EN

Instructions for Use

Weak D-TYPE 1-2-3 Q

Electronic instructions for use see www.bag-diagnostics.com

CE IVD

Test kit for second line determination of RHD alleles RHD*01W.1, *01W.1.1, *01W.2 and the *01W.3 on molecular genetic basis

ready to use

REF 728400

Contents

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1. Product description

The Weak D-TYPE 1-2-3 Q kit is an in vitro diagnostic medical device for use by qualified personnel. The kit is used as a second line test following discrepant or doubtful Rhesus D antigen determination yielded from serological typing and in the case of serological weak Rhesus D antigen expression.
The composition of the oligonucleotide mix allows the specific and qualitative typing of Rhesus D RHD*01W.1, *01W.1.1, *01W.2 and the *01W.3 alleles on molecular genetic basis as further determination test.

2. Test principle

The test is performed with genomic DNA as starting material. The DNA is amplified with sequence specific primers (SSP).The primers were specially developed for selective amplification of the exons 1, 6 and 9 of the RHD gene, which do only recognize the RHD*01W.1, *01W.1.1, *01W.2 and the *01W.3 types. The amplicons are detected with likewise gene locus specific hydrolyse probes (TaqMan® probes) using real-time PCR (RT PCR) technique, which increases the diagnostic sensitivity and specificity of the test compared to a conventional SSP-Technology.
If amplicons are present, the probes are hydrolysed by the Taq polymerase and a fluorescence signal is generated that increases proportionally to the amount of the PCR product. The flourescence signals are measured by optical detection unit of the real-time PCR Cycler. The test is performed in a single PCR reaction that detects the internal amplification control (IAC: human HBB gene) and the associated types with different fluorescent colours.

3. Material

3.1. Contents of the Weak D-TYPE 1-2-3 Q kit

- **230 µl Q Primermix WD123**, ready to use, contains primers and probes
- **230 µl Q Mastermix**, ready to use, contains dNTPs,Taq Polymerase, reaction buffer
- **Instructions for use**

3.2. Additionally required reagents and devices

- Reagents for DNA isolation (validated DNA isolation kits see 5.2)
- Real-Time PCR-Cycler (validated cycler see 3.3)
- RT PCR reaction tubes and closing system (validated products see 3.3)
- Aqua dest.
- Piston pipettes (0,5 – 1000 µl) and tips

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3.3. Recommendation for validated cyclers and reaction tubes

Cycler	RT PCR reaction tubes	RT PCR closing system
CFX96™ Real-Time PCR Detection System, Comp. Bio-Rad.	FrameStar® Break-A-Way PCR Plate, 96 white wells, black frame, Product No. 4ti-1201 Comp. 4titude/Brooks Life Sciences	4titude Crystal Strips, Product No. 4ti-0755 Optically clear adhesive film, Product No. 4ti-0560. Comp. 4titude//Brooks Life Sciences
MIC (Magnetic Induction Cycler) Comp. Bio Molecular Systems	Tubes and caps: MIC-TUBES No. 68MIC-60653 Exclusive distributor (D/A): Comp. Biozyme	
Rotor-Gene Q, Comp. Qiagen	Strip Tubes and Caps, 0.1 ml, Cat No./ID 981103 or 981106, Comp. Qiagen	

Special note: If other realtime cyclers, reactions tubes and closing systems are used they must be validated by the user.

4. Storage and stability

The kit is shipped at ambient temperature. Upon receipt store all reagents in temperature monitored devices at ≤ -20 °C. 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 15 cycles has no detrimental effects on the quality of the kit. The fluorophor labelled probes are very light sensitive. Store the reagents in dark.

5. Test procedure

5.1. Safety conditions and special remarks

Molecular genetic techniques are particularly sensitive and should be performed by well trained personnel experienced in molecular genetic techniques. The results of these techniques must not be used as sole basis for clinical decisions.

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

- Wear gloves during work (powder-free, if possible).
- Use new tips with each pipetting step (with integrated filter).
- If possible, use separate working areas for pre-amplification (DNA isolation and PCR set up) and post-amplification (detection).
- Use devices and other materials only at the respective places and do not exchange them.

5.2. DNA Isolation

The sample material for the isolation of genomic DNA must be sent in appropriate blood collection systems. For the test EDTA or Citrate blood is required. The presence of heparin potentially inhibits PCR; therefore blood collection systems with heparin are not suitable (3) and must not be used. It is recommended to use CE IvD certified kits for the DNA isolation.

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Validated DNA isolation kit:

- Qiagen QIAamp DNA Blood Kits (columns)

If the established standard method of the lab is used for gDNA isolation and this is not the validated kit above, it must be validated by the user.

A DNA concentration of 10-150 ng/µl is required to perform the Weak D-TYPE 1-2-3 Q test.

The DNA must have the following purity indexes:

- OD_{260}/OD_{280} = > 1.5 and < 2.0
  Higher values are an indicator for contamination with RNA, lower values for a contamination with proteins.
- OD_{260}/OD_{230} = > 1.8
  Lower values indicate a contamination with salt, carbohydrate or organic solvents.

5.3. Amplification

Reaction tubes recommended by the manufacturer of the realtime cycler or the materials recommended in chapter 3.3 should be used.

For each sample the following reagents are pipetted into a reaction tube:

2 µl Q Primermix
2 µl Q Mastermix
1 µl Sample DNA (10-150 ng/µl)
5 µl A. dest.

The reaction volume for each RT PCR test is 10 µl.

Exception: If the Rotor-Gene Q cycler is used, the reaction volume for each RT PCR preparation is 20 µl. Accordingly, the indicated volumes must be duplicated for each RT PCR preparation.

If a premix of Q Primermix, Q Mastermix and Aqua dest. is prepared for more than one sample please allow for a reasonable additional amount for pipetting losses.

If a **negative control (NTC)** should be performed prepare a PCR reaction with Aqua dest. instead of DNA.

Close the reaction tubes and briefly spin down the liquid. Ensure that no bubbles are present in the wells. If bubbles are observed, gently tap assay on the bench to remove the bubbles. Avoid direct sun light while pipetting. Start the PCR program with the following parameters.

Step	Time	Temperature	No. of cycles
Initial activation	10 min	96°C	1 cycle
Denaturation	20 sec	96°C	40 cycles
Annealing + Extension	40 sec + reading	64°C	

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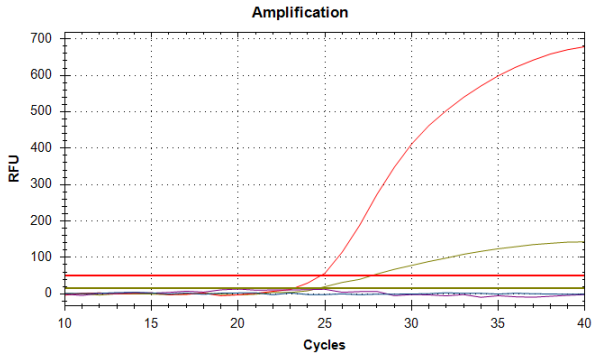
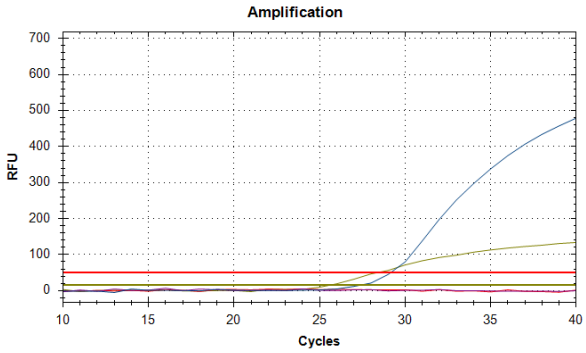
- If Magnetic Induction Cycler (MIC) or the CFX96™ Real-Time PCR Detection System is used the default settings should be used.
- If the Rotor-Gene Q cycler is used, the reaction volume for each RT PCR preparation is 20 µl. The function "Use noise slop correction" can be used for data analysis.

5.4. Interpretation of results

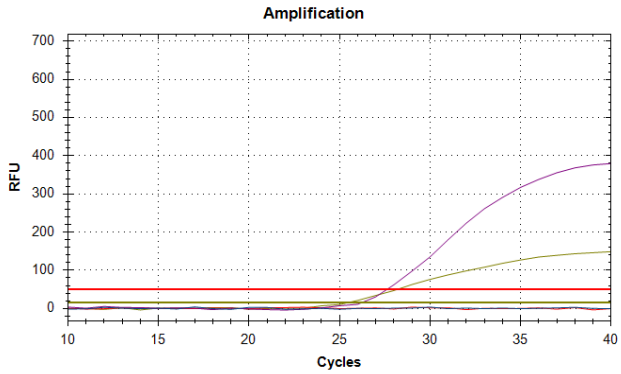
All tests with human gDNA must show a fluorescence signal in channel 2 (yellow- VIC channel) with the internal amplification control (IAC). RHD*01W.1 positive samples show a positive signal in channel 3. RHD*01W.2 positive samples show a positive signal in channel 1. RHD*01W.3 positive samples show a positive signal in channel 4. A RHD*01W.1.1 positive sample shows a positive signal in channel 3 and 4 as shown in the pictures below.



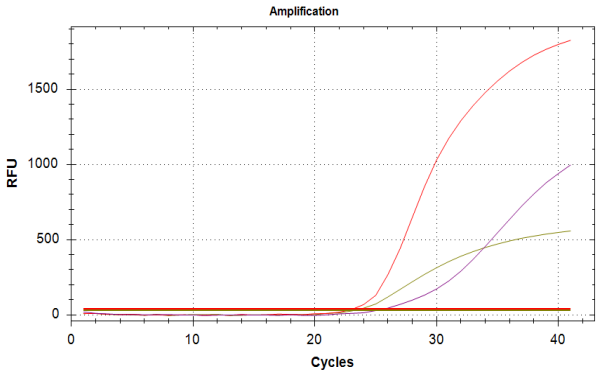
Picture 1: Weak D type 1 sample (RHD*01W.1) **red**- TexasRed Channel



Picture 2: Weak D type 2 sample (RHD*01W.2) **blue-** FAM Channel



Picture 3: Weak D type 3 sample (RHD*01W.3) **purple**- CY5 Channel



Picture 4: Weak D type 1.1* sample (RHD*01W.1.1) **red** and **purple** Channel

*Can not exclude
RHD*01W.1 in combination with RHD*01W.3

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Channel	Specificity[#]
Channel 3/Texas Red (weak D type 1 positive)	RHD*01W.1 RHD*weak D type 1
Channel 1/FAM (weak D type 2 positive)	RHD*01W.2 RHD*weak D type 2
Channel 4/Cy.5 (weak D type 3 positive)	RHD*01W.3 RHD*weak D type 3
Channel 3+4/ Texas Red+Cy.5 (weak D type 1.1 positive)	RHD*01W.1.1 RHD*weak D type 1.1

[#] The following alleles can not be excluded : RHD*01W.1.2; RHD*01W.2.1, 2.2; RHD*01W.3.1, 3.2.

The amplification signals for samples which has no weak D type 1, 1.1, 2, or 3 should be outside the defined Cq values for the channels 1,3 and 4. A negative control (No Template Control = NTC) with Aqua dest. should not show any fluorescent signal during the complete RT-PCR run and represents a contamination control. Fluorescence signals within the defined Cq values with the negative control with Aqua dest. indicate contamination. Flourescence signals outside the defined Cq values can occur due to the very sensitive test method in case of inaccurate pipetting. If this occurs, the test should be repeated.

The following signals are rated as positive:

	Channel/Dye	Pre-defined threshold	Cq-Level	LOD-Cq	Wave lenght in nm
RHD*01W.1, positive	Channel 3/ Texas Red	100	21	29	Excitation: 597 Emission: 616
RHD*01W.1.1, positive	Channel 4/ Cy. 5	100	25	34	Excitation: 651 Emission: 674
	Channel 3/ Texas Red	100	21	29	Excitation: 597 Emission: 616
RHD*01W.2, positive	Channel 1/ FAM	100	25	31	Excitation: 495 Emission: 520
RHD*01W.3, positive	Channel 4/ Cy. 5	100	25	34	Excitation: 651 Emission: 674
Internal amplification control	Channel 2/ VIC	50	19	29	Excitation: 538 Emission: 554

Special Note: The pre-defined threshold should only be chosen for the combination of a CFX96 cycler with white PCR reaction tubes. The channels 1 - 4 refer to the CFX96 real time cycler and may differ to other real time cycler systems. If use Magnetic Induction Cycler the auto set threshold should be chosen.

Cq-level is the PCR cycle that shows a positive detection against the background.

LOD-Cq is the latest PCR cycle that can be correctly rated as a positive detection against the background.

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6. Warnings and precautions

Weak D-TYPE 1-2-3 Q is designed for in vitro diagnostic use and should be used by properly trained, qualified staff only. All work should be performed using Good Laboratory Practices.

Biological material used for extraction of DNA, e.g. blood, should be handled as potentially infectious. When handling biological material appropriate safety precautions are recommended (do not pipet by mouth; wear disposable gloves while handling biological material and performing the test; disinfect hands when finished the test).
Biological material should be inactivated before disposal (e.g. in an autoclave). Disposables should be autoclaved or incinerated after use.
Spillage of potentially infectious materials should be removed immediately with absorbent paper tissue and the contaminated areas swabbed with a suitable standard disinfectant or 70% alcohol.
Material used to clean spills, including gloves, should be inactivated before disposal (e.g. in an autoclave).
Disposal of all samples, unused reagents and waste should be in accordance with country, federal, state and local regulations.
Microbial contamination of the reagents while taking aliquots should be avoided. It is recommended to use sterile one way pipettes and tips. Reagents that look cloudy or show any signs of microbial contamination must not be used.

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

7. Specific performance characteristics

The combination of primers and probes ensures a reliable identification of the Rhesus D alleles specified in chapter 5.4. The accuracy and reproducibility of the specificity of the kit is verified for each lot with pre-typed reference samples.

For the Weak D-TYPE 1-2-3 Q kit performance evaluation studies a total of 526 pre-typed DNA samples were performed. The results from the study were compared to the results that were obtained with other CE certified typing reagents (amongs others serology, SSO, SSP) and/or sequencing. A concordance of 99,8 % was achieved.

	Tests In total without NTC	WD1	WD1.1	WD2	WD3	IAC	Concordance to reference [%]
Internal study	**335**	**18**	**2**	**6**	**5**	**335**	**100**
External study	**191**	**60**	**11**	**39**	**14**	**190**	**99,5**
Total	**526**	**78**	**13**	**45**	**19**	**525**	**99,8**

Table: Summary of the internal and external study results with percentage concordance to the reference typing and detection of Weak D type 1, 1.1, 2 and 3.

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8. Limitations of the method

Because of the high susceptibility of the RT PCR method for cross contaminations special care should be taken during DNA isolation.Validation tests in the course of performance evaluation study of the Weak D-TYPE 1-2-3 Q kit have shown that a variation of the amount of DNA used for the amplification between 10 ng and 150 ng do not have a significant influence on the detection of the weak D type 1, 1.1, 2, or 3 alleles.

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

In the negative control with Aqua dest, there must not be any fluorescent signal (Cq > N.A.). In the case of signal development in the negative control (channel 2) the PCR working place has to be decontaminated and the reagents have to be exchanged if necessary.

All instruments (e.g. pipettes, realtime cyclers) must be calibrated according to the manufacturers instructions.

9. Internal quality control

Internal quality control of new lots of the Weak D-TYPE 1-2-3 Q kit can be performed using a combination of DNA samples with known RH weak D types. An internal amplification control for successful amplification is contained in the Q Primermix. Negative controls to detect possible contaminations are recommended. Use a PCR reaction without DNA for this purpose.

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10. Troubleshouting

Symptom	Possible reason	Potential solution
Bad or no signal	Presence of an inhibitor.	Use fresh reagents.
	No gDNA in the reaction.	Repeat test. Take care of correct pipetting .
	Wrong amplification parameters.	Check PCR program and ramp rate.
	Contaminated or degraded DNA.	Check DNA concentration and quality. Check DNA on a gel. Repeat DNA isolation.
	Fluorescent probes or primers degraded.	Use fresh Q primermix. Avoid exposition to light and frequent thawing and freezing. Observe storage conditions!
	Bubbles in the PCR reaction / remaining liquid at the inner wall of the tube.	Careful pipetting. Spin down PCR plate.
	Incompatible or low quality RT-PCR plastic ware.	Use compatible and high quality plastic ware (see chapter 3.3)
	Wrong signal calculation due to abnormal amplification signals during the initial cycles of the run.	Application of corrective measures in the software (e.g. "apply fluorescence drift correction“ ;function from Bio-Rad or exclusion of the first five cycles from analysis)
	Evaporation of the reagents due to incorrect closing of the PCR tubes.	Make sure that the PCR tubes are closed properly. Be careful at the edges of sealing foils.
Signal in the negative control	Contamination with DNA in the negative control	Repeat the negative control. Decontaminate the workplace.

11. Trademarks used in this document/product

TaqMan® is a trademark of Roche Molecular Systems Inc.

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12. Explanation of symbols used on the labels

	Sufficient for n tests
	Storage temperature / Lower limit of temperature
	Use by
	Consult instructions for use
	Manufacturer
BLOOD TYPING	Intended purpose: Blood typing
IFU	Instructions for use
IVD	For in vitro diagnostic use
LOT	Batch code
Q Primermix | WD123	Oligonukleotide mix for typing weak D 1, 1.1, 2 and 3
Q Mastermix	Mastermix for the Weak D-TYPE 1-2-3 Q kit
REF	Catalogue number

13. Literature

1. Wagner FF et. al. Blood. 1999 Jan 1; 93(1):385-93.
2. Geoff Daniels, Human Blood Groups, 3rd edition.
3. Beutler, E. et al., 1990. BioTechniques 9:166

Find further information and instructions for use in other languages on our website http://www.bag-diagnostics.com or contact us directly at info@bag-diagnostics.com or phone: +49 (0)6404-925-125

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