



CTS *Collaborative Transplant Study*

WORKING INSTRUCTION FOR THE
CTS-PCR-SSP BULK KITS

MAIN MANUAL (GENERAL INFORMATION)

To be applied to the following products:

Product No.	Description	
401	HLA-A* CTS-PCR-SSP BULK KIT (24 mixes)	
402	HLA-B* CTS-PCR-SSP BULK KIT (48 mixes)	
404	HLA-DRB1* CTS-PCR-SSP BULK KIT (24 mixes)	
403	HLA-Cw* CTS-PCR-SSP BULK KIT (24 mixes)	

Introduction

- Intended use: The CTS-PCR-SSP BULK KITS are designed for molecular typing of various Human Leukocyte Antigen (HLA) Class I and Class II gene loci based on the PCR-SSP method (see references).
- The kit contains combinations of allele- or group-specific primer mixes aliquoted in 580 µl frozen portions.
- Setup includes pipetting the primer mixes into the PCR-Tray, mixing a reaction buffer (= Mastermix) with a human genomic DNA sample and Taq DNA Polymerase and adding the mixture to the primer mixes in the PCR-Tray.
- Closing the tray 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 a tables which are provided in the locus- and lot-specific manual.
- The test can be completed within approx. 1.5 hours after 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 BULK KITS have been designed, produced and extensively tested with well characterized reference DNAs (DNAs which have been serologically and molecularly e.g. by PCR-SSP/PCR-SBT) in our laboratory.

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Please note:

The usage of the CTS-PCR-SSP BULK KITS requires detailed knowledge of PCR techniques!

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

1. KIT COMPOSITION

1.1. General description

- The CTS-PCR-SSP BULK KIT contains PCR-primer mixes (here commonly called “mixes”) prepipetted in plastic tubes and stored in a rack. Specificities of the reagents contained in each tube are given in the locus- and lot-specific manual sent along with the kit you ordered.
- If you order a CTS-PCR-SSP BULK KIT, you will receive an aluminum pouch containing a plastic rack filled with 1.2 ml-tubes containing frozen primer mixes. Each tube contains 580 µl of primer mix sufficient for the typing of at least 106 individuals.
- Each position in the rack is labelled with a digit-letter combination from H1 to A12. Digits are visible on the top, letters on the left edge of the rack. The mixes are placed from the **starting position at H1**, followed by G1, F1 etc. (Figure 1). The mixes are numbered, starting with Mix No. 1 and ending either with the last mix number or with the Negative Control Mix (some kits do not have a Negative Control Mix). Please refer to the locus- and lot-specific manual to obtain information on the number of mixes provided by the kit you received and whether this particular kit contains a Negative Control Mix.
- An index card shows the Mix numbers and Mix positions, the name and the amount of the CTS-PCR-SSP BULK KITS provided in a rack.
- Along with the CTS-PCR-SSP BULK KITS, you will receive an adequate amount of Mastermix (see section 1.4). The Mastermix is a buffer which contains crucial ingredients required for a successful amplification (see section 1.3). DNA-Polymerase is not included in the kit.

In general, **7.5%** Mastermix must be used for the amplification of HLA-class **I** product, whereas **5.0%** Mastermix is optimal for HLA-class **II** amplification.

- In a **combination** kit (HLA-class I and HLA-class II typing), **7.5%** Mastermix is required.
- All reagents contained in the CTS-PCR-SSP BULK KITS have been prepared and extensively quality-controlled at the University of Heidelberg and are ready for use.

Figure 1

	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									

1.2 Kit Components

<i>Description of the component</i>	<i>Quantity</i>	<i>Storage Conditions</i>
96-well racks containing plastic tubes (bulk tubes) with 580 µl of frozen primer mix each.	For the exact number of mixes in a particular kit, please refer to the locus- and lot-specific manual.	Store at -20...-80°C until expiration date.
Mastermix	For the exact volumen supplied in the kit, please refer to section 1.4. of this manual.	Store at -20°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.
Working instruction/ product information	Each kit: <ul style="list-style-type: none"> • 1 Main Manual • 1 Locus- and lot-specific Manual • 1 Material Safety Data Sheet (MSDS) 	-----

1.3 Information on Ingredients

<i>Component</i>	<i>Chemical</i>
BULK tubes	PCR-primers = DNA Oligonucleotides
	Cresol Red
Mastermix	Ammonium Sulfate
	Tris Buffer
	Magnesium Chloride
	Glycerol (glycerin)
	Cresol Red
	Deoxyribonucleotides (dNTPs)

1.4 Mastermix

Number of Primer Mix tubes in the kit	Volumen of Mastermix in the kit
8	2.5 ml
16	5 ml
24	7 ml
32	9.5 ml
40	12 ml

48	14 ml
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1.5 Storage of Primer Mixes

1.5.1 Non-dried aliquots

Upon receipt, we strongly recommend to set up small individual portions (non-dried aliquots) (5 µl – 50 µl) of the different primer mixes in PCR-Trays or Eppendorf tubes. The aliquots should be stored until use at -20...-80°C. The mixes should be frozen and thawed no more than 3 times.

1.5.2 Dried aliquots

The mixes can also be pipetted into the PCR-Trays (5 µl in each well) and put out to dry (e.g. on a water bath or in an incubator). After the reagents have dried, the PCR trays with the dried aliquots can be stored at 2...8°C for at least 6 months.

1.6 Storage of Mastermix

We strongly recommend to set up small aliquots of the Mastermix. For the appropriate amount please refer to Table 4.1 and Table 4.2, Section 4 (Preparation of the PCR Reaction Mix). Aliquots can be stored at -20°C until expiration date. Thawed aliquots can be stored at 2... 8°C for at least 1 month.

2. MATERIALS, REAGENTS, AND EQUIPMENT NOT SUPPLIED

- 2.1 The following enzymes are validated for use with the CTS-PCR-SSP BULK KIT:
 - 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 Multipipette Type 4720 with 0.5 ml Combitips or Gilson® Distriman™ No. 11800 with DistriTips™ Minichannel pipettor: 5 - 50 ml adjustable volume: Finnpiquette, ThermoLabsystems, Cat. No. 4510 020
- 2.4 1.5 ml polypropylene safe-lock tubes:

Eppendorf, Cat. No 0030 120.086.
- 2.5 Thermo-Fast Non-Skirted, Low Profile PCR-Trays, Abgene, 25 plate Cat. No.: AB-0700
- 2.6 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
Use of other thermal cyclers must be validated by the user!
- 2.7 TAE electrophoresis buffer (1x TAE)
 (TAE = Tris, concentrated acetic acid (CH₃COOH), 0.5 M EDTA pH 8)

- 2.8 Ultra Pure Agarose, Gibco-BRL, Cat. No. 5510UA
- 2.9 Ethidium bromide (10 mg/ml)
Caution: ethidium bromide is mutagenic! Handle with appropriate personal protective equipment.
- 2.10 Lysis Buffer I:
0.3 M sucrose
10 mM Tris-HCl (pH 7.5)
5 mM MgCl₂
1% Triton x-100 (C34 H62 O11; MW= 646.87 g/mol).
Store in a dark place at +4°C.
- 2.11 Lysis Buffer II:
0.075 M NaCl
0.024 M Na-EDTA.
Adjust pH to 8 with 4 M NaOH and store at room temperature.
- 2.12 10% SDS
- 2.13 5 M sodiumperchlorate
- 2.14 6 M NaCl
- 2.15 Absolute isopropanol (99.9%)
- 2.16 70% ethanol
- 2.17 Electrophoresis equipment
Submarine DNA electrophoresis set:
CTS electrophoresis chamber and combs (CTS Product No.: 501)
- 2.18 Gel Documentation System:

UV-light transilluminator (312 nm):
Renner, D-6701 Dannstadt, Germany, Cat. No. 35 2452.

Polaroid camera with UV-light filter:
Renner, D-6701 Dannstadt, Germany, Cat.No. 35 497
(Use with polaroid films No. 667).
- 2.19 Photometric DNA measurement:
Lambda Scan 200, MWG, www.THE.MWG.com
PC operated photometer program: KC 4, Bio-Tek Instruments, Inc. www.biotek.com

The use of other equipments requires validation by the user!

3. SAMPLE REQUIREMENTS

- 3.1. DNA Sample in TE buffer or sterile water.
- 3.2. Use a total amount of 10 - 15 µg of genomic DNA for one full 96-well PCR tray (please refer to Table 4.1 and 4.2, Section 4. Preparation of the PCR Reaction Mix).

3.3. DNA Isolation

- 3.3.1 Collect 10 ml blood with EDTA anticoagulant or use 20 - 50 x10⁶ 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 2.10) 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 2.11), 125 µl of 10% SDS (see 2.12), and 1.1 ml of 5 M sodiumperchlorate (see 2.13). Shake vigorously for 10 sec at room temperature.
- 3.3.4 For extraction of proteins, add 2 ml of 6 M 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 (ddH₂O) by incubation at 55°C for 10 - 20 min.
- 3.3.8. Dilute 5 µl of the DNA solution in 495 µl of ddH₂O 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 **0.1– 0.15 µg/µl** with ddH₂O.
- 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 will go 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 If you use non-dried aliquots of primer mixes (as described in 1.5.1):

- Pipette 5 µl of each PCR-SSP-Primer Mix into a thermocycler plate (e.g. see 2.5)

- In a clean Eppendorf tube, set up a mixture of an appropriate amount of Mastermix, Aqua dest. and Taq Polymerase depending on the number of PCR (primer mixes). Refer to the Table 4.1 for exact amount of the reaction volume to be set up. Vortex well before pipetting!

Table 4.1 Amounts of reagents to be used for a PCR setup with non-dried aliquots

Number of PCR	Mastermix (µl)	Aqua dest. (µl)	Taq-Polymerase at 5 U/µl* (µl)	DNA at 0.1-0.15 µg/µl (µl)
8	23	11.5	0.48	8
16	46	23	0.96	16
24	69	34.5	1.45	25
32	92	46	1.93	33
48	138	69	2.9	50
72	207	103.5	4.35	75
96	276	138	5.8	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.

- Add the required amount of DNA (refer to Table 4.1). There are **two options**:
- **DNA included in the mixture:** If a Negative Control Mix is included in the BULK KIT you received (please refer to the locus- and lot-specific manual whether a Negative Control is included in your test kit), pipette 5 µl of the Mastermix-Taq-mixture (without DNA) *first* into the Negative Control well, *then* add the DNA to the mixture. Vortex well. Using an electronic dispensing pipettor, dispense 5 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! The total reaction volume in each well should be 10 µl.
- **DNA pipetted separately:** Using an electronic dispensing pipettor, dispense 4 µl of the reaction mixture (= Mastermix-Aqua dest.-Taq) into each well (5 µl into the Negative Control well, if this is included in your test 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. Add 1 µl of DNA (0.1 – 0.15 µg/µl) to each well (except the Negative Control well, if a Negative Control Mix is included in your test kit). The total reaction volume in each well should be 10 µl.
- Close the tubes tightly, using either Strips of 8 Domed Caps or adhesive PCR Film. Spin the PCR-tray briefly in an ELISA-tray centrifuge, and place it in the thermocycler immediately. **Ensure caps completely seal the wells to prevent evaporation!**

4.2 If you use dried aliquots of the primer mixes (as described in 1.5.2)

- Depending on the number of PCR (primer mixes), add the corresponding amounts of Mastermix, Aqua dest. and Taq Polymerase in a clean Eppendorf tube and vortex well before pipetting. Refer to the Table 4.2 for exact amount of the reaction volume.

Table 4.2 Amounts of reagents to be used for a PCR setup with dried aliquots

Number of PCR	Mastermix (µl)	Aqua Dest. (µl)	Taq-Polymerase at 5 U/µl * (µl)	DNA at 0.1 - 0.15 µg/µl (µl)
8	23	54.8	0.48	8
16	46	109.6	0.96	16
24	69	164.5	1.45	25
32	92	219.3	1.93	33
48	138	329	2.9	50
72	207	493.5	4.35	75
96	276	658	5.8	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.

- Add the required amount of DNA (refer to Table 4.2).
- **DNA included in the mixture:** If a Negative Control Mix is included in your BULK KIT (please refer to the Work Sheet of the kit for the information whether there is a Negative Control in your kit), pipette 10 µl of the Mastermix-Taq-mixture *first* into the Negative Control well, *then* add the DNA to the mixture. 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. The total reaction volume in each well should be 10 µl.
- **DNA pipetted separately:** Using an electronic dispensing pipettor, dispense 9 µl of the reaction mixture (= Mastermix-Taq) into each well (10 µl into the Negative Control well). Be careful to dispense the drops onto the side walls, near each well's top, allowing the dispensed drop to slide down. Add 1 µl of DNA (0.1 – 0.15 µg/µl) to each well (except the Negative Control well). Do not allow the pipette tip to touch the bottom of wells. The total reaction volume in each well should be 10 µl.
- Close the tubes tightly, using either Strips of 8 Domed Caps or adhesive PCR Film, spin the PCR-tray briefly in an ELISA-tray centrifuge, and place it in the thermocycler immediately. **Ensure caps completely seal the wells to prevent evaporation!**

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 in the holder against the bench. Sufficient volume is supplied to allow for pipetting losses.

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

5. Thermal Cycler: CTS-PCR-SSP BULK 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 BULK 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 of agarose and 7 ml of 50x TAE buffer to 350 ml of ddH₂O.
- 6.1.3 Boil to dissolve the agarose, using a magnetic stirring hot plate or a microwave oven.
- 6.1.4 Cool to 60°C, add 17 µl of 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 from the thermocycler.
- 6.2.2 Carefully remove the strip caps from the PCR-Tray.

Caution: Sudden movement of the tray 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 <small>(Pos.1-8)</small>	Lane 1 <small>(Pos.9-16)</small>	Lane 1 <small>(Pos.17-24)</small>	Lane 2 <small>(Pos.25-32)</small>	Lane 2 <small>(Pos.33-40)</small>	Lane 2 <small>(Pos.41-48)</small>	Lane 3 <small>(Pos.49-56)</small>	Lane 3 <small>(Pos.57-64)</small>	Lane 3 <small>(Pos.65-72)</small>	Lane 4 <small>(Pos.73-80)</small>	Lane 4 <small>(Pos.81-88)</small>	Lane 4 <small>(Pos.89-96)</small>
B	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
C												
D												
E												
F												
G												
H												

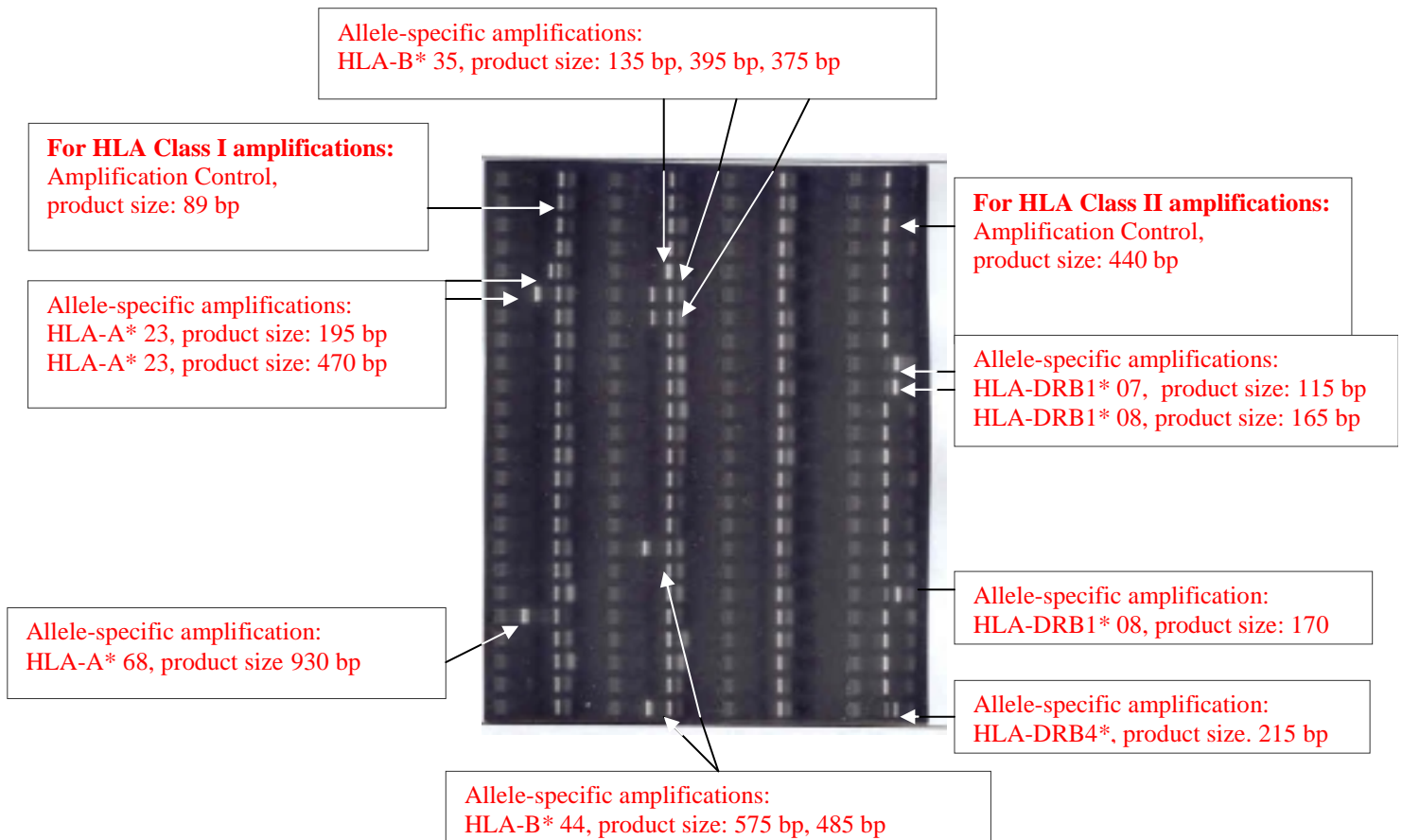
Note: The word 'well' refers to the Tray/Minitray/String location assignment, while the word 'lane' refers to a well's corresponding gel lane.

6.2.5 Run the electrophoresis for 20 min at 170 V (approx. 0.4 V/cm²).

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)

- 7.1.1 Each of the primer mixes contains a non-allelic amplification control primer pair which amplifies either a part of the β -globin gene (HLA Class I PCR-SSP Mixes) or a part of the C-reactive protein gene (CRP) (HLA Class II PCR-SSP Mixes).
- 7.1.2 The β -globin amplification control primers (only relevant for HLA-class I typing) should produce a **89 bp** fragment in all tubes in which an allele- or group-specific amplification is not present. 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 CRP amplification control primers (only relevant for HLA-class II typing) should produce a **440 bp** 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 locus- and lot-specific 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 test kit you received)

Any amplification band in the Negative Control lane 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.
- 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 manuals for more information on result interpretation of the kit you obtained.

7.5 Evaluation

- 7.5.1 Check the approximate size against the Primer Mix Specificity Table in the locus- and 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. In general, those 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 89 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.

- 7.5.2 Use the Typing Tables in the locus specific manual to make allele assignment, 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 allele-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 (> 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 general weak amplification might indicate that less than the required amount of sample DNA was used in the reaction (< 75 ng/µl per reaction) which may cause false negative reactions.
- 8.1.2 Degraded DNA may not amplify reliably with the CTS-PCR-SSP BULK KITS. 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 \Rightarrow verify activity of Taq with a known reference DNA sample.

8.2.7 Degraded primer mix or Mastermix \Rightarrow check expiration date of 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 failed lane)

Possible causes:

8.3.1 DNA is not evenly re-suspended in diluent \Rightarrow pipette DNA up and down several times to aid mixing.

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

8.3.3 Thermocycler defect \Rightarrow Check cycling conditions in failed cycler positions.

8.4 Problem: False positive results

Possible causes:

8.4.1 Excess of DNA or Taq Polymerase \Rightarrow measure DNA concentration with UV spectrophotometry and adjust the DNA concentration to 0.1 to 0.15 $\mu\text{g}/\mu\text{l}$; check or validate the amount of Taq Polymerase required.

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

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

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

8.4.5 Contaminations with PCR-product \Rightarrow perform wipe test.

8.5 Problem: False negative results

Possible causes:

8.5.1 Improperly calibrated thermal thermal cycler \Rightarrow recalibrate thermal cycler.

8.5.2 Incorrect order in gel loading \Rightarrow 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 \Rightarrow compensate for lost volume by adding water.

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

8.6.3. Overheating gel, too high voltage \Rightarrow lower voltage

8.6.3 Heavy streaking in random wells can be caused by uneven suspensions of DNA \Rightarrow 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 (10 mg/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(s), please do not hesitate to contact me or my coworkers at:

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