EN

Instructions for Use

HISTO TYPE Rainbow

Test kit for the determination of HLA alleles on a molecular genetic basis

IVD

CE 0123

REF 728220

Contents

Changes to version 01/2020 are marked in yellow

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1. INTENDED USE

The intended use of the HISTO TYPE Rainbow kit is the identification of HLA Class I and II alleles. HISTO TYPE Rainbow is an in vitro diagnostic test for tissue typing based on a molecular genetic basis (see Product Description).

2. PRODUCT DESCRIPTION

HISTO TYPE Rainbow kits are used for the molecular genetic determination of HLA Class I and II alleles at 11 loci: HLA-A, B, C, DRB1/3/4/5, DQA1, DQB1, DPA1 & DPB1. Kits are designed to generally detect all alleles at the 11 loci; if any rare alleles are not detected the alleles are listed in Kit Specific Information documents (KSI). The kit provides low to medium typing results of the common and well documented alleles based on the CWD 2.0.0 catalogue (1). The confirmed diagnostic results of HLA-alleles are a prerequisite for a successful organ transplantation.

3. TEST PRINCIPLE

The test is performed with genomic DNA as starting material. The DNA is amplified in a real-time PCR with sequence-specific primers (SSP). The primers were specially developed for the selective amplification of segments of specific HLA alleles or allele groups. The amplicons are detected using sequence-specific fluorescence dye-labelled hydrolysis probes (TaqMan®-probes), which increases the sensitivity and specificity of the test compared to the classical SSP.

If amplicons are present, the probes are hydrolysed by the Taq polymerase and a fluorescence signal is generated to enable detection of the amplicon. Five different wavelength ranges of fluorescence signals are measured by the optical detection unit of the real time PCR cycler. The presence of a positive reaction is determined primarily by the Cq point, which is the point where fluorescence signal increases beyond the baseline threshold. For amplification to be valid the amplification must also achieve a certain threshold of fluorescence at the end of the PCR process. This is to prevent false positive reactions.

Each PCR reaction also contains an internal amplification control (Human Growth Hormone gene (HGH)) which is detected in a specific fluorescent channel.

To distinguish positive reactions from negative or irrelevant amplifications the ratio of the Cq of the specific reaction compared to the Cq of the internal amplification is calculated. The thresholds for these Cq ratios (CqR) vary from reaction to reaction and hence the PlexTyper software is required for the analysis of amplification data.

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4. MATERIAL

4.1 Contents of the Rainbow kit

- **10x 230 µl Plex Mix**, ready to use, contains dNTPs, Taq Polymerase, reaction buffer.
- **10x HISTO TYPE Rainbow plates** for HLA-typing. The pre-pipetted and dried reaction mixtures contain HLA-specific primers and probes as well as HGH-specific control primers and probes (oligomixes).
- **10x qPCR Seal**

4.2 Additionally required reagents and devices

- Reagents for DNA isolation (validated extraction kits see 6.2)
- Real time-PCR Cycler (validated cycler see 4.3)
- Variable pipettes (0.5 – 1000 µl) and pipette tips
- Application spatula for qPCR Seal
- Molecular grade DNAse free water.
- Centrifuge (e.g. PlateFuge – MicroCentrifuge von Benchmark Scientific)

4.3 Validated real time-PCR Cycler

Real time-PCR Cycler
CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad)

The following fluorophores are used.

Fluorophore	Wave length in nm
FAM	Excitation: 495 Emission: 520
CAL Fluor® Orange 560	Excitation: 538 Emission: 559
CAL Fluor® Red 610	Excitation: 590 Emission: 610
Quasar® 670	Excitation: 647 Emission: 670
Quasar® 705	Excitation: 690 Emission: 705

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5. STORAGE AND STABILITY

The kits are shipped on blue ice. All reagents must be stored at ≤ -20°C in temperature-controlled devices. The expiry date is indicated on the label of each reagent. The expiry date indicated on the outer label refers to the reagent with the shortest stability contained in the kit. The freeze-thaw cycle testing has shown that up to 6 cycles for the Plex Mix has no detrimental effects on the quality of the kit.

6. TEST PROCEDURE

6.1 Precautions and special remarks

Molecular genetic techniques are extremely sensitive methods and should only be performed by qualified personnel with experience in molecular genetic techniques.

Special precautions must be met to to avoid contamination and thus false reactions:

- Wear suitable gloves (preferably powder-free) during work.
- Use tips with filter insert or integrated stamp.
- Work in two different areas for pre-amplification (DNA-isolation, preparation of the reactions) and post-amplification (detection); use two separate rooms if possible.
- Use devices and other materials only at the respective workplaces and do not exchange them.

6.2 DNA Isolation

The specimen material for the isolation of the genomic DNA must be sent in appropriate collection systems. For genomic material from whole blood use only EDTA or Citrate anticoagulants. The presence of Heparin may potentially inhibit the PCR reaction (2), therefore such collection systems are not suitable and must not be used. It is recommended to use CE IvD certified kits for the DNA isolation.

Validated DNA Extraction Kits:

- Qiagen QIAamp DNA Blood Kits (columns)
- ~~Automated DNA isolation with QIAcube~~

Both, the manual isolation and automated DNA isolation (QIAcube) are validated.

If the standard method established in the laboratory shall be applied for isolation of gDNA without using one of the specified test kits, it must be validated by the user.

The HISTO TYPE Rainbow test requires 10 – 20 ng DNA per well.
The purity indices must be in the following range:

- OD_{260} / OD_{280} = > 1,5 and < 2,0
  Higher values are an indicator for the presence of RNA, lower values indicate protein contamination.
- OD_{260} / OD_{230} = > 1,8
  Lower values indicate contamination with carbohydrates, salts or organic solvents.

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6.3 Amplification

A pre-mix consisting of Plex Mix, water and DNA is made for the amplification that is subsequently dispensed in the wells 1-95. In well 96 there is the negative control (no template control = NTC) which should only contain water and Plex Mix. For other DNA concentrations the pre-mix has to be modified accordingly (see below).

- The reaction volume for each PCR preparation is 10 µl.
- For a single well the following reagents must be pipetted into a reaction tube:

2 µl Plex Mix
1 µl DNA specimen
7 µl Molecular grade water

A **negative control (NTC)** should be performed. Therefore prepare a PCR reaction with molecular grade water instead of DNA.

2 µl Plex Mix
8 µl Molecular grade water

DNA concentration 10-20 ng/µl

- Add **805 µl** molecular grade water to the vial with 230 µl Plex Mix and mix (vortex briefly 1-3 sec).
- After mixing, pipette **10 µl** of the mix into the NTC well (well 96; position H12 -see also Figure 1 and 2).
- Then pipette **115 µl** DNA into the vial with the remaining Plex Mix-water-mixture and mix (vortex briefly 1 – 3 sec).
- Distribute 10 µl of the DNA/Plex Mix/water solution into each of the wells 1-95 of the HISTO TYPE Rainbow plate (please note figure 1 and 2). The NTC well (well 96; position H12) must not be filled with the DNA mix as this will cause the NTC to be positive and can invalidate the test!

DNA with other concentrations

- Add **8 µl** Molecular grade water and **2 µl** Plex Mix to the NTC (well H12). The NTC well (well 96; position H12) must not be filled with the DNA mix!
- Pipette the DNA and the molecular grade water into the remaining 228 µl Plex Mix according to the following table and mix (vortex briefly 1-3 sec).

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Depending on the concentration of DNA, pipette the applicable volumes in the table below to the 228 µl Plex Mix remaining in the vial after setting up the NTC:

Concentration of the DNA [ng/µl]	Molecular grade water [µl]	DNA volume [µl]
2	342	570
5	684	228
50	889	23
80	898	14
100	901	11
150	904	8
200	906	6
250	907	5
300	908	4
500	910	2

- Distribute **10 µl** of the DNA-Plex Mix-water solution into each of the wells 1-95 of the HISTO TYPE Rainbow plate.

Please note: When pipetting into the PCR wells it is important not to allow the pipette tip to contact the dried mix (dyed blue) in the bottom of the well. It is advisable to pipette to the side of the well to allow the 10 µl to mix by gravity with the dried mix (see Figure 1).

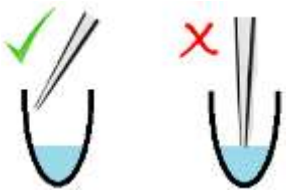

Figure 1

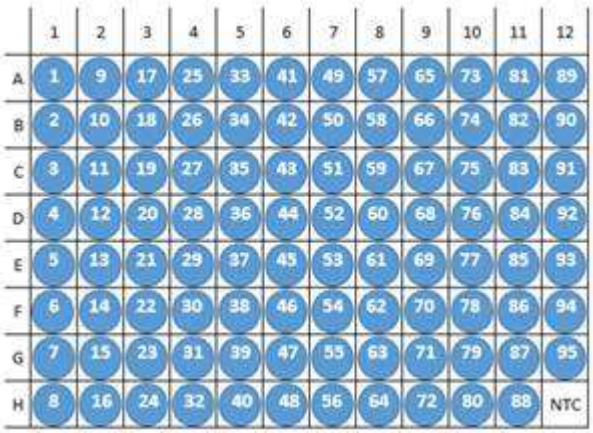

Figure 2: HISTO TYPE Rainbow plate. In wells A1 to G12 are the dried specific mixes (blue coloured). In well H12 there is the dried NTC (also blue).

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- Seal the PCR plate with the supplied qPCR seal and briefly spin the liquid down. Make sure that the plate is **completely sealed**, particularly at the edge of the plate. The seal must be free of bubbles to prevent evaporation during PCR. Make sure that the liquid has contacted the dried mix and there are **no bubbles** or air gaps in the reaction wells. If bubbles appear, gently tap the tubes on the laboratory bench to remove them, or preferably briefly spin it down (10 sec).

PCR-Program

Following the manufacturer's user guide of the real-time PCR cycler, set up a PCR run with the settings outlined below and using Scan Mode: **ALL CHANNELS**

Then perform the PCR reaction using the following parameters:

Step	Time [s]	Temperature [°C]	Ramp rate [°C/s]	Plate read	Cycles
Initial activation	120	96	2,5	-	1
Denaturation	5	98	2,5	-	13
Annealing + Extension	25	68	2,2	-	
Denaturation	5	98	2,5	-	37
Annealing + Extension	25	68	-	Yes	

The following real-time device is validated for use:

Bio-Rad: CFX96 Touch™ Real-Time PCR Detection System

Note With the CFX96 Touch™ Real-Time PCR Detection System a **modified heating rate** of the device (ramp rate) must be used. These are listed in the PCR program table above ("Ramp rate" column).

6.4 Evaluation and interpretation of the results

For evaluation and interpretation of the data it is mandatory to use the PlexTyper software (available free of charge from BAG Diagnostics) in conjunction with the kit specific data files. The kit files required for the evaluation are available from the BAG Diagnostics download server (www.service.bag-diagnostics.com).

Please make a note of the lot number of the kit. The interpretation kit files are product and lot specific. Use of incorrect kit files could result in incorrect genotyping. For interpretation of the results the raw data must be transferred from the thermocycler to a computer running the PlexTyper software (e.g. with a suitable USB drive).

Please follow the instructions for use for PlexTyper for interpretation of the data.

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It is possible, but not essential to perform a broad review of the data on the thermocycler software. For example, valid amplification must show suitable fluorescence signals for the internal amplification control in the FAM channel.

A negative control (NTC) is used as contamination control. If DNA or contaminating amplicon is inadvertently added to the NTC reaction a positive signal will occur. If the Cq is less than 36 it will be detected as possible contamination by the PlexTyper software and a warning message is generated. Amplification signals above Cq 36 in the NTC are regarded as PCR artefacts and are disregarded. If PCR contamination is suspected, it is advisable to follow local decontamination guidelines and to exchange the reagents.

The raw data collected from the cycler-specific software will be imported into the PlexTyper software. Based on the Cq values, RFUs (relative fluorescence units), quality scores and the curve progression the PlexTyper software determines the molecular genetic HLA pattern of the specimens used (see instructions for use for PlexTyper for details).

7. WARNINGS AND DISPOSAL INSTRUCTIONS

HISTO TYPE Rainbow is **designed for in vitro diagnostic use**. The kit should only be used by specially trained, qualified personnel. All work should be performed in accordance with Good Laboratory Practice.

All materials of biological origin used in the test to obtain DNA (e.g. blood) should be considered as potentially infectious. Therefore, appropriate safety precautions are recommended when handling biological materials as required by Good Laboratory Practice standards.

Biological materials must be inactivated before disposal (e.g. by autoclaving). Disposable materials must be autoclaved or incinerated after use.

Spilled potentially infectious material should be removed immediately with an absorbent paper towel and the contaminated area disinfected with an appropriate disinfectant or 70% Ethanol. Material used to remove spills must be inactivated before disposal (e.g. by autoclaving).

Disposal of all specimens, unused reagents and waste should be in accordance with the legislation of the respective country and the local authorities.

Microbial contamination of reagents while taking aliquots should be avoided. The use of sterile disposable pipettes and pipette tips is recommended. Do not use reagents looking cloudy or showing signs of microbial contamination.

A Material Safety Data Sheet (MSDS), respectively a declaration on Material Safety Data Sheets is available for download at **www.bag-diagnostics.com.**

8. KIT SPECIFICATIONS

The combination of primers and probes allows the determination of human HLA class I and II alleles according to lot-specific data (low to medium resolution, detection of all alleles with the exception of single rare alleles). The accuracy and reproducibility of the reactivity of the test kit is checked for

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each lot with control specimens with known HLA-alleles. The kit determines the HLA-Loci A, B, C, DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPA1 and DPB1.

8.1 SPECIFIC PERFORMANCE CHARACTERISTICS

A certain number of DNA samples pre-typed for all or some of the analysed HLA loci were tested with the HISTO TYPE Rainbow kit to show the correct reactivity. There was a 100% concordance of results with the pre-typings.

Locus	No of samples with previous result for the locus	Concordant	% Concordance
A	177	177	100%
B	177	177	100%
C	177	177	100%
DRB1	174	174	100%
DRB3	165	165	100%
DRB4	165	165	100%
DRB5	165	165	100%
DQA1	161	161	100%
DQB1	171	171	100%
DPA1	117	117	100%
DPB1	141	141	100%

Validation tests have shown that variation of the amount of DNA from 5 ng to 50 ng per reaction has no significant effect on the specific detection of HLA alleles.

9. LIMITS OF THE METHOD

During DNA isolation, special attention must be paid to the fact that the RT-PCR method reacts very sensitively to cross-contaminations. Special care should be taken to avoid contamination of kit reagents and other laboratory materials with amplicons or DNA.

The performance of a negative control without DNA in well H12 is strongly recommended. No fluorescence signal below 36 Cq should be detected in the NTC (H12) with molecular grade water . In the case of signal development in the negative control, the PCR laboratory workplace may have to be decontaminated from DNA and the reagents exchanged if necessary.

All devices (e.g. pipettes, real-time cyclers) must be calibrated according to the manufacturer's specifications.

10. INTERNAL QUALITY CONTROL

Internal quality controls for new lots can be performed with a combination of DNA specimens with known HLA type. An internal control to verify successful amplification is included in the dried oligomixes.

Performance of negative controls (well H12) to detect possible contaminations is recommended. For this purpose, prepare a test without DNA (NTC), see chapter 6.3. Amplification.

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11. TROUBLESHOOTING

For interpretation trouble shooting please see the Instructions for Use for PlexTyper Software.

Symptom	Possible Reason	Potential Solution
FAM signals weak or absent across whole plate	Presence of an inhibitor in the DNA.	Try a different extraction or sample.
	Insufficient DNA in the reaction.	Repeat test with correct amount of DNA.
	Wrong amplification parameters.	Check PCR program.
	Contaminated or degraded DNA.	Check concentration / quality of DNA. Check DNA on a gel. Repeat DNA isolation.
	Degraded Fluorescent probes or primers.	Avoid exposure to light and frequent thawing and freezing. Observe storage conditions.
Poor or no FAM signal in individual wells	Bubbles in the reaction / residual liquid at the inner wall of the tube.	Careful pipetting. Spin down PCR plate.
	User error.	Ensure all wells receive the required volume of reagents.
	Evaporation of the reagents due to incorrect closing of the PCR tubes.	Make sure that the PCR tubes are closed properly. Caution with adhesive foils in the edge area.
Signal in the Negative Control	Contamination with DNA or amplicon in the negative control.	Repeat the test. Decontaminate the workplace.
	Amplification due to PCR artefact.	Review after import into PlexTyper, if the signal may be below the thresholds and the data may in fact be OK (please note the PlexTyper Instructions for Use).

12. TRADE NAMES USED

TaqMan® is a tradename of Roche Molecular Systems Inc.

® Cal Fluor & Quasar Dyes are the registered trademark of LGC Biosearch Technologies

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13. EXPLANATION OF THE SYMBOLS USED ON THE LABELS

	Sufficient for n tests
	Storage temperature / lower limit of temperature
	Consult to instruction for use
	Manufacturer
	Use by
CONT	Content, contains
eIFU	Electronic instructions for use
HLA TYPING	Intended use: HLA typing
IVD	For in vitro diagnostic use
LOT	Batch code
PCRFOIL	PCR foil
PCRPLATE	PCR plates
PLEX MIX	Plex Mix: Mastermix, contains dNTPs, Taq polymerase, reaction buffer
REACTIONMIX	Reaction mixes
REF	Catalogue number
RTU	Ready to use

14. Literature

1. Cano, P. et al, 2007. Human Immunology 68, 392–417
2. Beutler, E. et al., 1990. BioTechniques 9:166

For further information please refer to our website www.bag-diagnostics.com or contact us directly at info@bag-diagnostics.com

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