EN

Instructions for use

BAGene SSP Kits

CE IVD

Test kits for determination of ABO blood groups, RH types, Kell, Kidd and Duffy systems, MNS system, rare blood group systems, HPA and HNA specificities on a molecular genetic basis

ready to use pre-aliquoted

REF	6640	**ABO-TYPE**
REF	6641	**ABO-TYPE variant**
REF	6645	**RH-TYPE**
REF	6646	**Partial D-TYPE**
REF	6647	**Weak D-TYPE**
REF	6648	**D Zygosity-TYPE**
REF	6650	**KKD-TYPE**
REF	6652	**MNS-TYPE**
REF	6653	**Rare-TYPE**
REF	6660	**HPA-TYPE**
REF	66701	**HNA-TYPE**

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1. Product description

The BAGene kits are used to determine blood group specificities of donors, recipients and pregnant women on a molecular genetic basis. The ABO-, ABO variant-, RH-, Partial D-, Weak D-, D Zygosity- and KKD-TYPE kits serve to complete, clarify and confirm serological results. The MNS-, HPA-, HNA- and Rare-TYPE kits can be used for molecular typing without additional serological tests, unless stated otherwise (consult your national regulations).
The basic material for typing with BAGene kits is purified leucocytic DNA. The test procedure is performed by using the Sequence Specific Primers (SSP)-PCR (see Fig. 1). This method is based on the fact that primer extension, and hence successful PCR, relies on an exact match at the 3'-end of both primers. As a result, the amplification is obtained only if the primers entirely match the target sequence. The product of the amplification is subsequently visualized by agarose gel electrophoresis.

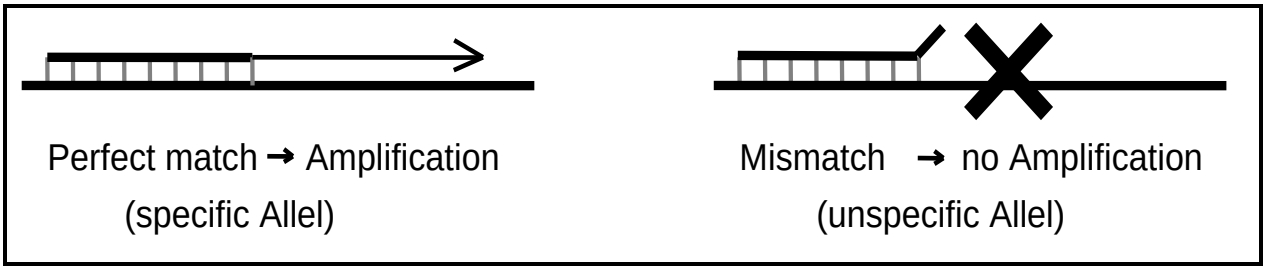


Fig. 1: Principle of SSP-PCR

The composition of the individual primer mixtures allows the clear identification of the ABO, RH, KEL, JK, FY, MNSs, rare blood groups, HPA and HNA genotypes indicated in the respective worksheet. Per typing a certain number of pre-aliquoted reaction mixes is used. An internal amplification control is included in each reaction mix.

2. Material

2.1 Contents of the BAGene kits

- PCR plates/strips for the blood group genotyping. The pre-aliquoted and dried reaction mixtures consist of allele specific primers, internal control primers (specific for the HGH gene (Human Growth Hormone) or for a sequence of the chromosome I (90 kbp 5' of Rhesus Box)) and nucleotides. The reaction mix No. 1 is marked. The lot number is printed on each plate/stripe.
- 10x PCR buffer
- 8 strip caps
- Instructions for use, worksheet
- Information for evaluation (only ABO-TYPE variant)

2.2 Required but not included material

- Happy Taq (REF 70976) (or another Taq Polymerase, validated with the BAGene kits by the user).The Happy Taq is supplied free of charge when ordering a BAGene Kit.
  Do not use a Hot-start Taq Polymerase (e.g. Ampli Taq Gold)!
- **EXTRA GENE I** kit (REF 7059) (optional) for DNA extraction from blood/lymphocytes/ leucocytes or material for other DNA extraction methods
- Piston pipettes (0.5 - 250 µl)
- Sterile tips with integrated filter
- Thermal Cycler (list of validated thermal cyclers, please see page 5)
- DNA agarose
- 0.5x TBE buffer (45 mM of Tris, 45 mM of boric acid, 0.5 mM of EDTA)
- Ethidium bromide (EtBr)
- Submarine electrophoresis unit
- Power supply (200 - 300 V, 200 mA)

- DNA-length standard (REF 7097)
- UV source (transilluminator, 220-310 nm)
- Gel documentation system

2.3. Storage and stability

The BAGene kits are delivered at ambient temperature. The Happy Taq will be shipped with dry ice. After delivery, store all reagents at ≤ -20°C. The expiry date is indicated on the label of each reagent and is also valid for opened reagents. The expiry date indicated on the outer label refers to the reagent with the shortest stability contained in the kit.
Thaw the 10x PCR buffer shortly before use.

3. Data of performance

The composition of the primer mixture guarantees a reliable identification of the alleles indicated in the worksheet based on the sequence data currently known.
The accuracy and reproducibility of the specificity of each primer mix were verified for each lot with DNA control samples with known specificities. Alleles, which are not included and that were currently not tested due to their rareness, are indicated on the worksheet (nt = not tested currently).
Performance studies with previously typed DNA samples were conducted for all BAGene kits. Some mixes could not be tested for a positive reaction because they are specific for rare alleles that were not available for testing. This is indicated on the evaluation diagram or specificity table. The investigations showed clear results in accordance with serological and/or genomical pretypings. There were no discrepancies during the studies.
The DNA samples used for the evaluation and quality control of the mixes were extracted with EXTRA GENE I (salting out method) or Qiagen kits (column based method).
The BAGene kits are validated with the Happy Taq (REF 70976). By using another Taq Polymerase, the enzyme must be validated with the BAGene kits by the user.
A reliable typing is guaranteed by using 50 - 100 ng DNA per reaction mix. An exception is represented by D Zygosity-TYPE. Due to a longer PCR program, for this product a lower DNA concentration of 30 - 50 ng per reaction mix should be used.

4. Test procedure

4.1 Safety conditions and special remarks

PCR is a highly sensitive method, which should be performed by well trained personnel with experience in molecular genetic techniques and blood group testing. Up to date guidelines on transfusion medicine, determination of blood groups and transfusion anamnesis should be observed in order to reduce the risk of false typings, especially when differing results are obtained with serological and molecular genetic methods. The genotyping of ABO, RHD/RHCE, Kell, Kidd and Duffy specificities has to be performed after a serological test.
Special safety conditions must be noted in order to avoid contamination and hence false reactions:

- Wear gloves during work (powder-free, if possible).
- Use new tips with each pipetting step (with integrated filter).
- Use separate working areas for pre-amplification (DNA isolation and preparation of the reactions) and post-amplification (gel electrophoresis, documentation); preferably use two separate rooms.
- Use devices and other materials only at the respective places and do not exchange them.

4.2 DNA isolation

The **EXTRA-GENE I** kit is suitable for the DNA isolation since pure DNA can be obtained from whole blood in a short time without the use of toxic chemicals or solvents. Furthermore commercial

column- or beads-based methods or other methods described in literature are suitable to provide DNA of sufficient purity. The presence of heparin potentially inhibits PCR. Therefore EDTA or Citrate Blood is recommended for typing.

DNA should have the following purity indexes:

- OD_{260}/OD_{280} = >1.5 and <2.0 (indicator for contamination with RNA/proteins)
- OD_{260}/OD_{230} = >1.8 (indicator for contamination with salt, carbohydrate or organic solvents)

4.3 Amplification

All pre-aliquoted reaction mixtures already contain allele and control specific primers and nucleotides. These are supplied dried down in the reaction tubes. Amplification parameters are optimized to a final volume of 10 µl.

1. Remove the required number of plates/strips from ≤ -20°C and thaw the 10x PCR-buffer.
2. Pipet the mastermix, consisting of 10x PCR-buffer, DNA-solution, Taq-Polymerase and Aqua dest. and mix well. The different BAGene kits work with the same mastermix and can therefore be combined, except the D Zygosity-TYPE for which another DNA concentration is recommended. The composition of the master mix is given in Table 1.

Table 1: Composition of the master mix depending on the number of reaction mixes

No. of mixes	Aqua Dest.	10x PCR buffer	DNA solution (50-100 ng/µl) ♦	Happy Taq (5 U/µl)	total volume	
1	8	1	1	0,08	10	µl
2	16	2	2	0,2	20	µl
6☆	50	7	7	0,5	65	µl
7	70	9	9	0,7	90	µl
8	80	10	10	0,8	100	µl
9	88	11	11	0,9	110	µl
10	96	12	12	1,0	120	µl
11	104	13	13	1,0	130	µl
12	112	14	14	1,1	140	µl
13	128	16	16	1,3	160	µl
14	136	17	17	1,4	170	µl
15	144	18	18	1,4	180	µl
16	152	19	19	1,5	190	µl

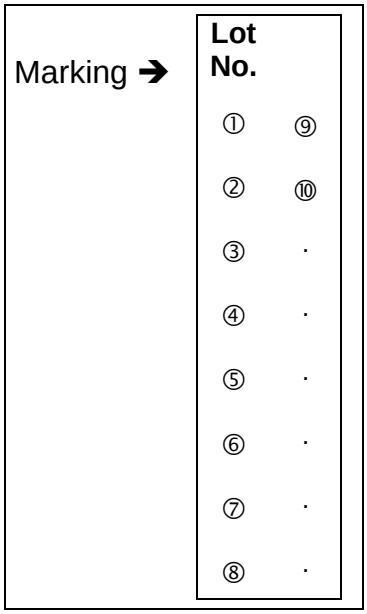
⇨ For different DNA concentrations, the quantities of DNA solution and water must be varied accordingly (e.g. for 12 mixes: DNA (120 ng/µl): use 5,8 µl DNA and 119 µl Aqua dest.).
If another Taq Polymerase shall be used, the enzyme must be validated with the BAGene kits by the user.

☆ Minimum preparation of master mix for 6 reaction mixes is recommended, due to the small volume of Taq-Polymerase.

♦ For **D Zygosity-TYPE** a DNA concentration of **30 – 50 ng/µl** is recommended.

3. After vortexing add 10 µl of this mixture immediately to the pre-aliquoted and dried reaction mixtures. Change the tip after each pipetting step. Tightly close the tubes with the respective caps. Ensure that you do not touch the inner side of the caps and the upper edges of the tubes with the fingers so that contamination is avoided. If thermal cyclers with tightly closable lid are used, it is also possible to use reusable PCR mats. Slightly shake the plate downwards to dissolve the pellet at the bottom of the plate. The complete PCR-solution should settle on the bottom.

4. Place the reaction tubes firmly into the thermal cycler and tighten the lid so that the reaction tubes do not warp in heating. Start the PCR program. Overlaying of the reaction mixtures with mineral oil is not required if a heated and adjusted cover is used!



Amplification parameters for all BAGene kits except D Zygosity-TYPE

Program-Step	Time	Temp.	No. of Cycles
First Denaturation	**5 Min**	96°C	1 Cycle
Denaturation	10 Sec	96°C	5 Cycles
Annealing+Extension	60 Sec	70°C	
Denaturation	10 Sec	96°C	10 Cycles
Annealing	50 Sec	65°C	
Extension	45 Sec	72°C	
Denaturation	10 Sec	96°C	15 Cycles
Annealing	50 Sec	61°C	
Extension	45 Sec	72°C	
Final Extension	5 Min	72°C	1 Cycle

Validated Thermal Cyclers

PTC 200 / C1000 (MJ Research/ BioRad)

GeneAmp PCR-System 9700 (use heating rate of 9600), Veriti (ABI)

Mastercycler epGradient S (use "simulate Mastercycler gradient" function) (Eppendorf)

Tprofessional (Biometra)

ATTENTION: Different PCR-program!
Amplification parameters for D Zygosity-TYPE

Program-Step	Time	Temp.	No. of Cycles
First Denaturation	**10 Min**	95°C	1 Cycle
Denaturation	20 Sec	92°C	35 Cycles
Annealing	30 Sec	64°C	
Extension	5 Min	68°C	
Final Extension	5 Min	72°C	1 Cycle

Validated Thermal Cyclers

See amplification parameters for the other BAGene kits

Please do not use an aluminium heating block (e.g. GeneAmp PCR-System 9700)!

By using thermal cyclers with a very fast heating and cooling rate, it is recommended to use a slower heating and cooling rate (~2.5°C/sec).

Since thermal cyclers of different manufacturers perform differently and even individual devices of one type may be calibrated differently, it may be necessary to optimize the amplification parameters.

To optimize your device use the following guidelines:

With **false positive** reactions (unspecific bands, additional types): Increase the annealing temperature by 1°C per step.

With **false negative** reactions (missing bands): Decrease the annealing temperature by 1°C per step and/or increase the annealing periods by 5 seconds per step and/or increase the denaturation periods by 5 seconds per step.

It is recommended to use only regularly calibrated thermal cyclers. For thermal cycler check we recommend the CYCLER CHECK kit (REF 7104, 71044).

4.4 Gel electrophoresis

Separation of the amplification products is done by electrophoresis via a horizontal agarose gel. As electrophoresis buffer, 0.5x TBE (45 mM of Tris, 45 mM of boric acid, 0.5 mM of EDTA) buffer is recommended. The gel concentration should be 2.0 - 2.5% of agarose. Allow the gel to polymerize at least 30 minutes before loading the samples.
After the amplification has been finished, take the samples out of the thermal cycler and load the complete reaction mixtures carefully in each well of the gel. In addition, apply 10 µl of the DNA length standard for size comparison. Electrophoretic separation is done at 10 - 12 V/cm (with 20 cm distance between the electrodes approx. 200 - 240 V), for 20 - 40 minutes. 40 minutes run is recommended in order to improve the separation of bands using D Zygosity-TYPE. After the run has been completed, the gel is stained in an ethidium bromide (EtBr) solution (approx. 0.5 µg/ml of EtBr in H_2O or TBE buffer). As an alternative, EtBr (0.5 µg/ml) can also be added to the electrophoresis buffer or the agarose gel. If required, excess of EtBr can be removed by soaking the gel in H_2O for 20 - 30 minutes.

4.5 Documentation

For documentation, visualize the PCR amplification using an UV transilluminator (220-310 nm) and photograph it with a suitable gel documentation system. Choose exposure time and aperture such that the bands are drawn sharp and stand out against the dark background (approximates aperture 11, exposure time 1 second). The results are documented on the provided worksheet (see point 4.6)

4.6 Interpretation of the results and limitations of the method

4.6.1 General

The results obtained with the BAGene kits are documented on the provided worksheets. In the worksheets the characteristics, specificities, phenotypes and genotypes are listed in a table and an example of a reaction pattern serves to support the interpretation. The PCR preparations have reaction numbers (e.g. ABO-TYPE reaction no. 1 - 8). Under the reaction numbers in the worksheet the fragment length of the specific PCR products is indicated in bp. Possible band patterns in the gel are shown in the lines below. Specific PCR products (positive reactions) are designated as + and the corresponding boxes of the diagram have a coloured background. ABO-TYPE, ABO-TYPE variant, Partial D-TYPE, Weak D-TYPE, D Zygosity-TYPE, KKD-TYPE, MNS-TYPE, Rare-TYPE, HPA-TYPE and HNA-TYPE are highlighted in **grey**, RH-TYPE additional in **red, green** and **blue**. The evaluation of the reaction patterns is carried out in the lines from left to right.
Only bands which show the correct size in correlation to the DNA length standard should be considered as positive. The correct sizes of the specific amplificates can be found in the work-sheet. In all lanes without allele-specific amplification the internal control has to appear at **434 bp**.

Exceptions are **D Zygosity-TYPE** and the PCR reaction with **mix no. 2 of RH-TYPE** which show an internal control of **659 bp**. In most cases where there is allele-specific amplification the internal control is weaker or absent! For improper results see troubleshooting (see point 6.).

If no clear result can be obtained with the BAGene kits (e.g. due to unknown alleles which cannot be detected with the existing primers), national transfusion guidelines should be followed in accordance with the serological typings. Sequencing analysis of those samples is recommended. The typing results should be interpreted taking into consideration the genetic variance of different ethnic groups. In case of doubt, the phenotype is valid.

4.6.2 ABO-TYPE and ABO-TYPE variant

The homozygous expression of the alleles *ABO*O01, ABO*O03, ABO*B101, ABO*A201* is indicated by means of bands in the corresponding PCR reaction (1, 3, 5, or 7). In heterozygosity all four „non-reactions“ have to have a band in the gel (2, 4, 6, and 8) in addition to two specific PCR preparations (1, 3, 5, 7). Homozygosity of allele *ABO*A101* is indicated only by bands in all four “non-reactions” (2, 4, 6, 8), since there is no specific preparation for *ABO*A101*. The heterozygous constellation of *ABO*A101* can be recognized by an additional band of the allele-specific reactions (1, 3, 5, 7, 9, 10, 11, 12, 13, 14, 15 or 16).

Since only a selection of variant *A* alleles can be detected by ABO-TYPE variant, other variant *A* alleles can be hidden by the PCR result ***ABO*A101***. Since only a selection of variant *B* alleles and no variant A^2 alleles can be detected by ABO-TYPE variant, other variant *B* alleles or variant A^2 alleles can be hidden by the PCR-results ***ABO*B101*** and ***ABO*A201*** respectively. The most $B^{(A)}$ and *cis AB* alleles also show a positive result in the ABO**B101* reaction.

A band which is specific for HGH with a fragment length of 434 bp appears as internal control.

Detailed explanations are provided in an additional information for the evaluation of ABO-TYPE variant enclosed with each kit. Please consult the special remarks on the worksheets of ABO-TYPE and ABO-TYPE variant as well.

4.6.3 RH-TYPE

The molecular genetic determination of standard *RHD* as well as of some *RHD* variants (*RHD* positive haplotypes in serological D negative specimens, partial D) are performed in designated PCR reactions.
Preparations 1 and 2 are Multiplex-PCR reactions for examining five *RHD* polymorphisms (*RHD* intron 4 and 7, exon 7, as well as the specific detection of *RHD* (W16X) and *RHD*Ψ). This means, that in contrast to all other BAGene kits (except for the internal control band) not only one, but also two specific amplicons may occur in one PCR reaction. To facilitate the evaluation, the respective boxes are divided when two possible bands appear and have a bi-coloured background. The fragment lengths of the PCR product and the polymorphisms are also identified with a specific colour according to the boxes for the reaction pattern.

Example *RHD*Ψ:

Preparation No. 1: Two specific bands have to appear in the gel.

- PCR product **224 bp – green** identification, reaction pattern  in a box with **green** background.
- PCR product **123 bp – blue** identification, reaction pattern  in a box with **blue** background.

Preparation No. 2: Two specific bands have to appear in the gel.

- PCR product **154 bp – red** identification, reaction pattern  in a box with **red** background
- PCR product **390 bp – green** identification, reaction pattern  in a box with **green** background.

Designated PCR reactions are intended for the molecular genetic determination of the characteristics of the *RHCE* gene locus. A band, which is specific for HGH, with a fragment length of 434 bp, appears as internal control. An exception is PCR reaction no. 2 where a control band appears at 659 bp (specific for genomic sequence of chromosome I, 90 kbp 5‘ of *Rhesus Box*).

If the reaction pattern indicates a D category, further examination using **Partial D-TYPE** should be performed in order to exclude point mutations as a cause for these results.

4.6.4 Partial D-TYPE

A missing band in reaction no. 4 may indicate DFR (serology: weak positive with anti-D) or *RHD*Ψ (hemi- or homozygous D negative in serology). If serological information is lacking, confirmation or exclusion of *RHD*Ψ can be obtained using RH-TYPE. In presence of weak D type 41 and 45 a missing reaction of mix no. 9 may occur. Mutations of intron sections may also lead to a missing reaction in mix no. 8 or 9. In presence of weak D type 20 the reaction no. 10 normally shows no band, but sometimes a weak band appears.

A molecular genetic differentiation of the D variants ***DCS***, ***DFW***, ***DIM***, ***DNU*** from standard *RHD* is currently not possible. The consideration of the haplotypes is useful.

4.6.5 D Zygosity-TYPE

For *RHD* alleles, which cannot be determined serologically (RhD neg.), a discrepancy between the serological test result and genotyping may occur. The positive detection of the Downstream *Rhesus Box* shows the presence of an *RHD* allele (*RHD* pos.), except *RHD*Ψ homozygous and hemizygous respectively. The reaction hereby is negative although an *RHD* allele is present.
In addition, the result with a genetically modified Downstream *Rhesus Box* may be also false negative, although the specimen is serologically D-positive. Thus, with a serologically D positive result and positive PCR for the Hybrid *Rhesus Box,* the outcome is “Dd.” It is “DD” when a negative PCR result for the Hybrid *Rhesus Box* occurs.
Due to a distinctive polymorphism in the Hybrid *Rhesus Box* of Africans, a false positive result may occur in presence of *RHD*Ψ and another *RHD* allele.
In case of a missing Hybrid *Rhesus Box* in the black population, the results for the *RHD*Ψ and *Cde*^{s} alleles obtained by **RH-TYPE** have to be considered. Further D antigen negative *RHD* alleles cannot be excluded with currently available test kits. This must be considered in the interpretation of the results. However the incidence of these alleles in the white population is quite low.

Degraded DNA may lead to false negative results. This is shown either by the only presence of the internal control bands or by complete absence of bands.

5. Warnings and Precautions

Ethidium bromide is a powerful mutagen. Avoid contact with skin and contaminations. Consult the instructions for use and the warnings and precautions of the manufacturer. The transilluminator radiates very short wavelenght UV light which may cause burns of the skin and the retina. Use a UV face safety mask!
Biological material used for the DNA extraction, e.g. blood or human tissue, 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. by means of 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. by means of an autoclave).

Disposal of all samples, unused reagents and waste should be in accordance with country, federal, state and local regulations.

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

6. Troubleshooting

Problem	Possible Reason	Solution
no amplification, length standard visible	DNA contaminated with PCR-inhibitors, DNA degraded	repeat DNA isolation, try different methods
	DNA concentration too high/too low	alter DNA concentration, repeat DNA isolation
	enzyme is missing or enzyme concentration too low	repeat typing, alter enzyme concentration
	DNA from heparinized blood	repeat typing with EDTA blood
	wrong amplification parameters	optimize the amplification parameters (see 4.3) ☆
repeated failure in single lanes (no amplification control)	leak in reaction tubes, water loss and change in concentration during PCR	tightly close tubes with caps
unspecific amplification, additional bands (additional bands of the wrong size must be neglected)	contamination with other amplification products	decontamination, repeat typing, ensure clean working conditions
	DNA contaminated with salts	repeat DNA isolation, try different methods
	DNA concentration too high	use less DNA
	enzyme concentration too high	use less enzyme
	wrong amplification parameters	optimize the amplification parameters (see 4.3) ☆
evaluation shows more than 2 specificities	carry-over contamination (amplification products!) new allele	check typing mixtures without adding DNA, decontamination, ensure clean working conditions
no or only very weak bands visible, length standard invisible	EtBr staining too weak	repeat staining
too bright gel background	EtBr staining too long, EtBr concentration too high	soak gel in H_2O or TBE, lower EtBr concentration
blurred band	electrophoresis buffer too hot or used up, wrong electrophoresis buffer, polymerisation of the gel not complete	lower the voltage, use 0.5x TBE buffer, use completely polimerised gel

☆ When using the equipment and materials listed, optimization of the amplification parameters should be looked as a last resort. In most cases, it is possible to evaluate the test by eliminating the additional bands due to size variation.

7. References

Green and Sambrook, 2012. Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbour Laboratory

Additional references see www.bag-healthcare.com.

8. Explanation of symbols used on Labelling

	Use by
	Storage temperature
	Consult instructions for use
	Sufficient for n tests
BLOOD TYPING	Intended purpose: Blood typing
CONT	Content, contains
HNA TYPING	Intended purpose: Determination of HNA specificities
HPA TYPING	Intended purpose: Determination of HPA specificities
IFU	Instructions for use
IVD	For in vitro diagnostic use
LOT	Batch code
PCRBUF | 10x	PCR buffer, 10x concentrated
PCRCAP	PCR caps
PCRPLATE	PCR plates
PCRSTRIP	PCR strips
REACTIONMIX	Reaction mixes
REF	Catalogue number
RTU	Ready to use
TAQ POLYMERASE	Taq-Polymerase
WORKSHEET	Worksheet

Instructions for use in other languages see:
http://www.bag-healthcare.com
http://service.bag-healthcare.com
or phone: +49 (0)6404-925-125

BAG

BAG Health Care GmbH
Amtsgerichtsstraße 1-5
35423 Lich/Germany
Tel.: +49 (0) 6404 / 925 - 0
Fax: +49 (0) 6404 / 925 - 250
www.bag-healthcare.com
info@bag-healthcare.com

Auftragsannahme/Ordering:
Tel.: +49 (0) 6404 / 925 - 450
Fax: +49 (0) 6404 / 925 - 460
verkauf@bag-healthcare.com

Customer Service:
Tel.: +49 (0) 6404 / 925 - 125
Fax: +49 (0) 6404 / 925 - 421
service@bag-healthcare.com