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CTS Collaborative Transplant Study

Manual No.: 100A
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MAIN MANUAL (GENERAL INFORMATION) FOR THE CTS-PCR-SSP TRAY AND MINITRAY KITS

To be applied to the following products:

Product No.	Description	
101	HLA-A* CTS-PCR-SSP TRAY KIT	IVD CE 0197
102	HLA-B* CTS-PCR-SSP TRAY KIT	
104	HLA-DRB1* CTS-PCR-SSP TRAY KIT	
120	HLA-A*+B*+C* CTS-PCR-SSP TRAY KIT	
121	HLA-A*+B*+DRB1* CTS-PCR-SSP TRAY KIT	
122	HLA-DRB1*+DQB1* Low Resolution CTS-PCR-SSP TRAY	
246	Celiac Disease CTS-PCR-SSP MINITRAY KIT	
347	Narcolepsy CTS-PCR-SSP MINITRAY KIT	
103	HLA-C* CTS-PCR-SSP TRAY KIT	IVD CE
340	HLA-C*04:09N CTS-PCR-SSP MINITRAY KIT	
119	HLA-DQB1*Low Resolution CTS-PCR-SSP TRAY KIT	
127	HLA-DQA1* Low Resolution CTS-PCR-SSP TRAY KIT	
128	HLA-DQB1*+DQA1* Low Resolution CTS-PCR-SSP	

Introduction

- Intended use: The CTS-PCR-SSP TRAY AND MINITRAY KITS are designed for molecular typing of various Human Leukocyte Antigen (HLA) Class I and II gene loci based on the PCR-SSP method (see references).
- One kit contains allele-specific or group-specific ready-for-use PCR primer mixes which are aliquoted and dried in 96-well trays or in 24-, 16- or 8-well Minitrays.
- Setup includes mixing a reaction buffer (= Mastermix) with a human genomic DNA sample and Taq DNA Polymerase.
- Dispensing the mixture into the tray or Minitray provided in the kit.
- Closing the wells with caps and then thermal cycling.
- The PCR products are loaded onto a 2% agarose gel for electrophoresis. After electrophoresis, the ethidium bromide stained gel is photographed and interpreted using tables which are listed in the locus- and lot-specific manual.
- The test can be completed in approx. 1.5 hours post DNA isolation.
- The primer sets amplify the alleles described by the WHO international nomenclature committee (http://www.anthonynolan.org.uk/HIG/index.html).
- The CTS-PCR-SSP TRAY and MINITRAY KITS have been designed, produced and extensively tested with well characterized reference DNAs (DNAs which have been serologically and molecularly typed e.g. by PCR-SSP/PCR-SBT) in our laboratory.

Content:

Please note:

The usage of the CTS-PCR-SSP TRAY AND MINITRAY KITS requires detailed knowlege of PCR techniques!

To avoid contamination with PCR amplification products, it is necessary to perform the test in two separate working areas: the pre-amplification area and the post-amplification area!

1. KIT COMPOSITION

1.1. General description

- The CTS-PCR-SSP TRAY AND MINITRAY KITS contain PCR primer mixes (here commonly called "mixes") prepipetted and dried in thin-walled plastic PCR-**Trays** (96 wells) or PCR-**Minitrays** (24, 16 or 8 wells). Specificities of the mixes are given in the locus- and lot-specific manual(s) sent along with the kits you ordered.

- If you order a CTS-PCR-SSP **TRAY** or **MINITRAY** KIT, you will receive 1 plastic pouch containing primer mixes already dispensed and lyophilized in PCR Trays or Minitrays. Each pouch includes 10 trays or 12 Minitrays.

Valid for TRAYS only:

- Each well of the tray (96 wells) is designated by a digit-letter combination from H1 to A12. Digits are visible on the top, letters on the left edge of the tray. For better orientation, the **lower** edge of the tray has been marked with a **black line**. **The starting position (mix no. 1) is at H1**, followed by G1, F1, etc. Mix no. 9 would be at position H2, followed by mix no. 10 at position G2, mix no. 11 at position F2 etc. (see Figure 1).

- The print on the right edge of the tray shows the product name as well as the lot number of the reagent used. For any question concerning the specific kit you received, please indicate this lot number!

- The label on the first tray indicates the name, the lot number and other characteristics (such as detectable specificities etc.) of the product.

Valid for MINITRAYS only:

- The **lower** edge of the Minitray (24, 16 or 8 wells) has been marked with a **black line**. **The starting position (mix no. 1) is the lower left well** (see Figure 1). Please disregard the numbers which appear on the upper edge of the Minitray!

- The Minitrays are stacked.

- The label on the pouch shows the name, the lot number and other characteristics (such as detectable specificities etc.) of the product. For any question concerning the specific kit you received, please indicate this lot number!

Figure 1

96-well tray

	1	2	3	4	5	6	7	8	9	10	11	12
A	Mix 8	Mix 16	Mix 24									
B	Mix 7	Mix 15	Mix 23									
C	Mix 6	Mix 14	Mix 22									
D	Mix 5	Mix 13	Mix 21									
E	Mix 4	Mix 12	Mix 20									
F	Mix 3	Mix 11	Mix 19									
G	Mix 2	Mix 10	Mix 18									
H	**Mix 1**	Mix 9	Mix 17									

e. g. HLA-A Lot# 10-1 601

Black marker line

24-well Minitray

n	n+1	n+2
Mix 8	Mix 16	Mix 24
Mix 7	Mix 15	Mix 23
Mix 6	Mix 14	Mix 22
Mix 5	Mix 13	Mix 21
Mix 4	Mix 12	Mix 20
Mix 3	Mix 11	Mix 19
Mix 2	Mix 10	Mix 18
Mix 1	Mix 9	Mix 17

16-well Minitray

n	n+1
Mix 8	Mix 16
Mix 7	Mix 15
Mix 6	Mix 14
Mix 5	Mix 13
Mix 4	Mix 12
Mix 3	Mix 11
Mix 2	Mix 10
Mix 1	Mix 9

8-well Minitray

n
Mix 8
Mix 7
Mix 6
Mix 5
Mix 4
Mix 3
Mix 2
Mix 1

(n, n+1, n+2, n+3, etc. = numbers which appear on the upper edge of minitrays can be ignored)

Valid for all KIT Formats:

- The specificities of the primer mixes contained in each well are given in the locus- and lot-specific manual.

- If requested by customer, you will find Tray or Minitray caps / foils (only for Trays) in separate pouches, which are needed for closing the wells tightly before starting the PCR. Alternatively, you can use a MicroAmp™ Full Plate Cover (only for Trays), which you may find to be more convenient.

- Along with the kits, you will receive an adequate amount of Mastermix (see section 1.2). The Mastermix is a buffer which contains crucial ingredients required for a successfull amplification (see section 1.3). DNA-Polymerase is not included in the kit.

- Mastermix SSP is used for the amplification of HLA-class **I**, HLA-class **II,** Celiac Disease and Narolepsy products.

- All reagents contained in the CTS-PCR-SSP TRAY and MINITRAY KITS have been prepared and quality-controlled in Heidelberg and are ready for use.

1.2 Kit Components

Description of the component	*Quantity*	*Storage Condition*
Polypropylene PCR Trays (96 wells) or Minitrays (24, 16 or 8 wells) containing dried primer	• Tray Kit: 10 Trays • Minitray Kit: 12 Minitrays	Store at +2°C…+8°C until expiration date (in originally sealed pouch or after opening the package)
3 ml / 1 ml / 0.6 ml/ 0.3ml aliquots of optimized Mastermix (without Taq Polymerase)	• Tray Kit: 1 tube (3ml) • 24-well Minitray Kit: 1 tube (1 ml) • 16-well Minitray Kit: 1 tube (0.6 ml) • 8-well Minitray Kit: 1 tube (0.3 ml)	Store at -15°C…-30°C until expiration date. An aliquot which is thawed and has been opened for use can be stored at +2…+8°C for 1 month.
Optional: **Strip caps** for sealing Trays/Minitrays **Alternative:** Foils for sealing Trays (not for Minitrays)	• Tray Kit: 11 packs of 12 • 24-well Minitray Kit: 4 packs of 12 • 16-well Minitray Kit: 3 packs of 12 • 8-well Minitray Kit: 2 packs of 12 • Tray Kit: 11 foils	Room temperature
Working instruction/product information	Each kit: • 1 Main Manual • 1 Locus- and lot-specific Manual • 1 Material Safety Data Sheet (MSDS)	--------

1.3 Information on Ingredients

Component	*Chemical(s)*
Trays/Minitrays	PCR primers = Oligonucleotides
	Cresol Red
Mastermix	Ammonium Sulfate
	Tris Buffer
	Magnesium Chloride
	Glycerol (glycerin)
	Cresol Red
	Deoxyribonucleotides (dNTPs)

2 MATERIALS, REAGENTS AND EQUIPMENT NOT SUPPLIED

2.1 The following enzymes are validated for use with the CTS-PCR-SSP TRAY and MINITRAY KITS :
- Taq DNA Polymerase, Fermentas, Cat.No EP0401/ EP0402, www.fermentas.com
- Axi Taq, Inno-Train, info@innotrain.de.
Use of other DNA polymerase enzymes must be validated by the user!

2.2 Sterile, molecular grade water

2.3 Pipettes (1-10 µl and 10-100 µl) and tips:
For quick pipetting use Eppendorf Multipette Type 4720 with 0.5 ml Combitips or
Gilson® Distriman™ No. 11800 with DistriTips™ Minichannel pipettor: 8 channel pipettor 5 - 50 ml adjustable volume: Finnpipette, ThermoLabsystems, Cat. No. 4510 020

2.4 1.5 ml polypropylene safe-lock tubes:
Eppendorf, Cat. No 0030 120.086.

2.5 The following thermal cyclers are validated for use with the CTS-PCR-SSP BULK KIT:
96 well thermal cycler with heated lid:
PE 2700, Applied Biosystems, www.appliedbiosystems.com,
PTC-100, MJ Research, Inc., www.mjr.com .
PTC- 200,MJ Research, Inc., www.mjr.com
The kit has been validated with the above thermal cyclers.
Use of other thermal cyclers must be validated by the user!

2.6 TAE electrophoresis buffer (1x TAE)
(TAE=Tris, concentrated acetic acid (CH_3C00H), 0.5 m EDTA pH 8)

2.7 Ultra Pure Agarose**,** Gibco-BRL, Cat. No. 5510UA

2.8 Ethidium bromide (10 mg/ml)
Caution, ethidium bromide is mutagenic! Handle with appropriate personal protective equipment!

2.9 Lysis Buffer I:
0.3 M sucrose
10 mM Tris-HCl (pH 7.5)
5 mM MgCl
1% Triton x-100 (C_{34} H_{62} 0_{11}; MW= 646.87 g/mol).
Store in a dark place at +4°C.

2.10 Lysis Buffer II:
0.075 M NaCl
0.024 M Na-EDTA.
Adjust pH to 8 with 4M NaOH and store at room temperature.

2.11 10% SDS

2.12 5 M Sodiumperchlorate

2.13 6 M NaCl

2.14 Absolute isopropanol (99.9%)

2.15 70% ethanol
Electrophoresis equipment
Submarine DNA electrophoresis set**:**
CTS electrophoresis chamber and combs (CTS order no.:501)

2.16 a) Gel Documentation System:
UV-light transilluminator (312 nm)
Renner, D-6701 Dannstadt, Germany, Cat. No. 35 2452.
b) Gel Documentation System:
Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software,
Cat. No.170-8195, Biorad, info@bio-rad.de

2.17 a) Photometric DNA measurement:
Lambda Scan 200, MWG, www.THE.MWG.com
PC operated photometer program: KC 4, Bio-Tek Instruments, Inc.www.biotek.com
b) Photometric DNA measurement:
Biophotometer 6131, Cat. No.: 6131 000.012, Eppendorf, vertrieb@eppendorf.de

Use of other equipments requires validation by the user!

3 SAMPLE REQUIREMENTS

3.1 DNA Sample in TE buffer or steril water.

3.2 Optimally, use a total amount of 10 - 15 µg of genomic DNA for one full 96-well PCR tray (see Table 1 at Section 4). Less DNA (e. g. down to 3 µg of DNA) may be used, but need to be validated under your working conditions.

3.3 DNA Isolation

3.3.1 Collect 10 ml blood with EDTA anticoagulant or use 20-50 x10^6 isolated lymphocytes.
EDTA blood or buffy-coat can be stored at 4°C for up to one week or at -80°C for approx. 6 months before DNA-isolation.
*Do **not** use heparinized blood!*
It is important to use intact blood samples as test interference by hyperlipidemia or hyperbilirubinemia etc. cannot be ruled out.

3.3.2 Transfer the whole blood or cells to a 50 ml tube and add cold Lysis Buffer I (see 2.9) to a final volume of 50 ml. Mix and centrifuge for 5 min at 2400 *g* and 4°C.

3.3.3 Carefully pour off the supernatant and resuspend the pellet in
4.5 ml of Lysis Buffer II (see 2.10)
125 µl of 10% SDS (see 2.11), and
1.1 ml of 5 M sodiumperchlorate.
Shake vigorously for 10 sec at room temperature.

3.3.4 For extraction of proteins add 2 ml of 6M NaCl and shake vigorously for 15 sec.
Centrifuge for 5 min at 1500 *g* and room temperature.

3.3.5 Carefully pour the supernatant into a clean 50 ml tube, avoiding the pellet.
Add 7 ml of absolute isopropanol. Cap the tube and mix gently.

3.3.6 Remove the precipitated DNA with a sealed Pasteur pipette and squeeze out the excess of isopropanol.

3.3.7 Wash the DNA twice in 3 ml of 70% ethanol and resuspend the DNA in 100 - 300 µl sterile water (ddH_20) by incubation at 55°C for 10 - 20 min.

3.3.8 Dilute 5 µl of the DNA solution in 495 µl of water (ddH_20) and measure the optical density at 260 nm in an UV spectrophotometer. The DNA concentration (in µg/µl) is 5 times the OD value.

3.3.9 For optimal reaction, adjust the **DNA concentration** to approximately **0.10 - 0.15 µg/µl** with ddH_20. As low as **0.03 µg/µl** of DNA have been successfully used by several laboratories.

3.3.10 Proceed with the PCR (Section 4) within 30 min. If it takes more than 30 min to set up the PCR, the DNA concentration may increase as more and more DNA goes into solution and the condition for amplification may become unfavorable. In this case it is recommended to readjust the DNA concentration before using it for the preparation of the PCR Reaction Mix.

4. PREPARATION OF THE PCR REACTION MIX

4.1 Thaw the frozen Mastermix

TRAY KITS:
We strongly recommend to prepare small aliquots of Mastermix:
276 µl of Mastermix for one Tray.
Freeze the aliquots and rethaw the number of tubes needed.

MINITRAY KITS:
We strongly recommend to prepare small aliquots of Mastermix:
23 µl (for one 8-well Minitray), 46 µl (for one 16-well Minitray) or 69 µl (for one 24-well Minitray).
Freeze the aliquots and rethaw the number of tubes needed.

4.2 Carefully remove one or more Trays/Minitrays from the pouch. Start with the bottom Tray. Reseal the pouch and place unused Trays/Minitrays back into the refrigerator (+2…+8°C)

If not a full Tray is needed, use scissors to cut the part of the Tray you need.
Note: When cutting a Tray, cut tests from the left to the right, avoiding cutting off the product name and the lot number! Place the remaining part of the tray into the pouch, reseal it carefully to avoid moisture and store it in the refrigerator (+2…+8°C).

4.3 Place the Tray/Minitray inside a sample holder, such as a clear microtiter plate or a 0.2 ml tube rack (8x12), and place it in a clean area during sample setup.

Carefully remove adhesive seal from the PCR Tray

4.4 Remove Taq DNA Polymerase from the freezer and keep it chilled during setup (e.g. on ice).

4.5 Add water and Taq DNA Polymerase to the aliquoted Mastermix and vortex thoroughly (see Table 1 for volumes of water and Taq).

4.6 Dispense 10 µl of this mixture to the Negative Control well, if this is included in your kit (some kits do not have a Negative Control Mix because the allele-specific mixes occupy all the cavities available on the Tray/Minitray). For position of the Negative Control please refer to the locus- and lot-specific manual.

4.7 Add the volume of DNA sample (10 - 15 µg needed for a full 96-well tray) to the Mastermix-Taq-Water mixture, as indicated by Table 1 and vortex thoroughly.

Table 1

Number of PCR	Mastermix (µl)	Taq DNA Polymerase at 5 U/µl* (µl)	Aqua dest. (µl)	DNA at 0.10-0.15 µg/µl** (µl)
2	**5,75**	**0,12**	**13,7**	**2**
8	**23**	**0.48**	**54.8**	**8**
16	**46**	**0.96**	**109,6**	**17**
24	**69**	**1.45**	**164.5**	**25**
48	**138**	**2.9**	**329**	**50**
96	**276**	**5.8**	**658**	**100**

*The exact amount of Taq-Polymerase needed may vary according to the brand and lot number and therefore should be established through your own validation.
**Several labs reported of having successfully used as low as 0.03 µg/µl of DNA (e. i. 3 µg of DNA for a 96-well tray). You may validate this option for your own lab.

Using an electronic dispensing pipettor, dispense 10 µl of the reaction mixture (= Mastermix-Taq-DNA) into each well. Be careful to dispense the drops onto the side walls near each well's top, allowing the dispensed drop to slide down. Do not allow the pipette tip to touch the bottom of wells.

Confirm that each well contains sample by noting the presence of the solution in each well. A settled sample will be indicated by a pink solution color on the bottom of wells. If a drop is hung up on the side of well, GENTLY tap the Tray/Minitray in the holder against the bench. Sufficient volume is supplied to allow for pipetting losses.

4.8 Close the Tray/Minitray. **Ensure the caps/foil completely seal the wells to prevent evaporation.**

4.9 Place the tray in thermal cycler and begin thermal cycling (see section 5. Thermal Cycler).

If you prefer to **pipette the DNA separately**, follow the description of Table 1 for the volumes of the Mastermix, Taq and water. Dispense 9 µl of the reaction mixture (= Mastermix-Taq-water) into each well. Dispense 1µl of DNA (0.10 - 0.15 µg/µl) into each well (except the Negative Control well, if it is included in your kit). Be careful to dispense the drops onto the side walls near each well's top, allowing the dispensed drop to slide down. Do not allow the pipette tip to touch the bottom of wells.

Sections 4.1 through 4.9 must be performed efficiently fast to minimize the time between sample addition and initiation of thermal cycling. Prolonged incubation at room temperature may cause mispriming and non-specific PCR products!

5. THERMAL CYCLER: CTS-PCR-SSP TRAY AND MINITRAY KIT AMPLIFICATION PROFILE

5.1 It is important to obtain rapid ramp times and precise temperature control for optimal results.

5.2 The following thermal cycler profile is optimized and validated with the thermocyclers given in 2.6 for use with the CTS-PCR-SSP TRAY AND MINITRAY KITS:

Initial denaturation:	94°C, 2min
Denaturation:	94°C, 15 sec
Annealing+Extension:	65°C, 1 min
	10 cycles
	followed by
Denaturation:	94°C, 15 sec
Annealing:	61°C, 50 sec
Extension:	72°C, 30 sec
	20 cycles.
Hold:	4°C, 15 min

5.3 After thermal cycling, remove tray and proceed to gel electrophoresis.

6. GEL ELECTROPHORESIS

Absence or presence of PCR products is visualized by submarine agarose gel electrophoresis. Conditions are described for the Pharmacia cell GNA 200 (gel size 20x25 cm).

6.1 Preparation of the agarose gel

6.1.1 While the PCR is running, pour a 2% agarose gel. If you use the CTS electrophoresis chamber and the CTS combs, proceed as follows:

6.1.2 Add 7 g Agarose and 7 ml 50x TAE buffer to 350 ml of ddH_20.

6.1.3 Boil to dissolve the agarose, using a magnetic stirring hotplate or a microwave oven.

6.1.4 Cool to 60°C, add 17 µl ethidium bromide, mix and pour the gel. Allow the gel to set for 1 hour at room temperature.

6.1.5. On a 20x25 cm gel you can place up to six CTS combs. These combs have a tooth distance corresponding to that of the channels of a standard 8-channel pipette. This allows the use of such a pipette for rapid loading of the samples onto the gel.

6.2 Electrophoresis

6.2.1 When the PCR is finished, remove the PCR Tray/Minitray from the thermocycler.

6.2.2 Carefully remove the strip caps from the PCR-Tray/Minitray.

Caution: Sudden movement of the Tray/Minitray can disperse amplified product, contaminating the laboratory and may require repetition of the test!

6.2.3 Remove the combs and place the gel into the electrophoresis cell. The level of electrophoresis buffer should be 2 - 3 mm above the gel surface. The electrophoresis buffer can be reused several times.

6.2.4 Load 10 µl of each PCR product onto the gel. Because of the glycerol and the cresol red included in the mix, there is no need for use of any additional loading buffer. Make sure that the order in which you load the samples is standardized, i.e. Gel Loading Template.

Gel loading template

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Lane 1 (Pos.1-8)**	**Lane 1 (Pos.9-16)**	**Lane 1 (Pos.17-24)**	**Lane2 (Pos.25-32)**	**Lane2 (Pos.33-40)**	**Lane2 (Pos.41-48)**	**Lane3 (Pos.49-56)**	**Lane3 (Pos.57-64)**	**Lane3 (Pos.65-72)**	**Lane4 (Pos.73-80)**	**Lane4 (Pos.81-88)**	**Lane4 (Pos.89-96)**
B	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲
C												
D												
E												
F												
G												
H												

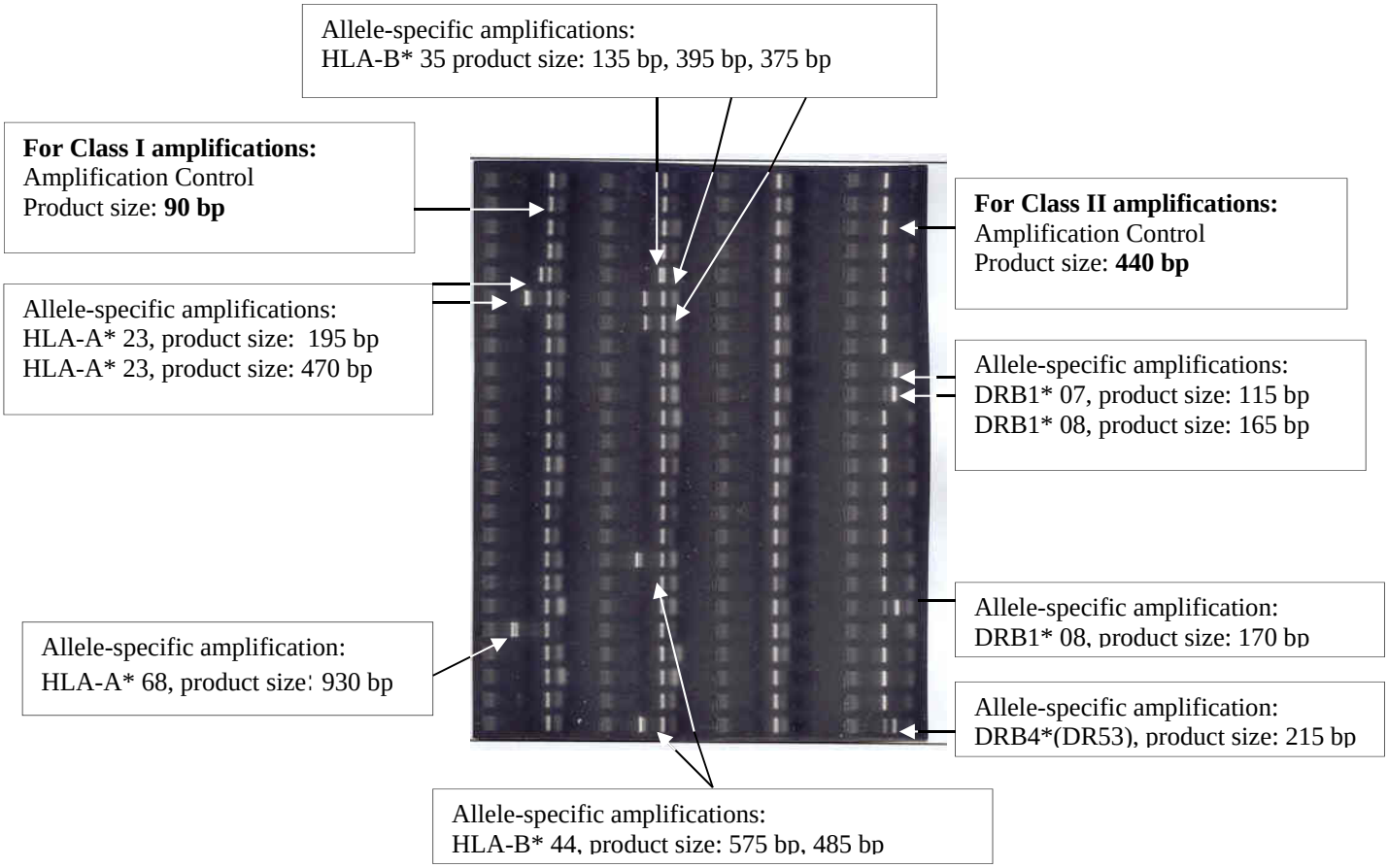
Note: The word 'well' refers to the Tray/Minitray location assignment, while the word 'lane' refers to a well's corresponding gel lane.

6.2.5 Run the electrophoresis for 20 min at 170 V (approx. 0.4 V/cm^2).

6.2.6 Turn off power, disconnect electrodes and remove gel.

6.2.7 Place the gel on a UV light transilluminator (312 nm) and take a polaroid picture for interpretation and documentation.

7. INTERPRETATION



Example of a HLA-A*B*DRB1* typing (Lot A09 B08 DR34)
(Results: HLA-A*23,68; HLA-B*35,44; HLA-DRB1*07,08)

7. 1 Amplification Control (Internal Positive Control)

7.1. Each of the primer mixes contains a non-allelic amplification control primer pair which amplifies either a part of the ß-globin gene (HLA-Class I CTS-PCR-SSP Mixes) or a part of the C-reactive protein gene (CRP) (HLA-Class II CTS-PCR-SSP Mixes).

7.1.2 The ß-globin amplification control primers (only relevant for HLA-class **I** typing) should produce a **90 base pairs** fragment, even in those which contain allele-specific PCR products. Please note that this amplification control band will appear just below the primer dimer cloud and is always shorter than the specific amplification bands!

7.1.3 The amplification control primers amplifying the CRP gene (only relevant for HLA-class **II** typing) should produce a **440 base pairs** amplicon in all tubes in which an allele- or group-specific amplification is not present.

7.2 Allele- or group-specific amplification

7.2.1 Slots in which a allele-specific PCR product is present (and no or only a weaker amplification control band is visible at the same time) indicate the presence of a specific allele in the tested individual. Most homozygous and heterozygous combinations can be detected with this system by unique amplification patterns.

7.2.2 Some lanes have two or more PCR products of different sizes. These wells have multiplexed specific primer pairs which give rise to different amplicons, depending upon the existing allele(s). Refer to the interpretation tables in the locus- and lot-specific kit manuals.

7.3 Negative Control (please refer to the locus- and lot-specific manual to find out whether such a control is included in the particular kit you received)

Any amplification band in the Negative Control well is evidence of contamination and the test result is invalid!

7.4 Interpretation Hints

7.4.1 If weak amplification bands of incorrect size are present and the overall strength and clarity of the assay is good, disregard these bands as they usually represent non-specific amplification.

7.4.2 Unused primers will form a diffuse cloud below 50 base pairs.

7.4.3 A primer dimer band (<80 base pairs) may be present. This **does not** invalidate the test. Primer dimers are known to occur occasionally.

7.4.4 False negative reactions can be caused by inefficient amplification, poor quality of DNA, uneven placement of the plate in the block, temperature variations across the wells of the thermal cycler itself, or inadequate cycler calibration etc.

7.4.5 Presumably “false negative” reactions may be due to a new or yet uncharacterized allele! In these cases we recommend to perform further investigation using other techniques such as sequencing etc.

7.4.6 Please refer to the enclosed locus- and lot-specific manual for more information on result interpretation of the kit you obtained.

7.5 Evaluation

7.5.1 Check the approximate size of the PCR products against the Primer Mix Specificity Table in the locus- andl lot-specific manual to confirm the correct product size.

7.5.2 As for HLA class I typing kits, some PCR products are shorter than 200 bp and might therefore be difficult to be distinguished from the amplification control bands (90 bp). In general, the specific amplification products will give a much stronger signal than that of the amplification control products, and they will not have migrated as far into the gel as the latter. If the amplification control band of one of those mixes appears to be very strong, you might let the gel run for another 15 minutes at a lower voltage. By this way the specific amplification primer band will be separated from the 90 bp amplification control band and you will be able to clearly see a double band in this area: a very strong specific amplification primer band and a shorter, weaker amplification control band.

7.5.3 Use the Typing Tables in the locus- and lot-specific manual to make allele assignments or use the SCORE Program (www.ihwg.org.) for detailed result interpretation.

7.6 **The test has to be repeated:**

7.6.1 If the amplification control bands as well as of specific amplification bands are absent. This is indicative of failed reactions.

7.6.2 If there is an apparent homozygous result and the missed reaction could change an allele assignment.

7.6.3 If the reaction pattern is inconclusive.

7.6.4 If the reaction pattern suggests the presence of three alleles.

7.6.5 If the Negative Control is positive.

7.6.6 However, if the result can be determined despite a failed PCR reaction (the positivity of which would not change the allele assignment), the test does *not* need to be repeated.

8. TROUBLESHOOTING

8.1 Problem: DNA-concentration and -quality

Possible causes:

8.1.1 Use of excess of DNA (e. g. > 0.2 µg/µl per reaction) may favor non-specific PCR products. An intense smear of high molecular weight DNA present on gel photos of amplified products may indicate that excess of DNA was used. A generally weak amplification might indicate that less than the required minimal amount of sample DNA was used in the reaction (e. g. < 30 ng/µl per reaction) which may cause false negative reactions.

8.1.2 Degraded DNA may not amplify reliably ⇒ obtain another blood sample and repeat the DNA extraction.

8.2 Problem: Overall poor or absent amplification indicated by weak or absent amplification control bands and absent specific amplification bands

Possible causes:

8.2.1 Inadequate contact between thermal cycler block and tray ⇒ DO NOT use the sample holder provided by Perkin Elmer with the PCR TRAYS in the PE9600.

8.2.2 Heparinized samples ⇒ use **EDTA** or **ACD** as anticoagulants.

8.2.3 Poor quality DNA ⇒ use the CTS DNA Extraction Technique (see section 3. Sample requirements/DNA isolation) . As a last resort, extract a fresh sample.

8.2.4 Degraded DNA samples (this is apparent by presence of smear in gel lanes) ⇒ isolate DNA from a fresh sample.

8.2.5 Improperly calibrated thermal cycler ⇒ recalibrate thermal cycler.

8.2.6 Lack of Taq DNA Polymerase activity ⇒ verify activity of Taq with a known reference DNA sample.

8.2.7 Degraded primer mix or Mastermix ⇒ check expiration date of the reagents, storage conditions and integrity of the kits. Repeat the test with an intact, well-characterized DNA as positive control. If necessary, discard the reagents and use another lot.

8.3 **Problem:** Random failures (more than one lane failed)

Possible causes:

8.3.1 DNA is not evenly re-suspended in diluent ⇒ pipet DNA up and down several times to aid mixing.

8.3.2 DNA not mixed adequately with Mastermix ⇒ vortex thoroughly before adding to tray.

8.3.3 Thermocycler defect ⇒ Check cycling conditions in failed cycler positions.

8.4 **Problem:** False positive results

Possible causes:

8.4.1 Excess of DNA or Taq Polymerase ⇒ measure DNA concentration with UV spectrophotometry and adjust the DNA concentration to 0.03 to 0.15 µg/µl; check or validate the amount of Taq Polymerase required.

8.4.2 Incorrect order in gel loading ⇒ check alignment of mixes and gel lanes.

8.4.3 Extensive delay between PCR setup and start of thermal cycling ⇒ no more than 5 minutes delay should be allowed before thermal cycling. Alternatively pipetted tray can be stored at 2°C to 8°C for up to 2 hours.

8.4.4 Mis-interpretation of primer dimer as specific amplification bands ⇒ check correct band size.

8.4.5 Contaminations with PCR-product ⇒ perform wipe test.

8.5 **Problem:** False negative results

Possible causes:

8.5.1 Improperly calibrated thermal thermal cycler ⇒ recalibrate thermal cycler.

8.5.2 Incorrect order in gel loading ⇒ check alignment of mixes and gel lanes.

8.6 **Problem:** Overall fuzzy bands, smeared lanes

Possible causes:

8.6.1 Gel is too thin due to excessive evaporation while heating ⇒ compensate for lost volume by adding water.

8.6.2 Agarose not completely dissolved ⇒ boil for an additional 30 seconds after melting.

8.6.3. Overheating gel, too high voltage ⇒ lower voltage.

8.6.3 Heavy streaking in random wells can be caused by uneven suspensions of DNA ⇒ using an 8 channel pipettor, mix the PCR product up and down two times before loading.

8.6.4 Rapid release of amplified product during gel loading can cause product to float out of well ⇒ use slow, steady pipetting when loading gel.

8.7 **Problem:** Gel picture too dark

Possible causes:

8.7.1 No or a wrong amount of ethidium bromide was added ⇒ use 5 µl of ethidium bromide (10mg/ml) for each 100 ml of agarose solution.

8.7.2 Gel tray not UV transparent ⇒ remove gel from tray before viewing.

8.7.3 Incorrect camera setting ⇒ increase exposure time or aperture setting.

8.8 Problem: Gel picture too bright

Possible causes:

8.8.1 Excessive amount of ethidium bromide ⇒ use 5 µl of ethidium bromide (10mg/ml) for each 100 ml of agarose solution. Ethidium bromide in the running buffer is not necessary.

8.8.2 Incorrect camera setting ⇒ decrease exposure time or aperture setting.

8.9 Problem: Occasional faint lanes

Possible causes:

8.9.1 Product floated out of well ⇒ pipette tips need to be properly aligned with gel wells.

9. REFERENCES

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10. CONTACT

If you have any particular questions concerning this kit, which are not answered in this or in the locus- and lot-specific manual, please do not hesitate to contact me or my coworkers at:

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