

EN

INSTRUCTIONS FOR USE

BAGene SSP Kits

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



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	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 in-vitro-diagnostics for use by qualified personnel. The kits are used to determine blood group specificities of donors, recipients and pregnant women on a molecular genetic basis. The ABO-, ABO variant-, RH (with D Zygosity)-, Partial D-, Weak D- 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.

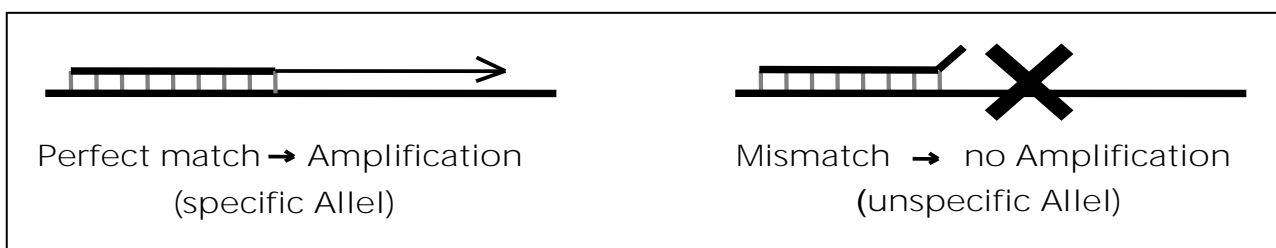


Fig. 1: Principle of SSP-PCR

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
- BAGene Information CD (contains instructions for use, worksheet and quality control certificate)

2.1. Required but not included material

- Taq polymerase (Happy Taq [REF] 66702 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 6)
- Aqua dest.
- 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
- UV source (transilluminator, 220-310 nm)
- Gel documentation system

2.2. 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 $\leq -20^{\circ}\text{C}$. 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 and the PCR plates/strips directly before preparing the PCR. Immediately place the prepared PCR plates/strips in the thermal cycler and start the PCR run.

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 worksheet. The results were compared to results with other blood group-SSP-Kits, sequencing or serological blood group test methods. The typing results showed 100% concordance with the pretyping results.

The evaluation and quality control of the mixes are done with DNA samples, which were extracted by EXTRA GENE I (salting out method) or Qiagen QIAamp DNA Blood Mini and Maxi kits (column based method). When another DNA extraction kit is used, the suitability of the extracted DNA for the application with the BAGene kits must be validated by the user.

The BAGene kits are validated with the Happy Taq (REF 66702). 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.

4. TEST PROCEDURE

4.1. Safety conditions and special remarks

PCR is a highly sensitive method, which must 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 sample material for the isolation of genomic DNA should be sent in appropriate blood collection systems. The presence of heparin potentially inhibits PCR; therefore blood collection systems with heparin are not suitable [2]. EDTA or Citrate blood is recommended for typing.

Validate DNA extraction methods:

- EXTRA GENE I (BAG)
- QIAGEN QIAamp DNA Blood Mini and Maxi Kit

Manual isolation or automated DNA isolation (QIAcube) are suitable.

Experiments with other kits revealed that elution should be performed with water (double distilled water, DNase free water) and not with elution buffers. Especially with magnetic beads isolation methods inhibition of the PCR reaction and, consequently, weak bands (bad amplification) or false negative results were observed occasionally when using elution buffers. Therefore, it is recommended to use the validated extraction methods.

Other standard methods for DNA isolation established in the laboratory must be validated by the user.

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 μ l.

1. Remove the required number of plates/strips from $\leq -20^{\circ}\text{C}$ 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.

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

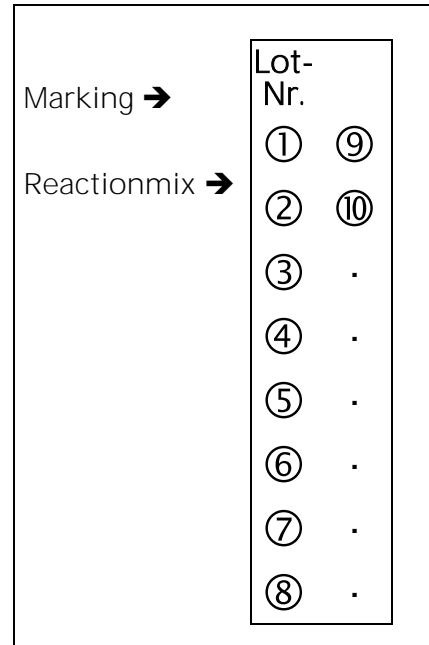
No. of mixes	Aqua dest.	10x PCR buffer	DNA solution (50-100 ng/ μ l)	Taq polymerase (5 U/ μ l)	total volume
1	8	1	1	0,08	10 μ l
2	16	2	2	0,2	20 μ l
6*	50	7	7	0,5	65 μ l
7	70	9	9	0,7	90 μ l
8	80	10	10	0,8	100 μ l
9	88	11	11	0,9	110 μ l
10	96	12	12	1,0	120 μ l
11	104	13	13	1,0	130 μ l
12	112	14	14	1,1	140 μ l
13	128	16	16	1,3	160 μ l
14	136	17	17	1,4	170 μ l
15	144	18	18	1,4	180 μ l
16	152	19	19	1,5	190 μ l
18	166	21	21	1,7	210 μ l

⇒ 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.

3. After vortexing add 10 μl of this mixture immediately to the pre-aliquoted and dried reaction mixtures (see figure). 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

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)

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 and/or amplification controls): 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 buffer (45 mM of Tris, 45 mM of boric acid, 0.5 mM of EDTA) 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 a 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. After the run has been completed, the gel is stained in an ethidium bromide (EtBr) solution for 30 - 45 minutes (approx. 0.5 µg/ml of EtBr in H₂O 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 H₂O 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, 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 amplicates can be found in the work-sheet. In all lanes without allele-specific amplification the internal control has to appear at **434 bp**. Exception is the PCR reaction with **mix no. 2 of RH-TYPE** which shows an internal control of **659 bp**. In most cases where there is allele-specific amplification the internal control is weaker or absent! If neither a specific band nor the internal control band appears, the result with the relevant mix cannot be used for evaluation.

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*O.01*, *ABO*O.02*, *ABO*B.01*, *ABO*A2.01* 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*A1.01* is indicated only by bands in all four “non-reactions” (2, 4, 6, 8), since there is no specific preparation for *ABO*A1.01*. The heterozygous constellation of *ABO*A1.01* 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*A1.01*. Since only a selection of variant *B* alleles and no variant *A²* alleles can be detected by ABO-TYPE variant, other variant *B* alleles or variant *A²* alleles can be hidden by the PCR-results *ABO*B.01* and *ABO*A2.01* respectively. The most *B^A* and *cis AB* alleles also show a positive result in the *ABO*B.01* reaction.

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

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, D_{el}) and the D Zygosity 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*01N.08* (W16X) and *RHD*08N.01* (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. It is recommended to use a DNA-length standard.

Example *RHD*08N.01* (Ψ):

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

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

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

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

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

D Zygosity

In the RH-TYPE kit it is additionally possible to type the RHD- Zygosity status DD, Dd or dd. Therefore the presense or absence of the Upstream *Rhesus-Box* (U_{BOX}) is requested in reaction no. 10 and the Rhesus *Hybrid-Box* (H_{BOX}) in reaction no. 11.

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 Upstream *Rhesus Box* shows the presence of an *RHD* allele (*RHD* pos.), except

*RHD*08N.01* (Ψ) homozygous and hemizygous respectively. The reaction of Mix 10 can be negative although an *RHD* allele is present.

In addition, the result of Mix 10 with a genetically modified Upstream *Rhesus Box* (e.g. for weak D 4.2 (DAR1)) may be also false negative, although the specimen is serologically D-positive. To check that kind of discrepancies the RH-TYPE contains reactions to detect *RHD*08N.01* (Ψ) and weak D 4.2 (DAR1). 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*08N.01* (Ψ) and another *RHD* allele.

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.

4.6.4. Partial D-TYPE

A missing band in reaction no. 4 may indicate DFR (serology: weak positive with anti-D) or *RHD*08N.01* (Ψ) (hemi- or homozygous D negative in serology). If serological information is lacking, confirmation or exclusion of *RHD*08N.01* (Ψ) can be obtained using RH-TYPE. In presence of *RHD*weak D type 41* (*RHD*01W.41*) und *RHD*weak D type 45* (*RHD*01W.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 *RHD*weak D type 20* (*RHD*01W.20*) the reaction no. 10 normally shows no band, but sometimes a weak band appears.

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

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 wavelength 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-diagnostics.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 ₂ O 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 polymerised 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
- Beutler, E. et al., 1990. BioTechniques 9:166

Additional references see www.bag-diagnostics.com.

8. EXPLANATION OF SYMBOLS USED ON LABELLING

	Use by
	Storage temperature / Upper limit of temperature
	Consult instructions for use
	Sufficient for n tests
	Manufacturer
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
BAGene INFORMATION CD	CD (contains instructions for use, worksheet, quality control certificate)
eIFU VXX/XXXX	Electronic instructions for use Version of the current 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

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