

EN

Instructions for Use

HISTO TYPE Rainbow LC480

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

IVD

CE 0123

REF 728222

Contents

1. INTENDED USE	2
2. PRODUCT DESCRIPTION	2
3. TEST PRINCIPLE	2
4. MATERIAL	3
4.1 Contents of the HISTO TYPE Rainbow LC480 kit	3
4.2 Additionally required reagents and devices	3
4.3 Validated real time-PCR Cycler	3
5. STORAGE AND STABILITY	4
6. TEST PROCEDURE	4
6.1 Precautions and special remarks	4
6.2 DNA Isolation	4
6.3 Entering sample information into PlexTyper® Software	5
6.4 Amplification	5
6.5 Export of results from LightCycler® 480 II	9
6.6 Evaluation and interpretation of the results	10
6.7 Import of the results file into the PlexTyper® software	11
7. WARNINGS AND DISPOSAL INSTRUCTIONS	11
8. KIT SPECIFICATIONS	12
8.1.1 Diagnostic sensitivity and specificity	12
8.1.2 Analytical sensitivity and measuring range	13
8.1.3 Analytical specificity / cross reactive substances	13
9. LIMITS OF THE METHOD	13
10. INTERNAL QUALITY CONTROL	13
11. TROUBLESHOOTING	14
12. TRADE NAMES USED	14
13. EXPLANATION OF THE SYMBOLS USED ON THE LABELS	15
14. LITERATURE	15

Version: 01/2021 / Issue: 2021-04

1. INTENDED USE

The intended use of the HISTO TYPE Rainbow LC480 kit is the identification of HLA Class I and II alleles using the Roche LightCycler® 480 II Real-Time PCR Detection System for PCR amplification. HISTO TYPE Rainbow LC480 is an in vitro diagnostic test for tissue typing on a molecular genetic basis (see Product Description).

2. PRODUCT DESCRIPTION

HISTO TYPE Rainbow LC480 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) which are available from the download section of the BAG website. The primer and probe binding sites are listed there as well. The kit provides low to medium resolution typing results of the common and well documented alleles using CWD list 2.1.0 which is largely based on CWD 2.0.0 list (1). The CWD list 2.1.0 used is available from the document download section of the BAG website. 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.

4. MATERIAL

4.1 Contents of the HISTO TYPE Rainbow LC480 kit

- **10x 230 µl Plex Mix**, ready to use, contains dNTPs, Taq Polymerase, reaction buffer.
- **10x HISTO TYPE Rainbow LC480 plates** for HLA-typing. The pre-pipetted and dried reaction mixtures in each well contain HLA-specific primers and probes as well as HGH-specific internal amplification 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)
- Plate holder LC480 (REF 726320)
- Variable pipettes (0.5 – 1000 µl) and pipette tips
- Application spatula for qPCR Seal
- Molecular grade DNase free water
- Suitable plate centrifuge

4.3 Validated real time-PCR Cycler

Real time-PCR Cycler
LightCycler® 480 II Real-Time PCR Detection System, Roche Molecular Systems Inc. (LC480)

Please note that the light sources utilised in LightCycler® 480 II instruments can either be xenon lamp or LED. From LightCycler® 480 II serial numbers 29001 onwards it is an LED lamp, before there instruments contained a xenon lamp. The test has been validated on an instrument with LED lightsources. PlexTyper® software utilises a built-in colour compensation file for LC480 analysis which may be sub-optimal for xenon lamp machines. Please contact BAG Diagnostics if you have an instrument with a xenon lamp or your results are sub-optimal.

The following fluorophores are used in the HISTO TYPE Rainbow LC480 kits.

Fluorophore	Wavelength in nm
A425 (ATTO 425)	Excitation: 436 Emission: 484
F495 (FAM)	Excitation: 495 Emission: 520
O560 (CAL Fluor® Orange 560)	Excitation: 538 Emission: 559
R610 (CAL Fluor® Red 610)	Excitation: 590 Emission: 610
Q670 (Quasar® 670)	Excitation 647 Emission: 670

5. STORAGE AND STABILITY

The kits are shipped on blue ice. All reagents must be stored at $\leq -20^{\circ}\text{C}$ 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 12 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 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)

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 LC480 test requires 10 – 20 ng DNA per well.

The purity indices must be in the following range:

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

6.3 Entering sample information into PlexTyper® Software

It is mandatory to use PlexTyper® Software to analyse HISTO TYPE Rainbow data. It is advisable to enter the sample information into PlexTyper® before setting up the PCR amplification to obtain the unique RUN ID. All import files must be prefixed with a PT number.

When saving a test in PlexTyper®, the software assigns a **Run ID** to the test which is displayed in red in the summary window shown below. This is a consecutive number prefixed with PT (PT1, PT2, PT3....) which is linked to the sample identity and is used as a prefix on the PCR machine export file to correctly link the PCR export to the sample identity.

This Run ID should be used when setting up the file identity in the PCR machine. If the PCR xml export file is not pre-labelled with the RUN ID the file can be manually prefixed with the correct PT Run ID before import into PlexTyper®.



6.4 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 LC480 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

For DNA of different stock concentrations use the dilution table below to adjust the DNA to working concentration.

- 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).

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 LC480 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).

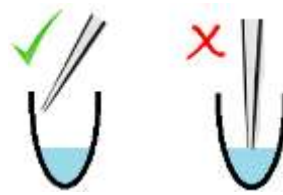


Figure 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	NTC

Figure 2: HISTO TYPE Rainbow LC480 plate. In wells A1 to G12 are the dried specific mixes (blue coloured). In well H12 there is the dried NTC (also blue).

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. 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 careful spin it down (10 sec).

Place the sealed PCR plate in the LC480 instrument. Please use the correct plate holder and orientation as per the notes below.

Note: Please make sure that you place the reaction plate into the cycler in the correct orientation as shown in Figure 2 above where the NTC well is in the bottom right corner, position H12.

Note: For the HISTO TYPE Rainbow plates a special plate holder for the Lightcycler 480 II is required, which is included in the first starter package (REF 726320). For more information please contact BAG Diagnostics.

PCR-Program

Following the manufacturer’s user guide for the LightCycler® 480 II, set up and save a PCR protocol with the following parameters:

Amplification parameters

Detection Format: HISTO TYPE RAINBOW, **Block size** 96, **Reaction volume** 10 µl

Step	Cycles	Analysis Mode	Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp rate (°C/s)
Hold	1	None	96	None	00:02:00	2.5
Cycle	13	None	98	None	00:00:05	2.5
			68	None	00:00:25	2.2
Cycle	37	Quantification	98	None	00:00:05	2.5
			68	Single	00:00:25	2.2

Channels for LightCycler® 480 Real-Time PCR Detection System

Use the following channel set up.

Excitation	Emission					
	488	510	580	610	640	660
440	✓					
465		✓				
498						
533			✓	✓		
618						✓


Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
440	488	A425 (ATTO425)	1	10	1
465	510	FAM	1	10	1
533	580	O560 (CalFluor Orange560)	1	10	1
533	610	R610 (CalFluor Red610)	1	10	1
618	660	Q670 (Quasar670)	1	10	1

Instrument Settings for Plate Type: White Plates

It is not necessary to utilise colour compensation facility in the LightCycler® 480 II program as the PlexTyper® software performs these calculations during analysis.

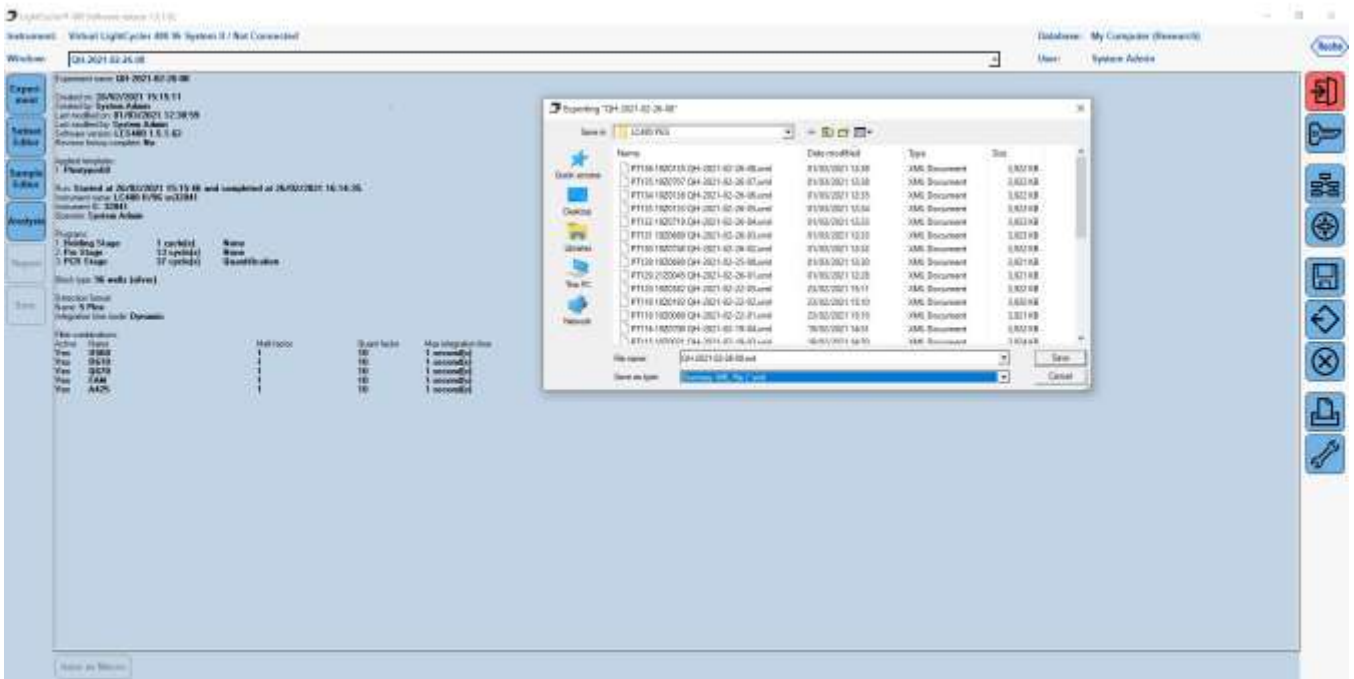
6.5 Export of results from LightCycler® 480 II

PlexTyper® utilises xml files from LightCycler® 480 II instruments. After the run is complete there is no requirement for analysis in the Roche software. Export the raw data in XML form with the following steps.

- 1) If not already open use the Roche software to open the Roche .IXO file.
- 2) Navigate to the export function  in the Roche software.



- 3) Select Export. The windows pop up will allow you to 'save as type'. Please select xml from the drop down menu. Export the run file to a suitable location for import into PlexTyper® software.



If the XML file has not been set up in the LightCycler® 480 II with the PT prefix number it should now be given the correct prefix so that it looks similar to the image below.

Name	Date modified	Type	Size
PT199 Rainbow run DNA 126F.xml	14/01/2021 16:24	XML Document	3,963 KB

6.6 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 file. 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).

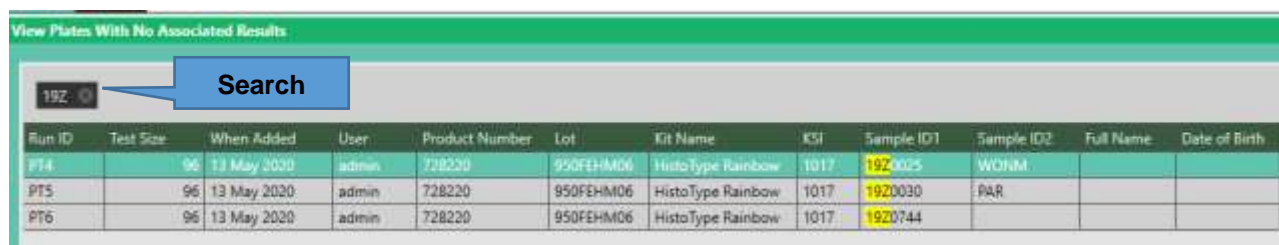
HISTO TYPE Rainbow LC480 is a 5-channel, 96 well multiplex genotyping test. It is possible to view data on the amplification machine software, but it is not possible to interpret without use of the PlexTyper® software. For LightCycler® 480 II analysis colour compensation must be applied, which occurs in the PlexTyper® software.



6.7 Import of the results file into the PlexTyper® software

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

Open the PlexTyper® software. From the home screen choose **View plates with no associated results** under **Plates**. A list opens with all tests that are not linked with raw data yet. There is a global **Search** field at the top of the table to search the whole table.



Run ID	Test Size	When Added	User	Product Number	Lot	Kit Name	KSI	Sample ID1	Sample ID2	Full Name	Date of Birth
PT4	96	13 May 2020	admin	728220	950FEHM06	HistoType Rainbow	1017	1920025	WONM		
PT5	96	13 May 2020	admin	728220	950FEHM06	HistoType Rainbow	1017	1920030	PAR		
PT6	96	13 May 2020	admin	728220	950FEHM06	HistoType Rainbow	1017	1920744			

Double click on the required test to be interpreted; this opens the results summary window. Select **Import File** and select the XML file with the correct PT RUN ID prefix (e.g. PT4.xml) exported from the LightCycler.

Initial loading of the kit file data takes approximately 45 seconds, unless it is already open, the bottom left corner of the screen shows a progress bar. After that the results for user review are presented.

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 LC480 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 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

8.1.1 Diagnostic sensitivity and specificity

A total of 114 of DNA samples pre-typed for the relevant HLA loci or international reference cell lines were tested with the HISTO TYPE Rainbow LC480 kit to show the correct reactivity. There was a 100% concordance of results with the pre-typings for all loci except DPA1. A single DPA1 discrepancy was identified in cell line IHW01059, where in addition to the expected DPA1*01:04 a DPA1*01:03 was identified as well. It is expected that in fact the cell line sample has only been reported for one allele previously and it is expected that this will be resolved by genotyping the sample on another CE marked system.

Locus	No of samples with previous result for the locus	Concordant	% Concordance
A	114	114	100%
B	114	114	100%
C	114	114	100%
DRB1	113	113	100%
DRB3	111	111	100%
DRB4	111	111	100%
DRB5	111	111	100%
DQA1	112	112	100%
DQB1	112	112	100%
DPA1	111	110	99%
DPB1	112	112	100%

8.1.2 Analytical sensitivity and measuring range

Validation tests have shown that variation of the amount of DNA from 8 ng to 30 ng per reaction has no significant effect on the specific detection of HLA alleles.

8.1.3 Analytical specificity / cross reactive substances

Eight substances that might interfere with the assay were tested and the following concentrations were shown to have no detrimental effect on the results:

Substance	Maximal non-inhibitory concentration
Protein (BSA)	0.2 mg/ml
TE (Tris/EDTA, pH 8.0)	7 mM Tris, 0.7 mM EDTA
NaCl	20 mM
Ethanol	1%
Haemoglobin	0.01 mg/ml
Sodium Citrate	7 mM
DNA extraction buffer 1 (Qiagen QIAamp DNA Blood Kits)	1%
DNA extraction buffer 2 (Qiagen QIAamp DNA Blood Kits)	2%

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 section 6.4 Amplification.

11. TROUBLESHOOTING

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

Symptom	Possible Reason	Potential Solution
ATTO 425 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 ATTO 425 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 or the data may in fact be OK (please note the PlexTyper® Instructions for Use).






12. TRADE NAMES USED

LightCycler® 480 II is a tradename of Roche Molecular Systems Inc.

TaqMan® is a tradename of Roche Molecular Systems Inc.

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

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. Mack, S.J. et al., 2013. Tissue Antigens 81, 194–203
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