

Instructions for use
BAGene DNA-SSP Kits



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

10/20 typings
ready to use prealiquoted

REF 6640: BAGene ABO-TYPE	(red)
REF 6641: BAGene ABO-TYPE variant	(red)
REF 6645: BAGene RH-TYPE	(purple)
REF 6646: BAGene Partial D-TYPE	(yellow)
REF 6647: BAGene Weak D-TYPE	(white)
REF 6648: BAGene D Zygoty-TYPE	(blue)
REF 6650: BAGene KKD-TYPE	(green)
REF 6652: BAGene MNS-TYPE	(yellow)
REF 6660: BAGene HPA-TYPE	(blue)
REF 6670: BAGene HNA-TYPE	(white)

Contents

1. Product description	2
2. Material	10
2.1 Contents of the BAGene DNA-SSP kits	10
2.2 Requirements and supplementary material	10
2.3 Storage and stability	11
3. Data of performance	11
4. Test procedure	12
4.1 Safety conditions and special remarks	12
4.2 DNA isolation	14
4.3 Amplification	14
4.4 Gel electrophoresis	17
4.5 Documentation and interpretation	18
4.6 Explanation of Worksheets and Evaluation diagrams	18
5. Warnings and precautions	21
6. Troubleshooting	22
7. References	23
8. Explanation of symbols	28

1. Product description

With the use of Polymerase Chain Reaction (PCR) in blood group and platelet diagnosis, much progress has been made recently. The technique is suitable to complete, clarify and confirm serological results. Sequencing of *ABO* [1-13], *RHD/RHCE* [14-38], *Kell*, *Kidd*, *Duffy* [39-56], *MNSs* [57-60], *HPA* [61-66] and *HNA* alleles [67-69] has facilitated clear typing of donors, recipients and pregnant women at the DNA level with a high resolution and has many advantages over the traditionally used serology methods.

The basic material for typing with **BAGene DNA-SSP kits** is purified leucocytic DNA. The test procedure is done by using the Sequence Specific Primers (SSP)-PCR (see Fig. 1) [72, 73]. 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. Therefore, only if the primers entirely match the target sequence amplification is obtained which is subsequently visualised by agarose gel electrophoresis.

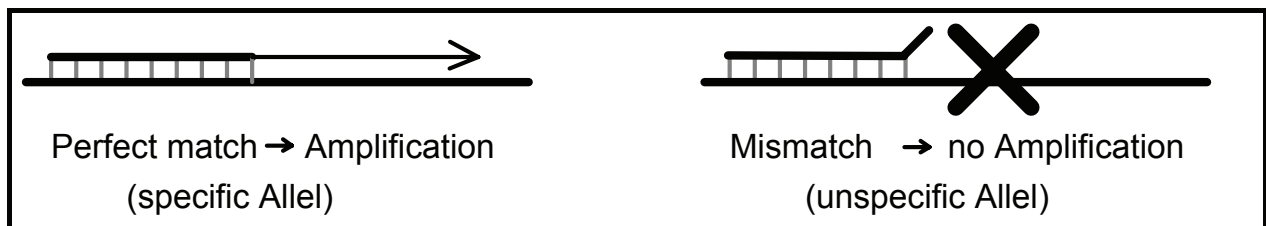


Fig. 1: Principle of SSP-PCR

The composition of the individual primer mixtures makes clear identification of the *ABO*, *RH*, *KEL*, *JK*, *FY*, *MNSs*, *HPA* and *HNA* genotypes indicated in the respective evaluation diagrams possible. With each typing a certain number of **prealiquoted** and **dried** reaction mixes including internal amplification control with a final volume of 10 µl are used.

Molecular genetic determination of ABO Blood Group

The molecular genetic determination of the ABO blood group is applied

- in neonates, since the ABO antigen expression is incompletely developed and the antibodies may be passively acquired from the mother
- in masking of the original serotype after repeated or massive transfusions of foreign erythrocytes or
- for monitoring after ABO different bone marrow transplantation [9]
- in forensic examinations.

◆ **BAGene ABO-TYPE** and **BAGene ABO-TYPE variant**

The characteristics of the ABO blood group system are defined by terminal residual sugar of the carbohydrate chains of glycoproteins and glycolipids on the surface of cells. Specific glycosyltransferases catalyze these modifications. Therefore the antigen properties are not directly determined by an A, B, or O gene, but by the genetically determined presence and activity of the glycosyltransferases. The transfer of the substrate N-acetylgalactosamine via A transferase and D galactose via B transferase results in the blood group characteristics A or B. If a person shows both transferases, he/she belongs to the blood group AB, if he/she shows neither A nor B transferase, he/she belongs to blood group O. The genes for the A and B transferases are located on the long arm of chromosome 9 (9q34). They consist of seven exons with a total length of 1065 base pairs. The most significant mutations (base substitutions, deletions, insertions) are located on exon 6 and 7. Five main alleles are described in the literature: ***ABO*A101***, ***ABO*A201***, ***ABO*B101***, ***ABO*O01*** and ***ABO*O03*** and there are also numerous variants and subgroups [1-13]. **BAGene ABO-TYPE** and **BAGene ABO-TYPE variant** allow the molecular genetic determination of these 5 main alleles, the common ***ABO*O02*** allele as well as A and B subgroup variants (e.g. ***A³***, ***A^x***, ***A^{el}***, ***A^w***, ***B³***, ***B^x***, ***B^w***).

Currently there is no official nomenclature for *ABO** alleles. Further nomenclature exists besides the nomenclature described in this instruction for use.

The Blood Group Antigen Gene Mutation Database http://www.ncbi.nlm.nih.gov/gv/mhc/xslcgi.cgi?cmd=bgmutsystems_info&system=abo consists of constant updated listings of all published *ABO** alleles.

Molecular genetic determination of RH types

Due to the strong immunogenicity of the D antigen and the high rate of immunization of D negative individuals after the transfusion of D positive erythrocytes, the determination of the *RHD* alleles is of special significance [34].

The immunization of D negative persons occurs for example

- in transfusions of D positive erythrocytes
- in pregnancies of D negative mothers with D positive fetus

A reliable molecular genetic determination of the *RHD* type aids in the prevention of D immunizations. Furthermore, D negative red blood cell concentrates can be more specifically allocated. Last but not least, typing of the *RHCE* alleles aids in the validation of unclear and weak serological findings.

The differentiation of D+/D+ and D+/D- in partners of D negative mothers by means of determining D zygosity, is of clinical relevance for evaluating the risks of MHN (**M**orbus **h**aemolyticus **n**eonatorum) [38] and supports improved pregnancy care.

◆ **BAGene RH-TYPE**

The two *RH* genes *RHD* and *RHCE* are localized on the short arm of chromosome 1 (p34.3 to p36.1) [14]. Their 3'-ends are aligned to each other and separated by 30,000 base pairs. A further gene (*SMP1*) is localized in this section, which also encodes for a membrane protein [26].

The *RHD* gene encodes antigen D; the *RHCE* gene encodes antigens C, c, E, e. *RHD* and *RHCE* genes consist of 10 exons each, with a common size of 69 kilobase.

Approximately 18% of Europeans are serologically D negative. In almost all RhD negative Caucasians the *RHD* gene is generally completely deleted on both chromosomes [28].

In other ethnic groups (Africans, Asians), with a low frequency also in Europeans, the presence of a seemingly functionless *RHD* gene was determined in phenotype RhD negative individuals (*Cde^s*, *RHD Ψ*) [14, 15, 20, 21, 26-28, 30, 32-34].

BAGene RH-TYPE allows the molecular genetic determination of standard *RHD/RHCE* alleles as well as the typing of a few *RHD* variants such as **DVI**, **DIV type 3**, *Cde^s*, *RHD Ψ* , *RHD(W16X)*, *RHD-CE(8-9)-D*, *RHD-CE(3-7)-D* [33] and **DEL** types e.g. *RHD(K409K)*, *RHD(M295I)*, *RHD(IVS3+1G>A)* [35]. The determination of **C^w** is included as well. If the reaction pattern indicates a D category, further examination using **BAGene Partial D-TYPE** should be added in order to exclude point mutations as a cause for these results.

Figure 2 A shows the genomic structure and arrangement of the *RHD* and *RHCE* gene locus in the *RHD* positive haplotype

Figure 2 B shows a model of the gene structure in the *RHD* negative haplotype

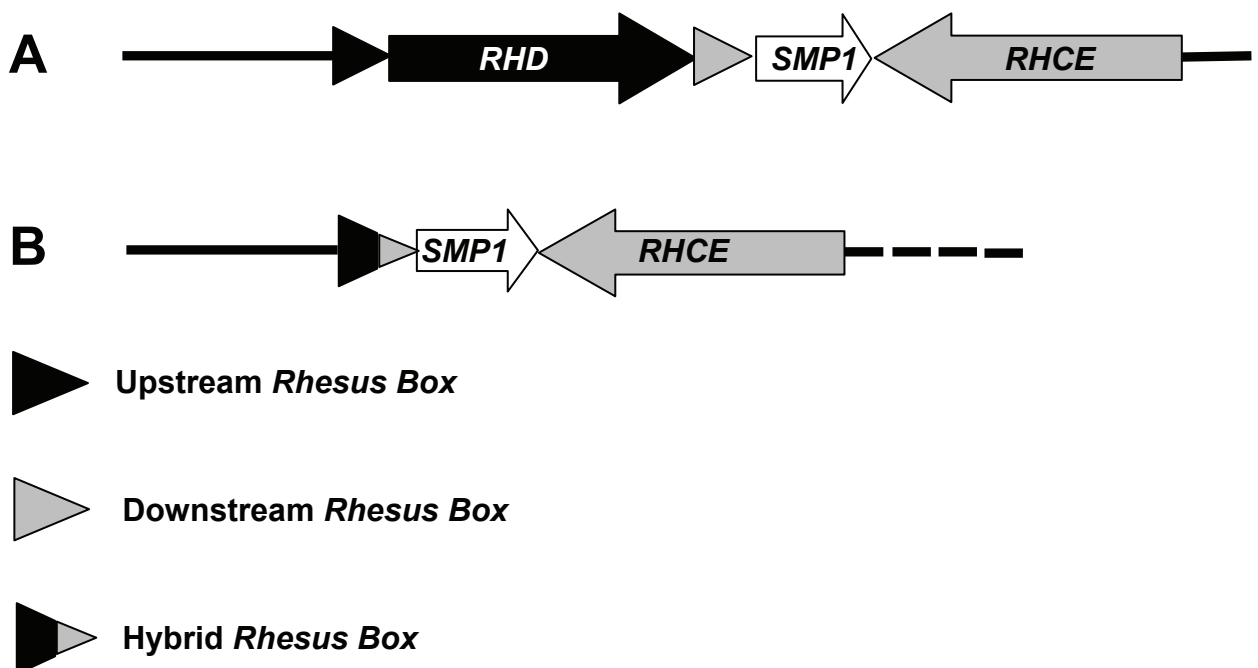


Figure 2: Model of the structure of the *RHD* and *RHCE* gene locus [acc. to 26, 27]

◆ **BAGene Partial D-TYPE** and **BAGene Weak D-TYPE**

Variations of the antigen structure of RhD result either in a partial (partial D) or a weak D phenotype (weak D).

Molecular genetic examinations of these D variants have shown that weak D phenotypes as well as some partial D types are caused by point mutations. In other partial D types, one or several exons of the *RHD* gene are exchanged for the corresponding segments of an *RHCE* gene, thus forming RhD-CE-D fusion proteins. In these fusion proteins, epitopes of the complete RhD protein are missing. Therefore, individuals with such partial D types (e.g. with the clinically relevant D category VI) may be immunized by means of transfusion of erythrocytes with the complete RhD protein [16, 22, 34].

As described in the literature, the substitution of amino acids in partial D phenotypes are localized mainly extracellular. The substitution of amino acids in weak D types generally is limited to intracellular or trans membrane sections of the RhD protein [22, 23, 25, 26, 28-31, 35].

BAGene Partial D-TYPE allows for the molecular genetic determination of partial D such as *DII*, *DIII*, *DIV*, *DV*, *DVI*, *DVII*, *DAU*, *DBT*, *DFR*, *DHMi*, *DHMii*, *DNB* and *DHAR (Rh33)* [16, 17, 19, 20, 22, 24, 25, 28, 36, 37]. 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.

BAGene Weak D-TYPE allows the molecular genetic determination of the weak D types *1*, *2*, *3*, *4.0/4.1*, *4.2*, *5*, *11*, *15* and *17*.

◆ **BAGene D Zygoty-TYPE**

In the RhD positive haplotype, the *RHD* gene is flanked by two highly homologous DNA segments, the so called *Rhesus Boxes*, which are localized 5' (Upstream *Rhesus Box*) and 3' (Downstream *Rhesus Box*) of the *RHD* gene (see Figure 2 A). In RhD negative Caucasians, the *RHD* gene is generally completely deleted on both chromosomes. This results in a Hybrid *Rhesus Box*, which comprises the 5'-end of the Upstream *Rhesus Box* and the 3'-end of the Downstream *Rhesus Box* (see

Figure 2 B) [26, 27, 38]. **BAGene D Zygoty-TYPE** allows for the determination of the D zygosity (homozygosity or hemizygoty of D) by means of the positive determination of the **Downstream Rhesus Box (DD)** or the **Hybrid Rhesus Box (dd)** or the **Downstream and the Hybrid Rhesus Box (Dd)** respectively. In case of a missing Hybrid Rhesus Box in the black population, the results for the *RHD* Ψ and *Cde*^s alleles obtained by **BAGene 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 results. However the incidence of these alleles in the white population is quite low [33, 38].

Molecular genetic determination of characteristics belonging to the Kell, Kidd and Duffy blood group system (KKD)

The most important antigens of the **Kell system** are *KEL1* (K) and *KEL2* (k – Cellano), whereas *KEL1* is of greatest interest due to its strong immunogenicity.

The antithetic alleles of the **Kidd system** are termed *JK*A* and *JK*B* (*Jk^a*, *Jk^b*).

The most significant characteristics of the **Duffy system** are *FY*A* and *FY*B* (*Fy^a*, *Fy^b*).

Antibodies, directed against antigens of the KKD system, cause either hemolytic transfusion reactions and/or severe cases of MHN.

A reliable determination of these antigens in samples of blood donors and patients is therefore of great interest.

◆ **BAGene KKD-TYPE** [39-56]

The significant difference between *KEL1* and *KEL2* (serological nomenclature K and k - Cellano), is caused by a single base substitution in exon 6 of its gene.

The Kidd system is located on chromosome 18 and consists of three different specificities *Jk^a*, *Jk^b*, *Jk-*. The alleles *JK*A*, *JK*B* of the Kidd-Systems differ in one single aminoacid substitution at position 280 of the antigen.

The FY gene is localized on chromosome 1. It consists of the alleles *FY*A*, *FY*B*, *FY*X* und *FY*null01* which may be represented mutually at the gene locus. Regarding serological nomenclature the *FY*A* allele corresponds to the Fy^a antigen and the *FY*B* allele to the Fy^b antigen. The weakly expressed allele *FY*X* (Fy^X) is serologically determined as Fy^{b weak}. In the african population, the phenotype Fy(a-b-) can be observed with a frequency of 68%, whereas in Europeans, Fy(a-b-) occurs extremely rare (< 0,1%). Individuals with this silent allele called *FY*null01* are resistant to the pathogene of Malaria tertiana (*Plasmodium vivax*) due to the lack of an antigen determinant.

In contrast to serological techniques **BAGene KKD-TYPE** allows a clear identification of the immunological relevant alleles Fy^{b weak} (*Fy*X*) and *FY*null01*.

The **KKD-TYPE** SSP kit consists at least of eight different PCR reaction mixes and enables to determine the following characteristics:

Kell (K, k) - *KEL*1* and *KEL*2*;

Kidd (Jk^a, Jk^b) – *JK*A* and *JK*B*;

Duffy (Fy^a, Fy^b, Fy^{null}, Fy^X) – *FY*A*, *FY*B*, *FY*null01* and *FY*X*

Molecular genetic determination of characteristics belonging to the MNS blood group system

The antigens **M** and **N** have been discovered in 1927 by Landsteiner and Levine [57], **S** and **s** in 1947 by Walsh und Montgomery [58]. Human glycophorins are the major sialoglycoproteins expressed on the red cell membrane that carry the antigens of the MNS blood group system [59]. Glycophorin A (GPA) has two allelic forms carrying the M or N antigen. Glycophorin B (GPB) also occurs in two allelic forms carrying the antigens S or s. GPA and GPB are encoded by ***GYPA*** and ***GYPB***, respectively, which are members of a gene family on chromosome 4 [60].

◆ **BAGene MNS-TYPE** [57-60]

The test kit allows the determination of the four main alleles of the MNS system (***M***, ***N***, ***S*** and ***s***) on a molecular genetic basis.

Molecular genetic determination of HPA specificities

Alloimmunothrombocytopenias such as neonatal alloimmunothrombocytopenia, post-transfusional purpura or refractory condition after thrombocyte transfusions are caused by antibodies directed against human platelet antigens which may be formed within the scope of transfusions or pregnancies. The reliable determination of the HPA specificities is necessary for diagnosing and providing suitable blood components for patients with these types of diseases [61-66].

◆ **BAGene HPA-TYPE**

The thrombocyte antigens HPA (**human platelet antigen**) represent a group of polymorphous allelic markers, localized on the human thrombocyte glycoproteins GPIIb/IIIa, GPIa, GPIb α , and GPIb β . The polymorphisms are based on point mutations, which result in the exchange of individual amino acids [65].

BAGene HPA-TYPE allows the molecular genetic determination of the *HPA* specificities *HPA-1a/b*, *HPA-2a/b*, *HPA-3a/b*, *HPA-4a/b*, *HPA-5a/b*, *HPA-15a/b*.

Molecular genetic determination of HNA specificities

Neonatal alloimmune neutropenia (NIN) or transfusion-related acute lung injury (TRALI) are caused by antibodies against human neutrophil antigens. Immunizations may occur within transfusions or pregnancies. The secure determination of the HNA specificities is necessary for diagnosis and availability of suitable blood components for patients suffering from these diseases [67-69].

◆ **BAGene HNA-TYPE**

The granulocyte antigens HNA (**human neutrophil antigens**) represent a group of polymorphic allele markers, localized on the surface of the human neutrophil granulocytes. Clinically important alloantigens are the specificities *HNA-1a* (NA1), *HNA-1b* (NA2) and *HNA-1c* (SH) of the Fc γ RIIIb glycoprotein [67]. Further clinically

relevant antigens are **HNA-4a** (Mart) and **HNA-5a** (Ond) which belong to the family of β_2 -integrines [68, 69].

BAGene HNA-TYPE allows the molecular genetic determination of the *HNA specificities HNA-1a/b/c HNA-4a/b, HNA-5a/b*.

2. Material

2.1 Contents of the **BAGene** / DNA SSP Kits

- ◆ 10/20 **BAGene** plates/strips sufficient for 10/20 typings. The prealiquoted and dried reaction mixtures consist of allele specific primers, internal control primers (specific for the HGH gene (**human growth hormone**) [74] and chromosome I genomic sequence, 90.000 bp 5' of *Rhesus Box* respectively) and nucleotides. The first reaction mix is marked (PCR strips: printed lot no. around the first reaction chamber, PCR plates: printed lot no. above the first reaction chamber).
- ◆ 10 x PCR buffer sufficient for 10/20 typings
- ◆ 8er strip caps à 12 sufficient for 10/20 typings
- ◆ Instructions for use, worksheet and evaluation diagram

2.2 Requirements and supplementary material

- ◆ Taq Polymerase (5 U/ μ l, e.g. Qiagen), **do not use hot start Taq Polymerase (e.g. Ampli Taq Gold)**
- ◆ **BAG EXTRA GENE** Kit (optional) for DNA extraction from blood / lymphocytes / leucocytes or material for other DNA extraction methods
- ◆ DNA length standard
- ◆ Piston pipettes (0,5-250 μ l)
- ◆ Sterile tips with integrated filter
- ◆ DNA Cycler (e.g. PTC 200 with a heated and adjusted cover, MJ Research/Bio-Rad)

Devices and material for gel electrophoresis

- ◆ DNA agarose
- ◆ 0.5 x 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)

Devices for interpretation and documentation

- ◆ UV source (transilluminator, 220-310 nm)
- ◆ Camera (e.g. Polaroid system) with films (Polaroid type 667)

2.3. Storage and stability

The kit is delivered unfrozen. Store all reagents at -20...-80°C in the dark. The expiry date is indicated on the label of each reagent. The shelf life indicated on the outer label is also valid for the time period after the first use and refers to the reagent with the shortest stability contained in the kit. Thaw the 10 x PCR buffer shortly before use.

3. Data of performance

analytical sensitivity: A reliable typing is guaranteed by using 50 - 100 ng DNA per reaction mix. Due to a longer PCR program for the use of **BAGene D Zygosity-TYPE** a lower DNA concentration of 30 - 50 ng per reaction mix is recommended.

diagnostical specificity: The composition of the primer mixture guarantees a reliable identification of the alleles indicated in the worksheet and evaluation diagram.

The diagnostical sensitivity and specificity of each primer mix was examined with DNA from reference samples. Alleles, which are not available and not have been tested caused by their rareness respectively, are indicated on the worksheet and evaluation diagram (*n.t.* = *not tested currently*). Studies of performance have been done for all **BAGene DNA SSP Kits** with previously typed reference samples. The investigations showed clear cut results in accordance with serological and/or genomical pretypings.

4. Test procedure

4.1 Safety conditions and special remarks

PCR is a highly sensitive method, which should be performed by skilled personnel with experience in molecular technology and blood group serology. Up to date guidelines on transfusion medicine and the determination of blood groups as well as transfusion anamnesis must be taken into account in order to reduce the risk of false typings, especially when differing results are obtained with serological and molecular biological test methods. Genotyping of *ABO* and *RHD/RHCE* as well as *Kell*, *Kidd*, *Duffy*, *MNSs*, *HPA* and *HNA* specificities has to be performed after the conclusion of the serological determination.

General limitations in molecular genetic typings using **BAGene** DNA SSP kits:

If no clear cut results can be obtained using **BAGene** DNA SSP Kits (e.g. due to unknown alleles that cannot be detected with existing primers), national transfusion guidelines should be followed in accordance with serological typings. Sequencing analysis of those samples is recommended. The typing results have to be interpreted in consideration of the genetic variation in different ethnic groups. In case of doubt the phenotype is authoritative.

Limitations in determining the ABO blood group:

Since only a selection of variant *A* alleles can be detected by **BAGene ABO-TYPE variant**, other variant *A* alleles can be hidden behind the PCR result ***ABO*A101***. Since only a selection of variant *B* alleles and no variant *A*² alleles can be detected by **BAGene ABO-TYPE variant**, other variant *B* alleles or variant *A*² alleles can be hidden behind 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.

Limitations and remarks in D zygosity typing:

- In *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 finding therefore is “Dd.” It is “DD” with a negative PCR for the Hybrid *Rhesus Box*.
- Due to a distinctive polymorphism in the Hybrid *Rhesus Box* of Africans, a false positive result may occur in presence of *RHD*Ψ and a further *RHD* allele .
- Degraded DNA may lead to false negative results of both the Downstream *Rhesus Box* and the Hybrid *Rhesus Box*. This is indicated either by bands of the internal control only, or by missing bands at all.

Limitations and remarks in determination of partial D:

A missing band in reaction no. 4 may indicate DFR (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 **BAGene RH-TYPE**.

Special safety conditions must be noted 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).
- ◆ 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

For **BAGene** typing 5-10 µg of DNA (corresponding to approximately 0.5 ml of blood) is required. E.g. the **BAG EXTRA-GENE** kit is most suitable for isolation since pure DNA can be obtained from whole blood in a short time without the use of toxic chemicals or solvents. Also, other methods described in literature [70] such as the chloroform-triethyl-ammonium-bromide (CTAB) method or phenol-chloroform purification are suitable to provide DNA of sufficient purity. The presence of heparin potentially inhibits PCR [71]. Therefore EDTA or Citrate Blood is recommended for typing. DNA should have a purity index (extinction ratio OD_{260}/OD_{280}) between 1.5 and 2.0.

4.3 Amplification

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

- 1.: Remove the required number of **BAGene** plates/strips from -20...-80°C and thaw the 10 x PCR buffer at room temperature.
- 2.: Pipet the master mix consisting of 10 x PCR buffer, DNA solution, Taq Polymerase and Aqua dest. and mix thoroughly. The different **BAGene** DNA-SSP Kits do all work with the same master mix and can therefore be combined. The composition of the master mix depending on the number of reaction mixes is given in Table 1.

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

No. of mixes	Aqua dest.	10 x PCR buffer	DNA sol. (50-100 ng/μl) ♠	Taq Polymerase (5 U/μl)	total volume, app.	
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

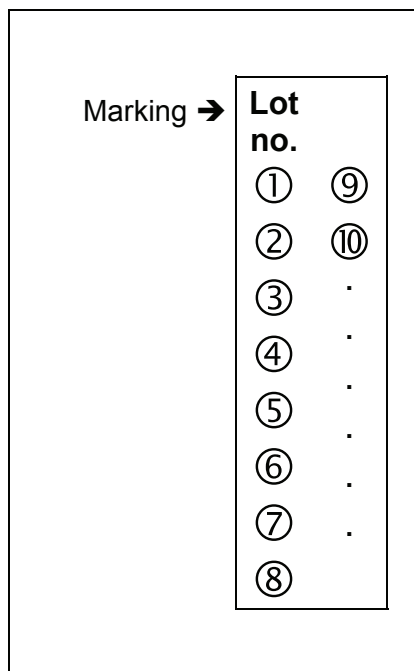
⇒ for different DNA concentrations, the quantities of DNA and water must be varied accordingly (e.g. for 12 mixes: DNA (120 ng/μl): use 5,8 μl DNA and 119 μl Aqua dest.).

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

♠ Using **BAGene 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 predropped 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 cyclers with tightly closable lid are used, it is also possible to use reusable PCR mats.

Slightly move the plate to dissolve the blue pellet at the bottom of the tube. All PCR solution should settle on the bottom.



4.: Place the reaction tubes into the thermocycler and tighten lid so that the reaction chambers 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 the BAGene DNA SSP 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

Cycler types:

e.g. PTC 100 / 200
(MJ Research/ Bio-Rad)
and GeneAmp PCR-System 9600 / 9700
(ABI Co.)

CAUTION: Different PCR-program !

Amplification parameters D Zygoty-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

Cycler types:

e.g. PTC 100 / 200

(MJ Research/ Bio-Rad)

and GeneAmp PCR- System

9600 / 9700 (ABI Co.)

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

To optimize your machine use the following guide:

With **false positive** reactions (unspecific bands, additional types): Increase of the annealing temperature in 1°C steps.

With **false negative** reactions (bands missing): Decrease of the annealing temperature in 1°C steps and/or increase of the annealing periods in 5 second steps and/or increase of the denaturation periods in 5 second steps.

It is recommended to use regularly calibrated cyclers only. E.g. the BAG-Cycler Check kit (Cat.-No.: 7104) is suitable for the calibration procedure.

The quality control tests were performed using the following cyclers: PTC 200 and PTC 100 respectively (MJ Research) and 9700 (ABI).

4.4 Gel electrophoresis

Separation of the amplification products is done by electrophoresis via a (horizontal) agarose gel. As electrophoresis buffer, 0.5 x 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 sample loading.

After amplification has been finished, take the samples out of the thermocycler and load the complete reaction mixtures carefully in each slot 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 **BAGene D Zygosity-TYPE**. After the run has been completed, the gel is stained in an ethidiumbromide (EtBr) solution (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 and interpretation

For documentation, visualize the PCR amplification using an UV transilluminator (220-310 nm) and photograph it with a suitable camera, film and filters (e.g. polaroid, film type 667). 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).

For interpretation use the evaluation diagram; only bands that have the correct size in correlation to the DNA length standard should be considered positive. The correct sizes can be found in the worksheet and evaluation diagram. In all lanes without allele-specific amplification the internal control has to appear at **434 bp** in all cases (except for **D Zygosity-TYPE** and the 2. PCR reaction of **RH-TYPE**: here, the fragment length of the internal control is **659 bp**). In the preparations with specific positive reactions the internal control is mostly weaker, or may completely disappear due to the competitive PCR conditions! For improper results see troubleshooting (6.).


4.6 Explanation of worksheets and evaluation diagrams


◆ General

The results of the molecular genetic determinations performed by means of the **BAGene DNA-SSP Kit** are documented on the provided worksheets.

The listing of characteristics, specificities, phenotype, genotype and the illustration of the reaction patterns, partially by means of examples in the evaluation diagrams, serve to aid

interpretation. The PCR preparations have reaction numbers (e.g. ABO-TYPE reaction no. 1 - 8).

The fragment length of PCR products (specific bands) is stated in bp. In the rows beneath, possible band patterns in the gel are shown. Specific PCR products (positive reaction) are designated as  and the corresponding boxes of the diagram are shaded in colours (ABO-TYPE, ABO-TYPE variant, Partial D-TYPE, Weak D-TYPE, D Zygosity-TYPE, KKD-TYPE, MNS-TYPE, HPA-TYPE, HNA-TYPE – **green**, RH-TYPE additional **red**, **pink** and **blue**).

The absence of specific amplicons is designated in white boxes as .

The evaluation of reaction patterns is performed in rows from left to right (marked with red arrows in the worksheet) and is inserted into the provided boxes together with the specimen data and gel image.

◆ **BAGene ABO-TYPE** [8] and **BAGene ABO-TYPE variant** [6,7,10,13]

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, and 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 oder 16). Detailed explanations are described in an extra instruction for the evaluation of **BAGene ABO-TYPE variant** which is enclosed with each kit. Please consult the special remarks on the worksheet of **BAGene ABO-TYPE** and the result protocol of **BAGene ABO-TYPE variant** as well.

Limitations in the molecular genetic *ABO** determination (see page 12) must be taken into account. A band which is specific for HGH with a fragment length of 434 bp appears as internal control.



◆ **BAGene RH-TYPE** [18, 20, 32, 33, 35]

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** DNA SSP 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 must appear in the gel.

- PCR product 224 bp – green identification, reaction pattern 
in box with green background
- PCR product 123 bp – blue identification, reaction pattern 
in 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 box with red background
- PCR product 390 bp – green identification, reaction pattern 
in 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: here, a control band appears at 659 bp (specific for genomic sequence of chromosome I, 90,000 bp 5' of *Rhesus Box*).

- ◆ **BAGene Partial D-TYPE** [20, 22, 24, 25, 28, 35-37], **Weak D-TYPE** [23, 25, 31], **D Zygosity-TYPE** [38], **KKD-TYPE** [56], **MNS-TYPE** [59], **HPA-TYPE** [66], **HNA-TYPE** [67]

Notice page 18 chapter „**General**“.

5. Warnings and Precautions

Ethidiumbromide 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-wave UV light which may cause burns of the skin and the retina. Use a UV face safety mask!

All for extraction of DNA used biological material, 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. 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).

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 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	close tubes tight with caps;
unspecific amplification, additional bands, (additional bands of the wrong size must be neglected)	contamination with amplification products	repeat typing, ensure exact working decontamination
	DNA contaminated with salts	repeat DNA isolation, try diff. 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 (no DNA added) ensure exact working decontamination
no or only very weak bands visible, length standard invisible	EtBr staining too weak	repeat staining
gel background shines too bright	staining was too long, EtBr concentration too high	soak gel in H ₂ O or TBE lower EtBr concentration
blurred band	- electrophoresis buffer too hot - wrong electrophoresis buffer - too little buffer	lower the voltage use 0,5x TBE buffer

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

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8. Explanation of symbols	
	For in vitro diagnostic use
	Batch code
	Catalogue number
	Storage temperature
	Use by
	Consult Instructions for use

Version 05/2009



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