

## HLA-DQA1 Hybridization Assay

### DELFLIA®

### Diabetes Reagents

This assay is intended for scientific research use only. Not for use in diagnostic procedures.

This assay should only be used by investigators certified by Wallac Oy.

These instructions for use apply to the following reagents:

<u>Product number</u>	<u>Description</u>	<u>DQA1 alleles recognized by the probe</u>
<b>AD0133</b>	Eu-DQA1 Control probe	all alleles
<b>AD0134</b>	Tb-DQA1*0201 probe	*0201
<b>AD0135</b>	Eu-DQA1*03 probe	*03011, *0302-03
<b>AD0136</b>	Sm-DQA1*05 probe	*05011-12, *0502-05
		<u>Complementarity to DQA1-probes</u>
<b>AD0138</b>	Biotinylated Hybridization control	Complementary to AD0133 - AD0136 probes

## INTRODUCTION

This set of three sequence-specific oligonucleotide probes contains reagents to define the presence of HLA-DQA1\*0201, 03 and 05 alleles. The additional alleles recognized by these probes are presented above. A consensus sequence-specific probe (Eu-DQA1 Control), is included in the set to control the success of the PCR<sup>1</sup> (polymerase chain reaction) amplification. The biotinylated hybridization control containing the complementary sequences of all probes can be used as an artificial positive sample to control the success of the hybridization reaction.

The assay is based on DNA amplification of HLA-DQA1 alleles and lanthanide(III) chelate labeled DNA probes specific for the alleles described above. Analysis of the amplified DNA is performed by collecting the biotin labeled DNA fragments onto streptavidin-coated

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<sup>1</sup> Since the PCR process is covered by patents owned by Hoffman-LaRoche, Inc., the process shall not be used without a license. The Buyer shall sign an undertaking stating that they have the license and agree to indemnify and hold the Seller harmless in case of any possible actions, suits or proceedings based on a claim of unauthorized use of the process.

microtitration wells, denaturing the collected fragments, and hybridizing them with lanthanide(III) chelate labeled probes (1).

Due to their unique properties, lanthanide(III) chelates provide a sensitive label technology. The DELFIA technology utilizes efficient labeling of biomolecules with lanthanide(III) chelates and highly sensitive time-resolved fluorometric detection after a dissociative fluorescence enhancement (2,3). The oligonucleotide probes are labeled with Eu(III) / Sm(III) / Tb(III) chelate of 2,2',2'',2'''-{{4-[2-(4-isothiocyanatophenyl)ethyl]pyridine-2,6-diyl}bis(methylenenitrilo)}tetrakis(acetate). The labeled oligonucleotides as such are non-fluorescent, but after hybridization, the lanthanides are dissociated to form highly fluorescent Eu(III), Sm(III) and Tb(III) chelates in DELFIA Enhancement Solution and DELFIA Enhancer.

The analysis with these probes requires a DNA amplification step and the amplification product should be biotin-labeled in order to collect the product onto streptavidin-coated wells. The amplification of the DQA1 alleles can be carried out by PCR using the following primers, 5' primer: Bio-TAT GGT GTA AAC TTG TAC CAGT, 3' primer: GGT AGC AGC GGT AGA GTT G. The reactions can be performed on PCR plates in a reaction volume of 50 µL using a blood spot or isolated genomic DNA as template. The optimal performance of PCR is reached with heat-treated blood spots. The PCR parameters should be established in each individual laboratory.

## VIAL CONTENT

Labeled reagents are supplied as ready-for-use solutions in 20 mmol/L Tris-HCl buffered 50 mmol/L sodium chloride (NaCl). One probe vial contains 10 µg of labeled oligonucleotide probe, in a concentration of 10 µg/mL, and a control vial 100 µL of biotinylated control.

## STORAGE

Store labeled oligonucleotide as such at -20°C. We recommend aliquoting the oligonucleotides for storage. Do not store labeled oligonucleotides in DELFIA Hybridization Buffer, phosphate buffer or any other buffer that contains chelating agents.

## MATERIALS REQUIRED BUT NOT SUPPLIED WITH THE PRODUCT

The DELFIA system requires the following items, which are available from PerkinElmer Life Sciences or its distributors.

1. Time-resolved fluorometer -1420 VICTOR™ Multilabel Counter<sup>2</sup>
2. Automatic shaker - DELFIA Plateshake (prod. no. 1296-001/002 or 1296-003/004)
3. Automatic washer - DELFIA Platewash (prod. no. 1296-026)

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<sup>2</sup> VICTOR is a trademark of Wallac Oy.

4. DELFIA system reagents:
  - DELFIA Streptavidin Microtitration Strips (prod. no. 4009-0010, 10 plates/package)
  - DELFIA Hybridization Buffer (prod. no. 4006-0010)
  - DELFIA Wash Concentrate (prod. no. 1244-114)
  - DELFIA Enhancement Solution (prod. no. 1244-105)
  - DELFIA Enhancer (prod. no. C500-100)
5. Pipette for dispensing DELFIA Enhancement Solution and the DELFIA Enhancer - Eppendorf Multipipette (prod. no. 1296-014) with 5 mL Combitips (prod. no. 1296-016) and 2.5 mL Combitips (prod. no. 1296-017), or alternatively DELFIA Plate Dispense (prod. no. 1296-041) (only for DELFIA Enhancement Solution).

In addition to the DELFIA system the following are required:

- reagents and equipment to perform DQA1-gene amplification
- +31°C incubator for incubating plates
- +39°C incubator for warming up the wash solution
- precision pipettes
- denaturation solution: 20 mmol/L NaOH
- distilled water
- adhesive tape or other cover for plates to avoid contamination by PCR products during collection and evaporation of hybridization solutions during incubation at + 31°C

## **WARNINGS AND PRECAUTIONS**

This assay is intended for scientific research use only. Not for use in diagnostic procedures.

Disposal of all waste should be in accordance with local regulations.

## **DQA1-TRF-HYBRIDIZATION PROTOCOL**

### **1. Collection of the DNA amplification product onto streptavidin coated wells:**

- 1.1. Pipette 10 µL of distilled water into four streptavidin-coated microtitration wells for controlling the hybridization background (e.g. to the first four wells of the plate, since hybridization is done in duplicates with two hybridization solutions).
- 1.2. Pipette 10 µL of diluted (1:100 in hybridization buffer) hybridization control into four wells (hybridized with two hybridization solutions in duplicate) for controlling the success of the hybridization reaction.
- 1.3. Pipette 10 µL of each amplification reaction product into four wells (each sample is hybridized with two hybridization solutions in duplicates). We recommend the use of PCR background sample (no template in PCR) in every PCR run to control the possible contamination. This contamination control should be treated as a normal sample in the hybridization assay.
- 1.4. Add 50 µL of DELFIA Hybridization Buffer into each well.

- 1.5. Seal the plate with an adhesive tape.
- 1.6. Incubate the plate for 30 minutes at room temperature (+20 - +25°C) using the DELFIA Plateshake (slow shaking).

## 2. Preparation of hybridization solutions

Each sample should be hybridized with two hybridization solutions (A and B) in duplicate. Prepare the hybridization solutions A and B as stated below. Please note that the hybridization solutions should be used as soon as possible (within 30 minutes even when stored on ice).

- 2.1. Add the correct volume of each probe to the hybridization buffer to prepare the respective hybridization solutions (A and B).

Hybridization solutions for one plate (96 wells):

Product no.	Description	A	B
4006-0010	Hybridization buffer	6 mL	6 mL
AD0133	Eu-DQA1 Control probe	30 ng	-
AD0134	Tb-DQA1*0201 probe	60 ng	-
AD0135	Eu-DQA1*03 probe		30 ng
AD0136	Sm-DQA1*05 probe		180 ng

(The volumes can be adjusted if the number of plates / strips is altered.)

## 3. Denaturation of double stranded DNA

- 3.1. Prepare wash solution by diluting DELFIA Wash Concentrate 25-fold with distilled water. Wash solution remains stable for 2 weeks at +2 - +25°C in a sealed container.
- 3.2. Wash the plate 3 times with wash solution.
- 3.3. Add 150 µL of 20 mmol/L NaOH into each well.
- 3.4. Shake the plate slowly on the DELFIA Plateshake for 5 minutes at room temperature.
- 3.5. Wash the plate 3 times with wash solution.

#### 4. Hybridization

- 4.1. Add 100 µL of the respective hybridization solutions (A or B; prepared as stated in section 2 above) into two wells of each sample on the plate (A into wells 1 and 2, B into wells 3 and 4, etc.; see below).

1	2	3	4	5	6	7	8	9	10	11	12	Strip
Bg A	Bg A	Bg B	Bg B	Ctrl A	Ctrl A	Ctrl B	Ctrl B	PCR A	PCR A	PCR B	PCR B	A
S1 A	S1 A	S1 B	S1 B	S2 A	S2 A	S2 B	S2 B	S3 A	etc.			B
Bg for hybridization background, Ctrl for hybridization control, PCR for PCR background sample, S1, S2, etc. for unknown PCR samples												C etc.

- 4.2. Seal the plate with an adhesive tape.

- 4.3. Incubate for 2 hours ( $\pm$  10 minutes) at  $+31^{\circ}\text{C}$  ( $\pm$   $1^{\circ}\text{C}$ ).

#### 5. Stringent washes

- 5.1. Wash the plate 6 times with wash solution preheated to  $+39^{\circ}\text{C}$  ( $\pm$   $1^{\circ}\text{C}$ ).

#### 6. Enhancement

- 6.1. Add 200 µL of DELFIA Enhancement Solution into each well.

- 6.2. Incubate the plate for 30 minutes at room temperature using the DELFIA Plateshake (slow shaking).

- 6.3. Measure Eu / Sm fluorescence with a time-resolved fluorometer. Select Diabetes DQA1EuSm MultiCalc<sup>® 3</sup> program for automatic measurement and result calculation or calculate the results manually.

- 6.4. Add 50 µL of DELFIA Enhancer into each well.

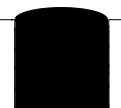
- 6.5. Shake the plate slowly on the DELFIA Plateshake for 5 minutes.

- 6.6. Measure Tb fluorescence with a time-resolved fluorometer. Select Diabetes DQA1Tb MultiCalc program for automatic measurement and result calculation or calculate the results manually.

<sup>3</sup> MultiCalc is a registered trademark of Wallac Oy.

## PROCEDURAL NOTES

1. The labeled probes are stable under normal hybridization conditions. For prolonged storage at  $-20^{\circ}\text{C}$  we recommend aliquoting the probes and control. Avoid unnecessary storage at room temperature.
2. Avoid exposure of lanthanide labeled probe to pH lower than 7 and EDTA concentration higher than  $200\ \mu\text{mol/L}$ .
3. When washing the wells, ensure that each well is filled up completely to the top edge as shown in the figure. After washing the strips, check that the wells are dry.



Pay special attention to rinsing the washing device thoroughly with distilled water after the hybridization washes have been completed. For detailed information on the cleaning and maintenance of the washing device, please refer to the DELFIA Platewash manual.

4. The avoidance of lanthanide contamination and resulting high fluorescent background demands high standard pipetting and washing techniques. Avoid contaminating pipettes with Eu / Sm / Tb-labeled reagents.
5. The DELFIA Enhancement Solution should be dispensed using only the recommended Eppendorf Multipette after the Combitip has first been flushed with Enhancement Solution according to the instructions for use. The same Combitip must not be used for pipetting any other reagent.

When using the DELFIA Plate Dispense, please refer to the manual.

## EXPECTED VALUES AND INTERPRETATION OF RESULTS

Before interpretation of the results the S/N ratios (signal-to-noise; specific signal divided by the hybridization background signal) of all samples should be calculated. Samples with known HLA-DQA1 genotypes assayed according to the protocol described in this insert are shown in the tables below.

The S/N ratio of the control probe reflects the amplification level. Generally, it can thus be said that samples with S/N ratios over 100 have been satisfactorily amplified. This also allows equalization of amplification levels, which is done by comparing the S/N ratio of each allele-specific probe to the S/N ratio of the corresponding control probe (reference value = 100 %). The results are expressed as percentages (Table 2). More information is thus obtained about probe-specific variation typical for positive samples facilitating the discrimination into the two categories (positive or negative).

The recommended intra-assay variation for a positive sample signal is  $< 15\%$ . When the signals of duplicate samples (positive sample) differ from each other more than  $25\%$ , we recommend repeating the hybridization assay.

Table 1. Time-resolved fluorescence signals and corresponding signal-to-noise ratios (S/N) obtained in a study performed at Wallac Oy. Positive results are **in bold**.

Sample no	Genotype	Eu-DQA1 Control		Tb-DQA1*0201		Eu-DQA1*03		Sm-DQA1*05	
		signal	S/N	signal	S/N	signal	S/N	signal	S/N
Bg		812	1	922	1	1939	1	322	1
Hybr. ctrl		1508477	<b>1859</b>	1470979	<b>1596</b>	1814408	<b>936</b>	58341	<b>181</b>
1	<b>02</b>	1637909	<b>2018</b>	108684	<b>118</b>	4691	2	291	1
2	<b>02</b>	1330611	<b>1640</b>	113344	<b>123</b>	2439	1	287	1
3	<b>02</b>	1976300	<b>2435</b>	190852	<b>207</b>	84983	44	817	3
4	<b>03</b>	1540333	<b>1898</b>	1693	2	1438643	<b>742</b>	335	1
5	<b>03</b>	1488879	<b>1835</b>	1831	2	1570155	<b>810</b>	308	1
6	<b>03</b>	1710866	<b>2108</b>	1842	2	1631781	<b>842</b>	387	1
7	<b>05</b>	2370315	<b>2921</b>	1358	1	11171	6	15638	<b>49</b>
8	<b>05</b>	2376281	<b>2928</b>	1165	1	14951	8	15027	<b>47</b>
9	<b>02,03</b>	1258530	<b>1551</b>	243519	<b>264</b>	1748815	<b>902</b>	394	1
10	<b>02,03</b>	1360220	<b>1676</b>	293089	<b>318</b>	1829193	<b>944</b>	244	1
11	<b>02,03</b>	1488295	<b>1834</b>	335665	<b>364</b>	1800621	<b>929</b>	421	1
12	<b>02,05</b>	2471464	<b>3046</b>	111258	<b>121</b>	7333	4	13558	<b>42</b>
13	<b>02,05</b>	2437409	<b>3004</b>	68415	<b>74</b>	4906	3	12925	<b>40</b>
14	<b>02,05</b>	2895357	<b>3568</b>	102609	<b>111</b>	34049	18	22325	<b>69</b>
15	<b>03,05</b>	2346036	<b>2891</b>	2814	3	1159952	<b>598</b>	12363	<b>38</b>
16	<b>03,05</b>	2451927	<b>3021</b>	2969	3	1463092	<b>755</b>	13764	<b>43</b>
17	<b>03,05</b>	2437638	<b>3004</b>	2278	2	1560849	<b>805</b>	13024	<b>40</b>

Table 2. The results of Table 1 expressed as percentages of reference (control probe). Positive results are **in bold**.

Sample no	Genotype	% of reference S/N ratio			
		Eu-ctrl	0201-Tb	03-Eu	05-Sm
Hybr. ctrl		<b>100</b>	<b>86</b>	<b>50</b>	<b>10</b>
1	<b>02</b>	<b>100</b>	<b>6</b>	0	0
2	<b>02</b>	<b>100</b>	<b>8</b>	0	0
3	<b>02</b>	<b>100</b>	<b>9</b>	2	0
4	<b>03</b>	<b>100</b>	0	<b>39</b>	0
5	<b>03</b>	<b>100</b>	0	<b>44</b>	0
6	<b>03</b>	<b>100</b>	0	<b>40</b>	0
7	<b>05</b>	<b>100</b>	0	0	<b>2</b>
8	<b>05</b>	<b>100</b>	0	0	<b>2</b>
9	<b>02,03</b>	<b>100</b>	<b>17</b>	<b>58</b>	0
10	<b>02,03</b>	<b>100</b>	<b>19</b>	<b>56</b>	0
11	<b>02,03</b>	<b>100</b>	<b>20</b>	<b>51</b>	0
12	<b>02,05</b>	<b>100</b>	<b>4</b>	0	<b>1</b>
13	<b>02,05</b>	<b>100</b>	<b>2</b>	0	<b>1</b>
14	<b>02,05</b>	<b>100</b>	<b>3</b>	0	<b>2</b>
15	<b>03,05</b>	<b>100</b>	0	<b>21</b>	<b>1</b>
16	<b>03,05</b>	<b>100</b>	0	<b>25</b>	<b>1</b>
17	<b>03,05</b>	<b>100</b>	0	<b>27</b>	<b>1</b>

## WARRANTY

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All information supplied with the product and technical assistance given is believed to be accurate, but it remains the responsibility of the investigator to confirm all technical aspects of the application. We appreciate receiving any additions, corrections, or updates to information supplied to the customer.

## REFERENCES

1. Sjöroos, M., Ilonen, J., Reijonen, H., Lövgren, T. (1998): Time-resolved fluorometry based sandwich hybridisation assay for HLA-DQA1 typing. *Disease Markers* **14**, 9-19.
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3. Hemmilä, I., Dakubu, S., Mikkala, V.-M., Siitari, H. and Lövgren, T. (1984): Europium as a label in time-resolved immunofluorometric assays. *Anal. Biochem.* **137**, 335-343.

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