

# Instructions for use

## HISTO SPOT<sup>®</sup> SSO Kits

Test kits for tissue typing of HLA alleles on a molecular genetic basis

IVD

CE 0123

<b>REF</b>	<b>726010: HISTO SPOT<sup>®</sup> A 4D (96 tests)</b>
<b>REF</b>	<b>726020: HISTO SPOT<sup>®</sup> B 4D (96 tests)</b>
<b>REF</b>	<b>726030: HISTO SPOT<sup>®</sup> C 4D (96 tests)</b>
<b>REF</b>	<b>726040: HISTO SPOT<sup>®</sup> DRB1 4D (96 tests)</b>
<b>REF</b>	<b>726045: HISTO SPOT<sup>®</sup> DRB3/4/5 (24 tests)</b>
<b>REF</b>	<b>726050: HISTO SPOT<sup>®</sup> DQB1 4D (96 tests)</b>
<b>REF</b>	<b>726060: HISTO SPOT<sup>®</sup> DPB1 (96 tests)</b>
<b>REF</b>	<b>726061: HISTO SPOT<sup>®</sup> DPB1 (24 tests)</b>
<b>REF</b>	<b>726098: HISTO SPOT<sup>®</sup> Reagent Kit</b>

**Version: 9 / 2013**

Changes to version 8 / 2013

highlighted in yellow.

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## 1. PRODUCT DESCRIPTION

The HISTO SPOT<sup>®</sup> SSO system is an in vitro diagnostic test for tissue typing of HLA alleles on a molecular genetic basis and provides low to medium resolution typing results. It consists of the HISTO SPOT<sup>®</sup> typing kits, the HISTO SPOT<sup>®</sup> reagent kit, the MR.SPOT<sup>®</sup> processor and the HISTO MATCH interpretation software.

The HISTO SPOT<sup>®</sup> typing kits contain all components required for the PCR reaction and testwells with immobilized sequence-specific oligonucleotide probes for the detection of the PCR products. The HISTO SPOT<sup>®</sup> reagent kit contains the reagents for the hybridisation and detection and can be used in combination with all HISTO SPOT<sup>®</sup> typing kits. The MR.SPOT<sup>®</sup> processor is specifically designed to be used with the HISTO SPOT<sup>®</sup> kits in order to process between 1 and 96 samples, automating the process from hybridisation, detection through to result interpretation. The HISTO MATCH software is required to interpret the results.

## 2. TEST PRINCIPLE

The test includes four basic steps:

- DNA isolation
- PCR amplification
- hybridisation and detection
- data interpretation

DNA isolation is performed on the clinical sample, using the DNA isolation method established in the laboratory or using commercial kits. Then the DNA is amplified in a locus specific PCR reaction using the mastermix and the MgCl<sub>2</sub> solution provided with the kit. The specificity of the amplification is governed by a set of biotinylated primers that have been designed to uniquely amplify the chosen HLA locus. After the PCR amplification process, the PCR plate containing biotin labelled amplicon is transferred to the MR.SPOT<sup>®</sup> processor. MR.SPOT<sup>®</sup> adds hybridisation buffer to each well and then transfers each amplicon plus hybridisation buffer to a test well containing an array of immobilized sequence-specific oligonucleotide (SSO) probes. These probes are either single oligonucleotide probes or a combination of 2 or more individual probes, immobilised in the same spot (Mosaic Probes) which have been designed to improve the identification of cis located polymorphisms.

The biotin labelled amplicon binds to those SSO probes that contain a complementary target sequence and can then be detected by a colourimetric reaction. In order to prevent unspecific binding of the amplicon on the surface of the test wells MR.SPOT<sup>®</sup> has blocked the wells with blocking buffer before transferring the amplicon.

After a stringent wash step to remove all unbound amplicon a streptavidin-alkaline phosphatase conjugate is added to the wells and binds to the biotin labelled amplicon captured by the SSO probe. After further wash steps, BCIP/NBT substrate is added which produces a blue-purple colour when converted by the alkaline phosphatase. The resulting coloured dots in the bottom of each test well are photographed by MR.SPOT<sup>®</sup> and the image is transferred into the HISTO MATCH software installed on the PC of the user. The image analysis program of the HISTO MATCH software determines the intensity of each spot in the array and compares it to the intensity of the background. From this data the positive and negative reactions are calculated. The pattern matching program of the HISTO MATCH software determines the HLA type of the sample based on the specific hybridisation pattern.

### 3. MATERIAL

#### 3.1 Reagents provided with the locus specific HISTO SPOT® typing kits

**Testwells I X**      **Testwells**, individually packed, each strip containing 8 tests, contains immobilized, sequence-specific oligonucleotide probes

**Mastermix I X**      **Mastermix**, ready to use, contains biotinylated primers for the chosen locus, dNTPs, Taq polymerase, reaction buffer, 0.05% sodium azide

**MgCl<sub>2</sub>**              **Magnesium Chloride**, 6 mM, ready to use, contains 0.001 % Proclin® 300

**X = A, B, C, DRB1, DRB3/4/5, DQB1 or DPB1**

With each kit there is a CD containing the batch file that has to be stored within the database of the HISTO MATCH interpretation software (for details see: Instructions for use for HISTO MATCH). For each kit there are lots and batches:

- **Kit:** e.g. **HISTO SPOT® A**, defines the locus tested
- **Lot:** e.g. **A084, A085**, defines the layout and specificity of the probes that are contained in the kit. A single lot can contain many different batches.
- **Batch:** e.g. **A085-1, A085-2, A085-3**, defines how a probe reacts in comparison to the control probes (cut off values), and defines the manufacture and expiry date of the strips. **Single probes may be switched off in some batches within the same lot. This information can be found on the lot-specific CD supplied with the kits (Hit Tables – Excel file).**

#### 3.2 Reagents provided with the HISTO SPOT® Reagent kit

The reagents contained in one kit are sufficient for 96 tests. Each reagent set contains:

**BLOCKBUF**          **Blocking Buffer**, ready to use, contains 0.001% Proclin® 150I

**HYBBUF**            **Hybridisation Buffer**, ready to use, contains 0.001% dye, 0.1% sodium dodecyl sulphate, 0.001% Proclin® 150

**STRGWASH**        **Stringent Wash Buffer**, ready to use, contains 0.001% dye, 0.1% sodium dodecyl sulphate, 0.001% Proclin® 150

**TBSWASH**         **TBS Wash Buffer** (Tris Buffered Saline), ready to use, contains 20 mM Tris, 0.003% dyes, 0.001% Proclin® 150

**SUBS**                **BCIP® / NBT Substrate**, ready to use, (5-bromo-4-chloro-3-indolyl phosphate / nitroblue tetrazolium chloride)

**CONJ**                **Conjugate**, Streptavidin Alkaline Phosphatase, concentrate, contains < 0.1% sodium azide (to be diluted 1:1666 in Blocking Buffer)

#### 3.3 Reagents and equipment required but not provided

- MR.SPOT® processor, including HISTO MATCH software, [REF] 726100
- Pipette tips for the MR.SPOT® processor, 1000 µl [REF] 726099 and 200 µl [REF] 726097
- DNA extraction reagents (no salting out method)
- Skirted PCR plates with lids or adhesive film  
(HISTO SPOT® PCR Frameplates, [REF] 726220, HISTO SPOT® PCR Caps, [REF] 726090, HISTO SPOT® PCR Foils, [REF] 726089)
- Thermal cycler

- Deionized water
- Variable pipettes (range 0,5 – 1000 µl) and disposable tips

#### 4. STORAGE AND STABILITY

All reagents and components of the kits should be stored at 2...8°C. The expiry date is indicated on the label of each reagent and is valid for the originally sealed reagents. The expiry date indicated on the outer box label refers to the reagent with the shortest stability contained in the kit. Individual strips of 8 wells may be opened, the required number of wells for a test run can be snapped off and the unused wells returned to the opened foil bag and stored for future use with the kit. Test wells stored in open foil pouches should be used within 30 days of being opened. The other opened reagents should be used within 3 months. The conjugate dilution must always be prepared afresh for each test run.

#### 5. TEST PROCEDURE

##### 5.1 Safety conditions and special remarks

Molecular genetic techniques are particularly sensitive methods and should be performed by well trained personnel, experienced in molecular genetic techniques and histocompatibility testing. The results from these tests must not be used as the sole determinant for making clinical decisions. Transplantation guidelines as well as EFI standards should be followed in order to minimize the risk of false typings, in the particular case of discrepancies in serological and molecular genetic methods.

Special safety conditions must be noted in order to avoid contamination and thus false reactions:

- ◆ Wear gloves during work (powder-free, if possible).
- ◆ Use new tips with each pipeting step (with integrated filter).
- ◆ Use separate working areas for pre-amplification (DNA isolation and preparation of the reactions) and post-amplification (hybridisation and detection). Preferably use two separate rooms.
- ◆ Amplicon should not be taken back into PCR set up area.
- ◆ Use devices and other materials only at the respective places and do not exchange them.

##### 5.2 DNA isolation

Prepare sample DNA by the laboratory standard method for DNA isolation for use in PCR (preferably no salting out method).

##### Validated DNA extraction methods:

- Qiagen columns

##### Methods successfully tested in the field:

- EZ-1 / Geno M6 (Qiagen beads)
- Promega Maxwell 16
- QuatroProbe (BeeRobotics)

The presence of heparin potentially inhibits PCR. Therefore, EDTA or Citrate Blood is recommended for typing. The sample DNA should have a concentration of 15-30 ng/µl.

The purity indexes should be the following:

- extinction ratio  $OD_{260}/OD_{280}$ : > 1.5 and < 2.0  
Higher values indicate the existence of RNA, lower values mean contamination with protein.
- extinction ratio  $OD_{260}/OD_{230}$ : > 1.8  
Lower values indicate a possible contamination with carbohydrates, salts or organic solvents.

### 5.3 Amplification

Use skirted PCR plates for the amplification, because they have to be held down at the skirt by a clamp in the MR.SPOT<sup>®</sup> processor afterwards. HISTO SPOT<sup>®</sup> PCR Frameplates have been validated for this application, plates from other suppliers have to be validated by the user. For each sample to be amplified add the following reagents to each PCR tube:

- 10  $\mu$ l Mastermix
- 5  $\mu$ l  $MgCl_2$
- 5  $\mu$ l Sample DNA (15-30 ng/ $\mu$ l)

Total volume for each amplification reaction is 20  $\mu$ l.

<u>Premix for multiple samples:</u>	no. of samples+2	x 10 $\mu$ l Mastermix	} use 15 $\mu$ l premix per sample
	no. of samples+2	x 5 $\mu$ l $MgCl_2$	

**Note:** It is important that the DNA concentration is in the range between 15 and 30 ng/ $\mu$ l. Higher concentrations may result in false-positive probe reactions and lower concentrations may cause amplification failures.

If a **negative control** should be done, prepare one PCR reaction with distilled water instead of sample DNA.

Seal the amplification tubes with lids or adhesive film and shortly spin down the liquid. Place in the thermal cycler and amplify under the following conditions:

Programme-Step	Time	Temperature	No. of Cycles
First Denaturation	2 Min	96°C	1 Cycle
Denaturation	15 Sec	96°C	10 Cycles
Annealing + Extension	60 Sec	65°C	
Denaturation	10 Sec	96°C	20 Cycles
Annealing	50 Sec	61°C	
Extension	30 Sec	72°C	
Hold	$\infty$	22°C	

The conditions are the same for all thermal cyclers however the overall time required for this step will vary according to the ramping speed of the specific thermal cycler.

The following thermal cycler models have been validated with HISTO SPOT SSO:

Applied Biosystems: PE 9600, PE 9700 (use ramp rate of PE 9600), Veriti<sup>™</sup>

Biorad: PTC 100 / PTC 200, Mycycler

Eppendorf: Mastercycler EP Gradient S

If other thermal cyclers are used, the validation has to be done by the user. It is generally recommended to use a ramp rate of 1-2°C/sec.

Once the amplification step is complete, the samples may be tested immediately or stored at 2...8°C for up to 5 days.

It is not necessary to make a gel to control the amplification. It is also not always helpful because assay results may be good although there was only a very faint band visible on the gel.

If a gel should be done anyway, you should not take more than 2-3 µl of the amplicon to do this. The amplicon sizes for the different kits are given on the information CDs that can be found in every kit (Hit Table in Excel format: Second sheet "Notes").

## **5.4 Automated hybridisation assay on the MR.SPOT<sup>®</sup> processor**

### **5.4.1 Reagent preparation**

Take HISTO SPOT<sup>®</sup> reagents and HISTO SPOT<sup>®</sup> testwells out of the fridge and allow them to warm to room temperature.

Salt crystals may be observed in the hybridisation buffer and in the stringent wash solution. If crystals are present, warm reagents up to 30°C to dissolve. Warm the whole content of the bottle, not an aliquot.

The conjugate has to be diluted 1:1666 in blocking buffer. The conjugate dilution must always be prepared afresh for each test run.

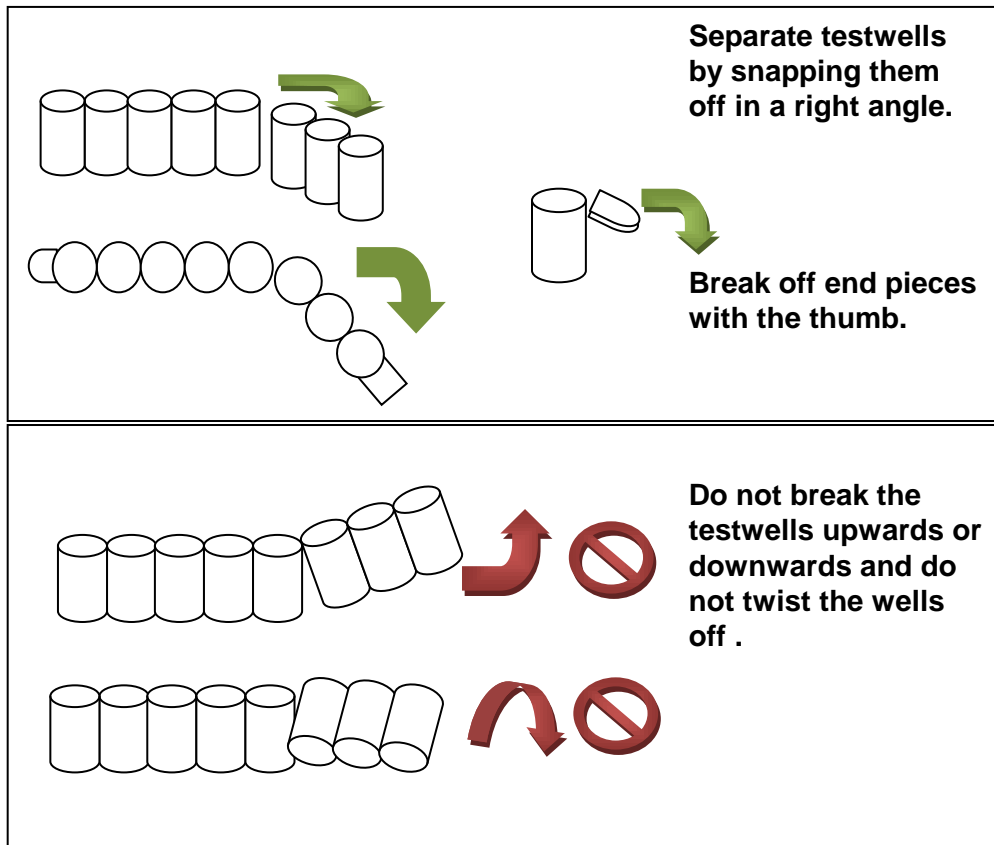
### **The conjugate has to be vortexed and spun down each time before the dilution step!**

The required volumes of the reagents will vary depending on the number of strips to be tested. MR.SPOT<sup>®</sup> displays the required volumes for the chosen number of strips. Fill the required volumes of the reagents into the corresponding labelled reservoirs.

Place the test wells and the PCR plate into the appropriate blocks of the MR.SPOT<sup>®</sup> processor. Note the correct arrangement of the PCR plate.

Please make sure that there is no dirt or plastic particles in the reaction plate holder, because this may disturb the heat transfer during hybridization.

The teststrips can be separated in single wells according to figure 1, if less than eight tests should be run. If you are using separated wells be careful that they are sitting properly in the reaction plate holder and are not twisted against each other.



**Fig. 1: Separating single testwells.**

#### **5.4.2 Setup of the MR.SPOT® processor**

Switch on the MR.SPOT® processor. The start up screen will appear. Follow the process as indicated on the screen. Details are described in the User Manual for the MR.SPOT® processor.

#### **5.4.3 Transfer of results to a PC for interpretation**

Transfer the data to the HISTO MATCH software via network or USB stick as described in the manual for the HISTO MATCH software.

#### **5.4.4 Interpretation of results**

Open the HISTO MATCH software (if this is not already installed, it can be installed from the CD delivered with the MR.SPOT® processor) and interpret the data as described in the manual for the HISTO MATCH software.

The images should look like the example shown in figure 2 and figure 3 gives a schematic illustration of the result and the functions of the different probes.

The colour of the circles around the probes indicate their function (see IFU for the HISTO MATCH software for details).

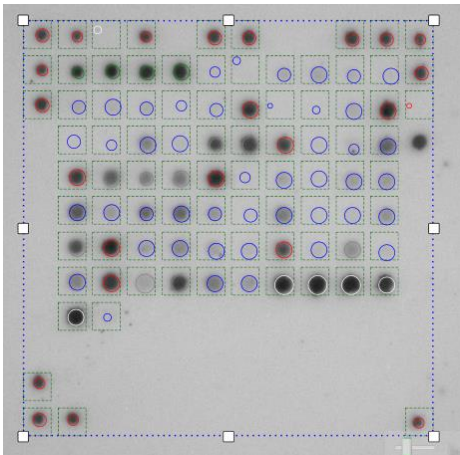


Figure 2: Image of a result for HLA A

●	●		●		●	●			●	●	●
●	●	●	●	●	○	○	○	○	○	○	●
●	○	○	○	○	●	○	○	○	○	○	
	○	○	○	●	●	●	○	○	○	●	●
	○	○	○	○	○	○	○	○	○	○	
	○	●	○	○	○	○	○	○	○	○	
	○	●	○	●	○	○	○	○	○	○	
	○	○									
	○	○									
●											
●	●										●

●: Positional probes: They are reacting with the amplification primers in the mastermix and indicate that mastermix was added and that all reagents during the SSO assay were added correctly. Furthermore, they allow the software to locate the image. The pattern is specific for the batch.

●+●: Amplification control for Exon 2 and Exon 3 in duplicate. Those probes are universal for all alleles of the respective locus and show that the PCR was successful. They are also functioning as a reference for the allele specific probes.

●: Positive allele specific probe

○: Negative allele specific probe

Figure 3: Schematic illustration of the result and function of the probes

## 6. WARNINGS AND PRECAUTIONS

HISTO SPOT® is designed for in vitro diagnostic use and should be used by properly trained, qualified staff. All work should be performed using Good Laboratory Practices.

Biological material used for extraction of DNA, e.g. blood or human tissue, should be handled as potentially infectious. When handling biological material appropriate safety precautions are recommended (do not pipet by mouth; wear disposable gloves while handling biological material and performing the test; disinfect hands when finished the test).

Biological material should be inactivated before disposal (e.g. in an autoclave). Disposables should be autoclaved or incinerated after use.

Spillage of potentially infectious materials should be removed immediately with absorbent paper tissue and the contaminated areas swabbed with a suitable standard disinfectant or 70% alcohol.

Material used to clean spills, including gloves, should be inactivated before disposal (e.g. in an autoclave).



Blocking Buffer, Hybridisation Buffer, Stringent Wash Buffer and TBS Wash Buffer contain ProClin®150 and the Magnesium Chloride Solution contains ProClin®300. The reagents contain 0.001% preservative only, nevertheless avoid contact with the skin and mucous membranes.

Mastermix and Conjugate contain the preservative sodium azide. The reagents contain < 0.1% sodium azide which is not considered to be a harmful concentration. Nevertheless avoid contact with the skin and mucous membranes. Sodium azide may react with lead and copper plumbing to form explosive metal azides. While disposing of sodium azide containing solutions down laboratory sinks, flush the drains with a large volume of water to prevent azide build-up.

All work with reagents should be handled with the appropriate precautions. Wear eye protection, laboratory coats and disposable gloves when handling the reagents. Avoid contact of these materials with the skin, eyes or mucous membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated.

If spills of reagents occur, dilute with water before wiping dry. Do not expose substrate to metals, oxidising agents.

Disposal of all samples, unused reagents and waste should be in accordance with country, federal, state and local regulations.

Avoid microbial contamination of reagents when removing aliquots from reagent bottles. The use of sterile disposable pipettes and pipette tips is recommended. Do not use reagents with evidence of turbidity or microbial contamination.

**Material Safety Data Sheets (MSDS)** are available to download at [www.bag-healthcare.com](http://www.bag-healthcare.com) .

## **7. SPECIFIC PERFORMANCE CHARACTERISTICS**

### **7.1 Evaluation**

For all HISTO SPOT® SSO kits evaluation studies with pre-typed DNA samples has been performed. The results were compared to other typing methods (e.g. SSP, sequencing). No discrepancies were observed between the typing methods.

For every lot the specificity of each probe was verified with DNA from reference samples.

### **7.2 PCR Amplification reaction**

The alleles amplified with each HISTO SPOT® SSO kit, the HLA nomenclature release referred to and the exons that are amplified are given in the respective lot specific information. This is found on a CD in each kit.

### **7.3 Assay resolution**

The HISTO SPOT® SSO typing system is designed to provide unambiguous results at least at allele group level i.e for two digits.

Different combinations of alleles that cross allele groups but have the same positive probe pattern are considered as ambiguous.

**HISTO SPOT® 4D** typing kits usually give allele level results (4 digits) if a common allele filter is used. Criteria for the common allele filter are the following: allele frequency  $\geq 0,5\%$  in at least one population with a sample size of at least 1000 samples on the website [www.allelefreqencies.net](http://www.allelefreqencies.net) .

## 8. LIMITATIONS OF THE METHOD

Because of the high susceptibility of the PCR method to variations in DNA concentration and quality, only DNA samples should be used that have a concentration between 15 and 30 ng/μl and appropriate purity indices (extinction ratio  $OD_{260}/OD_{280}$ : between 1.5 and 2.0 / extinction ratio  $OD_{260}/OD_{230}$ : > 1.8).

Extreme care should be taken to prevent contamination of the kit reagents and other laboratory materials and equipment with amplicons or DNA. Regular wipe tests (e.g. BAG Wipetest, [REF] 7091) and negative controls with each assay are strongly recommended.

The hybridisation assay is a very temperature-sensitive process. Therefore, the HISTO SPOT<sup>®</sup> SSO kits should only be used in combination with the MR.SPOT<sup>®</sup> processor to ensure correct temperatures and incubation times.

All instruments (e.g. pipettes, thermal cyclers, heat blocks; MR.SPOT<sup>®</sup> processor) must be calibrated according to the manufacturers instructions. Accuracy and temperature uniformity of thermal cyclers may be tested with the BAG CycyclerCheck ([REF] 7104).

## 9. INTERNAL QUALITY CONTROL

Internal quality control of new lots of the HISTO SPOT<sup>®</sup> SSO kits can be performed using a combination of DNA samples with known HLA type.

Internal positive controls are contained in each test well to ensure successful amplification and hybridization.

Negative controls to detect possible contaminations are recommended. Use a PCR reaction without DNA in the subsequent hybridization assay as a negative control.

## 10. TROUBLESHOOTING

Symptom	Possible problem(s)	Potential Solution(s)
Instrument Malfunction	Numerous	Refer to MR.SPOT <sup>®</sup> manual
Error message at data transfer	Failure in data transfer	Manually transfer data using USB drive
No result	Failure to grid image	Perform manual gridding
No Spots in well	Failure to add mastermix to PCR	Repeat whole assay and check PCR product on gel
Only control spots positive	Failure to add DNA to PCR or amplification failure	Repeat whole assay and check PCR product on gel
False positive probes	Too much DNA used or conjugate concentration too high (not spun down).	Check DNA concentration. Spin down conjugate before use
Exon dropout	DNA concentration too high or DNA degraded	Check DNA concentration, run a gel with the DNA
No result / inconclusive result due to weak signals	Mistake in conjugate dilution or poor amplification Instrument malfunction	Repeat assay. Check hybridisation temperature on instrument




## 11. TRADEMARKS USED IN THIS DOCUMENT/PRODUCT

Proclin<sup>®</sup> is a trademark of Rohm and Haas company.

BCIP<sup>®</sup> is a trademark of Sigma Aldrich Co.

Veriti<sup>™</sup> is a trademark of Applied Biosystems.

**12. EXPLANATION OF SYMBOLS USED ON LABELING**

<b>IVD</b>	For in vitro diagnostic use
	Storage temperature
<b>LOT</b>	Batch code
	Use by
<b>REF</b>	Catalogue number
	Consult instructions for use
<b>HLA TYPING</b>	Intended purpose: HLA typing
<b>Mastermix   A</b>	Mastermix for amplification of the HLA-A locus
<b>Mastermix   B</b>	Mastermix for amplification of the HLA-B locus
<b>Mastermix   C</b>	Mastermix for amplification of the HLA-C locus
<b>Mastermix   DRB1</b>	Mastermix for amplification of the HLA-DRB1 locus
<b>Mastermix   DQB1</b>	Mastermix for amplification of the HLA-DQB1 locus
<b>Mastermix   DPB1</b>	Mastermix for amplification of the HLA-DPB1 locus
<b>Mastermix   DRB3/4/5</b>	Mastermix for amplification of the HLA-DRB3/4/5 loci
<b>Testwells   A</b>	Testwells with bound probes to type the HLA-A locus
<b>Testwells   B</b>	Testwells with bound probes to type the HLA-B locus
<b>Testwells   C</b>	Testwells with bound probes to type the HLA-C locus
<b>Testwells   DRB1</b>	Testwells with bound probes to type the HLA-DRB1 locus
<b>Testwells   DQB1</b>	Testwells with bound probes to type the HLA-DQB1 locus
<b>Testwells   DPB1</b>	Testwells with bound probes to type the HLA-DPB1 locus
<b>Testwells   DRB3/4/5</b>	Testwells with bound probes to type the HLA-DRB3/4/5 loci
<b>MgCl<sub>2</sub></b>	Magnesium chloride solution
<b>BLOCKBUF</b>	Blocking buffer
<b>HYBBUF</b>	Hybridisation buffer
<b>STRGWASH</b>	Stringent wash solution
<b>TBS</b>	Tris buffered saline
<b>SUBS</b>	Substrate
<b>CONJ</b>	Streptavidin Alkaline Phosphatase-Conjugate

Instructions for use in other languages see:

<http://www.bag-healthcare.com>

<http://service.bag-healthcare.com>

or phone: +49 (0)6404-925-125