left undetected.

The numbers of samples typed with each assay in all steps are shown in parentheses in the flowcharts presented in Figure 1 for the general population and in Figure 2 for the FDRs for the Finnish and German populations. The numbers of samples typed in the later stages are markedly smaller than those screened in the primary homogeneous assay for the DQB1 alleles *02, *0302, and *05/6, indicating an efficient selection by this multistage screening strategy. Only 16.5% of the general population samples and 45.8% of the FDR samples are typed in the stage 2 DQB1 step. For the DQA1 screening, DRB1*04 subtyping, and other DRB1 assays, the percentages for the general population and FDRs were 5.3% and 19.3%, 5.0% and 5.2%, and 1.7% and 15.1%, respectively. For the FDRs the percentages of samples continuing after the primary screening are to some extent higher than with the general population, as can be expected. Also, the numbers of newborn infants with different haplotypes eligible for the TEDDY study identified in the sample cohort are shown in Table 1 for the Finnish and German samples.

DISCUSSION

The HLA class II region acts as an oligogenic superlocus that defines approximately half of the genetic component of T1D. The genetic risk can accordingly be defined to a great extent by HLA typing. The problem inherent to HLA typing is the enormous polymorphism and multiplicity of the loci, and to reduce the costs of genetic risk estimation in HLA-associated diseases one can concentrate on the loci and alleles shown to be positively or negatively associated with the disorder.

Our research group has had a long-term interest in the development of high-throughput assays for screening population cohorts for recruitment of study subjects for natural history and prevention studies in type 1 diabetes. The original assay consisted of typing those DQB1 alleles found increased or decreased among T1D patients, and the procedure has thereafter been complemented by additional typing for relevant DQA1 and DRB1 alleles, which has increased its sensitivity and specificity and made it more applicable in various populations.[6]

It should be noted that it is possible to reduce substantially the number of necessary typings at some secondary loci because of the known strong linkage disequilibriums between alleles at different loci. Thus the DQB1*0302 allele is always associated with DQA1*03 and DRB1*04. Typing for various DRB1*04 alleles is, however, important because the risk of the haplotype is dependent of the exact DRB1*04 allele. DRB1*0403 and DRB1*0406 in the haplotype are strongly protective, while the other *04 alleles are associated with variable degree of risk. A contrasting scenario is present for DQB1*02, which is coding for a risk conferring molecule when combined with DQA1*05, whereas the other common haplotype combination, DQB1*02 with DQA1*0201, is coding for a neutral or protective molecule. Typing can thus be arranged in a stepwise manner where the result of one stage defines further typing. The current model addresses genetic risk assessment in a very robust and cost-efficient way.

The first step in the screening program for the TEDDY study includes a vast number of samples.[1] A simple, inexpensive, and rapid screening method is an absolute requirement for large-scale projects to be performed within a practical time frame and with reasonable costs. Here we present a screening system utilizing a homogeneous approach in the primary screening step including thousands of samples.

The specificity of the introduced homogeneous assay and specifically of the previously unpublished DQB1*05/6 probe was certified by typing a set of previously analyzed samples, and the results were completely concordant (data not shown). With the inclusion of the DQB1*05/6 probe into the primary homoge-

neous screening step the percentages of samples excluded from the study in the primary step are for the general population approximately 83.5% and for the FDRs 54.2%. With this selection of analytes in the primary screening step the numbers of samples requiring further genotyping were considerably reduced.

The homogeneous assay requires only a little hands-on time, and a single assay can be performed in only 4 h. The stability of the system allows the measurement of the signals to be performed either right after the thermal cycling or even 2 days after the cycling step, giving even more flexibility to the user. The reduction in the required hands-on time compared to the DELFIA-based assays consisting of separate steps for PCR and hybridization also translates into significant savings in the assay costs. Also, the reductions in the materials required for the assay contribute to the markedly lower costs of the homogeneous assay format when compared to DELFIA-based assays.[12,13,15] The simplicity of the assay is further increased by the use of blood dried on sample collection cards; no complicated DNA extraction steps are necessary. The pretreatment protocol of the samples enables a multitude of assays from a single sample even when the original sample material is scarce. A single blood spot 3 mm in diameter can be used for as many as 90 different assays if a 100-μL volume of H_2O is used in the final step of the pretreatment protocol and 1 μL of this used as a sample in the PCR.

After the primary DQB1 screening the DELFIA hybridization assay concept was used for additional typing except for the few DR assays where the presence of DR1, DR8, and DR9 was ensured in DQB1*0501, DQB1*04, and DQA1*03-DQB1*0303 haplotypes. These haplotypes were parts of a few eligible genotypes in family members only, and the conventional gel detection was used as it was not reasonable to establish a high-throughput method for such a small number of samples.

In summary, the presented risk assessment strategy employing a multistage screening procedure allows a high-resolution risk estimation that is critical when classifying subjects according to HLA-conferred susceptibility to T1D. In contrast to screening systems focusing only on DQB1 that give limited information,[15] our method defines genetic risk according to the best knowledge defined by previous population studies.[5,6,16] The designed system allows considerable flexibility in the sizes of sample collections under investigation; the system is as suitable for small collections with only a few samples to be genotyped as well as for large studies with thousands of samples.

Screening has here been performed in two European populations, but HLA associations between even remote populations are mainly consistent although disease-associated haplotypes are different, e.g., between the Oriental- and European-derived populations, because of the difference in haplotypes present in general populations.[17] The screening strategy can easily be altered to suit the risk haplotypes of other populations by changing the probes in the different stages of the screening. Importantly, modern technology enables reduction in assay time and costs, which is critical in high-throughput population screening studies.[10,18]

APPENDIX: THE TEDDY STUDY GROUP

Committees

[1]Ancillary Studies, [2]Diet, [3]Genetics, [4]Human Subjects/Publicity/Publications, [5]Immune Markers, [6]Infectious Agents, [7]Laboratory Implementation, [8]Maternal Studies, [9]Psychosocial, [10]Quality Assurance, [11]Steering, and [12]Study Coordinators.

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