Review Article

Challenges with assigning RH alleles and accurately predicting phenotypes using commercially-available genotyping kits: a narrative review

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Background and Objective: This work describes the concept of resolution of genotyping panels including those used to predict RH phenotypes. Commercially available panels are described. Challenges and limitations where low and medium resolution genotyping can yield equivocal or inaccurate results are presented with a discussion of the causes and implications. This review is directed towards the users of molecular genotyping panels and recipients of the results, including reference laboratory managers and technologists, blood bank managers and technologists as well as transfusion medicine physicians.

Methods: Peer-reviewed literature with key words RH, genotyping, limitations, antigen and blood published in English in PubMed from 1990 to April 2023 was reviewed. Commercial genotyping kit package inserts were reviewed including Immucor PreciseType HEA Molecular BeadChip, RHD and RHCE BeadChip, Grifols IDCoreXT. ISBT Working Party for Red Cell Immunogenetics and Blood Group Terminology allele tables for *RHD*, *RHCE* and *FY* were reviewed. Insights were derived from more than a decade managing a national reference laboratory of molecular immunohematology.

Key Content and Findings: The concept of resolution of genotyping methods is introduced. Limitations are presented including failure to identify a variant allele due to lack of a common, uncommon or rare variant. The need to investigate discrepancies between genotype and phenotype is discussed, including the use of high-resolution methods.

Conclusions: This work provides the reader with information that can be used to select genotyping panels as well as better understand the results of such panels, specifically what has been ruled in or out.

Keywords: Blood group antigen; allele; genotyping; test resolution

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Introduction

Background

Utility of molecular methods for predicting phenotype

Molecular methods have been used to catalogue (1) and explore blood group antigen genes and predict phenotypes for more than three decades (2-4) including a focus on the RH blood group system (5). There are multiple patient use cases for molecular immunohematology including after recent transfusion, when serologic reagents are not available, to resolve antigen typing discrepancies, to aid in complex antibody identifications, to assess the risk of alloimmunization including hemolytic disease of the fetus and newborn (HDFN) and fetal and neonatal alloimmune thrombocytopenia (FNAIT) as well as to determine zygosity status. Use cases for molecular immunohematology in blood donors include to efficiently predict multiple antigen phenotypes in multiple individuals simultaneously as well as to resolve antigen typing discrepancies and to assign RH alleles for purposes of RH allele matching (6-10). These use

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cases have been reviewed elsewhere (11).

The RH Blood Group System (ISBT 004)

The RH blood group system is encoded by two highly homologous genes (*RHD* and *RHCE*), both with 10 exons. The *RHD* gene encodes one common antigen, RhD or RH1, while the *RHCE* gene encodes two sets of antithetical common antigens, big C (RH2) and little c (RH4) and big E (RH3) and little e (RH5). Rare phenotypes include Rh_{null} with loss of all RH antigens (12), and D—, D.. (13) and those with loss of high prevalence antigens including hr^B (RH31) and hr^S (RH19) (14). Importantly, there are multiple genetic backgrounds that can result in loss of these antigens (15).

Rationale and knowledge gap

The purpose of this narrative review is to provide the reader with an understanding of how a molecular test used to predict RH blood group system phenotypes can yield an incorrect or equivocal result. The understanding of test limitations can be helpful in interpreting the results and determining if additional testing is warranted. This information is useful to molecular and serologic immunohematology reference laboratories as well as blood centers and hospital blood banks.

Objective

The objective of this review is to address the following questions

- What is the impact of resolution of genotyping methods on genotype-predicted phenotype?
- What red cell genotyping panels are commercially-available?
- What are the limitations of commercially-available red cell genotyping panels, including:
  - Failure to test for variants associated with most null alleles;
  - Failure to test for uncommon but clinically significant variants;
  - Failure to test for common variants with impact on predicted phenotype.
- What is the impact of inferences due to linkage disequilibrium and phase assumptions?
- What is the impact of equivocal allele assignments on antigen phenotypes?

I present this article in accordance with the Narrative Review reporting checklist (available at https://aob.amegroups.com/article/view/10.21037/aob-23-18/rc).

Methods

Peer-reviewed literature with key words RH, genotyping, limitations, antigen and blood published in English in PubMed from 1990 to April 2023 was reviewed (*Table 1*). Commercial genotyping kit package inserts were reviewed including Immucor PreciseType HEA Molecular BeadChip, RHD and RHCE BeadChip, Grifols IDCoreXT. ISBT Working Party for Red Cell Immunogenetics and Blood Group Terminology allele tables for *RHD*, *RHCE* and *FY* were reviewed (1). Insights were derived from more than a decade managing a national reference laboratory of molecular immunohematology.

Table 1 summarizes the literature search strategy used to gather information to include in this review.

Findings

Genotyping test resolution

Blood group antigens are determined by genetic variation, including copy number variants, insertion/deletion polymorphisms and single nucleotide variants (SNV). Currently, commercial genotyping panels that include prediction of RH antigens are based on SNV typing. Molecular testing methods can be classified as low-, medium- or high resolution. High resolution methods include those that interrogate all critical gene regions such as coding regions and splice sites. Examples of high-resolution test methods are Sanger sequencing (16) and next-generation sequencing (17). Though there are several reports of targeted NextGen panels for blood group antigen expression, currently, there are no commercial high-resolution kits for predicting red cell antigens including RH antigens. Low-resolution genotyping is characterized by using one or a limited set of markers to predict antigen expression. An example would be the PreciseType HEA Molecular BeadChip (Immucor, Norcross, GA, USA), that predicts RH antigen expression but does not include sufficient markers to detect variant antigens. Medium-resolution genotyping is characterized by inclusion of markers sufficient to detect common variants. Examples include the RHCE and RHD BeadChips (Immucor) that includes 25 and 35 genetic markers, respectively. A limitation of both low- and medium-resolution panels is the failure to detect variants that can impact antigen expression. These can be variants

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Table 1 Literature search strategy

Items	Specification
Date of search	April 29, 2023
Database searched	PubMed
Search terms used	RH, genotyping, limitations, antigen, blood
Timeframe	1990 to April 2023
Inclusion criteria	Peer-reviewed literature published in English
Selection process	Selection conducted by the author
Any additional considerations	Commercial genotyping kit package inserts were reviewed including Immucor PreciseType HEA Molecular BeadChip, RHD and RHCE BeadChip, Grifols IDCoreXT
	ISBT Working Party for Red Cell Immunogenetics and Blood Group Terminology allele tables for *RHD*, *RHCE* and *FY* were reviewed
	RHD blood group alleles v6.3 31-MAR-2023 (accessed on 09/04/2023)
	RHD blood group alleles v6.4 31-JUL-2023 (accessed on April 29, 2023)
	RHCE blood group alleles v6.2 31-MAR-2022 (accessed on April 29, 2023)
	FY blood group alleles v6.1 30-NOV-2021 (accessed on April 29, 2023)
	Insights were derived from more than a decade managing a national reference laboratory of molecular immunohematology

that result in weak or partial antigen expression, gain of low-prevalence antigen expression or loss of high prevalence antigen expression.

Available genotyping products that include RH antigens

All currently commercially available genotyping panels that predict RH blood group system antigens are low or medium resolution. *Table 2* lists the commercially-available genotyping panels that include RH antigens. Only two are approved for use by the US Food and Drug Administration (FDA), several are Conformité Européénne (CE)-marked and several are sold as "research use only" (RUO). These include bead-based fluorescence assays from Immucor and Grifols (Barcelona, Spain), as well as sequence-specific primer (SSP)-polymerase chain reaction (PCR)-based assays from inno-Train diagnostic GMBH (Kronberg, Germany) and BAG Diagnostics (Lich, Germany), as well as Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry from Agena Bioscience (San Diego, CA, USA).

Test limitations

The next sections will present examples of limitations of low and medium-resolution genotyping panels.

What's missing on panels: null alleles

Most low-resolution commercial panels and many medium resolution commercial panels do not include analytes that detect null alleles. This can lead to false positive predicted phenotypes. An example is found in the RH blood group system, specifically in the *RHCE* gene. A sample may be tested for the common antigens C, c, E and e using a low-resolution commercial RBC genotyping panel and predicted to express all of them. Even a medium-resolution genotyping panel focused on *RHCE* markers might be performed and would also predict expression of C, c, E and e. If the RBCs test C+ e+ c– E–, a null allele should be suspected since each *RHCE* allele would be expected to express one of each antithetical antigen pair (C/c, E/e). High-resolution testing can be used to rule this scenario in or out. A false positive prediction for c and E antigen expression would be expected in a sample carrying one *RHCE*Ce* allele and the other *RHCE*cEN.02* allele that includes a single nucleotide deletion associated with premature termination (18).

What's missing on panels: uncommon variants

Antigen typing discrepancies can identify samples that may

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Table 2 List of genotyping panels that include RH analytes with manufacturer, methodology and approvals

Test name	Manufacturer	Methodology	Approval
PreciseType HEA Molecular BeadChip	Immucor	Elongation-mediated Multiplexed Analysis of Polymorphisms (eMAP®)	FDA, CE certified
IDCoreXT	Grifols	Qualitative, PCR and hybridization-based genotyping test	FDA, CE certified
RHD BeadChip	Immucor	Elongation-mediated Multiplexed Analysis of Polymorphisms (eMAP®)	CE certified
RHCE BeadChip	Immucor	Elongation-mediated Multiplexed Analysis of Polymorphisms (eMAP®)	CE certified
ID RHD XT	Grifols	Qualitative, PCR and hybridization-based genotyping test	RUO
HemoID DQS	Agena BioScience	MALDI-TOF mass spectrometry	RUO
RBC-FluoGene	Inno-train diagnostic GMBH	TaqMan-based SSP-assay	CE certified
RBC-ReadyGene	Inno-train diagnostic GMBH	PCR-SSP-assay	CE certified
BAGene	BAG diagnostics	PCR-SSP	CE certified

FDA, Food and Drug Administration; CE, Conformité Européénne; PCR, polymerase chain reaction; RUO, research use only; SSP, sequence-specific primer; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight.

carry variant antigens that are expressed weakly and/or lack epitopes that result in differential reactivity with serologic reagents. RhD is the classic example (19). There are several medium-resolution commercial *RHD* genotyping panels. These panels are generally assembled to cover common variants and often are selected based on findings in Caucasian and in some cases populations of African descent. Use of these panels may result in failure to identify genetic variants that are associated with weakened antigen expression or loss of epitopes. This is a serious limitation when this information is used to determine candidacy for Rh immune prophylaxis in women of child-bearing age (20). An example of this is a female with trauma found to have differential reactivity with two anti-D reagents. The sample was tested using a medium resolution panel that interrogates 35 genetic variants. The patient was found to have no variants and predicted to type D+ and based on that result, would not be expected to be at risk of allo-anti-D formation. However, high-resolution Sanger sequencing was performed of all coding exons and the patient was found to carry *RHD* c.1195A associated with *RHD*01W.45*. This allele has been reported to be associated with allo-anti-D (21). This case highlights the need for caution when a medium-resolution testing platform does not resolve a serologic discrepancy. Such cases should be referred to a reference laboratory where high resolution testing can be performed.

What's missing on panels: common variants

There are several examples of common variants not included on commercial genotyping panels having an impact on the interpretation of the results.

One example is in *RHCE*, where the c.48G>C SNV (rs586178) is very common. It has a global minor allele frequency of 0.40 and a minor allele 0.20 in African Americans. *RHCE* c.48C is found on *RHCE* alleles that express the big C antigen as well as many variant *RHCE*ce* alleles (see *Table 3*). The presence of *RHCE* c.48C changes the phenotype in some instances, as shown in *Table 4*. The impact of c.48C alone is associated with a weak e phenotype as per ISBT (1) but has been found in individuals with allo-anti-e (22).

Therefore, when genotyping panels such as IDCore XT (Grifols) fail to test for *RHCE* c.48C, this may result in incorrect or incomplete predicted phenotypes. This is important in cases of RH allele matching (9,10), when RBC units are selected based on similarity to the patient RH alleles. As described in Keller 2022, donors are ranked based on the similarity of RH alleles between them and a specific patient (23). Tier 1 is the rank given to donors with probable RH alleles that are the same as the probable alleles assigned to the patient. Tier 2 is the rank given to donors

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Table 3 *RHCE*01* alleles carrying c.48C with the nucleotides that differ from *RHCE*01* and the impact on phenotype

RHCE allele name/*RHCE* allele alias	Nucleotides	Phenotype[†]
*RHCE*01.01/RHCE*ce48C*	c.48G>C	RH:5 (e+ weak)
*RHCE*01.02/RHCE*ceTI*	c.48G>C	RH:4 (c+ weak, partial)
	c.1025C>T	RH:5 (e+ weak, partial)
*RHCE*01.04/RHCE*ceAR*	c.48G>C	RH:4 (c+ partial)
	c.712A>G	RH:5 (e+ weak, partial)
	c.733C>G	RH:10,–20 (V+ weak, VS–)
	c.787A>G	RH:–18,–19 (Hr–, hr^S–)
	c.800T>A	
	c.916A>G	
*RHCE*01.05/RHCE*ceEK*	c.48G>C	RH:4 (c+ partial)
	c.712A>G	RH:5 (e+ weak, partial)
	c.787A>G	RH:–18,–19 (Hr–, hr^S–)
	c.800T>A	
*RHCE*01.07/RHCE*ceMO*	c.48G>C	RH:4 (c+ partial)
	c.667G>T	RH:5 (e+ weak, partial)
		RH:–19 (hr^S–)
		RH:–31 (hr^B–)
		RH:–61 (CEVF–)
*RHCE*01.08/RHCE*ceBI*	c.48G>C	RH:5 (e+ partial, weak to neg)
	c.712A>G	RH:–18,–19 (Hr–, hrS–)
	c.818C>T	RH:49 (STEM+)
	c.1132C>G	
*RHCE*01.09/RHCE*ceSM*	c.48G>C	RH:5 (e+ positive to negative)
	c.712A>G	RH:–18 (Hr–), inferred
	c.818C>T	RH:–19 (hr^S–)
		Rh:49 (STEM+ weak)
*RHCE*01.10/RHCE*ceSL*	c.48G>C	RH:5 (e+ weak)
	c.365C>T	Some monoclonal anti-D crossreact
*RHCE*01.12/RHCE*ceRA*	c.48G>C	RH:5 (e+ weak)
	c.538G>C	
*RHCE*01.16/RHCE*ce.16*	c.48G>C	RH:5 (e+ weak)
	c.1170C>T	
	c.1193T>A	
*RHCE*01.20.02/RHCE*ceVS.02*	c.48G>C	RH:4 (c+ partial)
	c.733C>G	RH:5 (e+ partial)
		RH:10,20 (V+VS+)
		RH:–31 (hr^B–)
*RHCE*01.20.03/RHCE*ceVS.03*	c.48G>C	RH:4 (c+ partial)
	c.733C>G	RH:5 (e+ partial)
	c.1006G>T	RH:–10,20 (V–VS+)
		RH:–31 (hr^B–)

Table 3 *(continued)*

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Table 3 *(continued)*

RHCE allele name/*RHCE* allele alias	Nucleotides	Phenotype[†]
*RHCE*01.20.04/RHCE*ceTI Type 2*	c.48G>C	RH:5 (e+ partial)
	c.733C>G	RH:10,20 (V+VS+)
	c.1025C>T	Probable RH:–31 (hr^B–)
*RHCE*01.20.06/RHCE*ceCF*	c.48G>C	RH:4 (c+ partial)
	c.697C>G	RH:5 (e+ partial, positive to negative)
	c.733C>G	RH:20 (VS+)
		RH:–19,–31 (hr^S–, hr^B–)
		RH:43 (Crawford+)
		RH:–58 (CELO–)
		Some monoclonal anti-D crossreact
*RHCE*01.20.08/RHCE*ceVS.08*	c.48G>C	RH:5 (e+ weak)
	c.733C>G	RH:10,20 (V+VS+)
	c.748G>A	Probable RH:–31 (hr^B–)
*RHCE*01.20.09/RHCE*ceVS.09*	c.48G>C	RH:5 (e+ weak)
	c.733C>G	RH:10,20 (V+VS+)
	c.941T>C	RH:31 (hr^B+ weak)
*RHCE*01.20.10/RHCE*ceVS.10*	c.48G>C	Probable RH:4 (c+ partial)
	c.712A>G	Probable RH:5 (e+ partial)
	c.733C>G	
*RHCE*01.20.11/RHCE*ceVS.11*	c.48G>C	Not reported
	exons 2-3 D	
	c.186G>T	
	c.410C>T	
	c.455A>C	
	c.733C>G	
	c.1006G>T	
*RHCE*01.20.12/RHCE*ceVS.12*	c.48G>C	Some monoclonal anti-D crossreact
	c.462G>T	
	c.733C>G	
	c.1006G>T	
*RHCE*01.20.13/RHCE*ceVS.13*	c.48G>C	RH:5 (e+)
	c.106G>A	RH:9 (CX+)
	c.733C>G	RH:20 (VS+)
*RHCE*01.21.02/RHCE*ce.21.02*	c.48G>C	RH:5 (e+ weak)
	c.187G>C	RH:48 (JAL+)
	c.341G>A	

[†], "weak" and "partial" phenotypes are not mutually exclusive, and for many alleles the associated phenotype(s) have not been extensively investigated or samples have not been informative. All partial antigens may not be indicated.

homozygous for one of the probable alleles of the patient. Tier 3 is the rank given to donors that carry one or both alleles that are distinct from those carried by the patient but with a similar phenotype. Donors that are ranked Tier 3 often carry RH alleles with additional amino acid substitutions that could have immunogenetic impact. An

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Table 4 *RHCE*ce* alleles with or without c.48C, and phenotypes that differ in bold

RHCE allele name	*RHCE* allele alias	Nucleotides	Phenotype
*RHCE*01*	*RHCE*ce*	N/A	RH:4 or c
			RH:5 or e
			RH:6 or f (ce)
*RHCE*01.01*	*RHCE*ce48C*	c.48G>C	RH:5 **(e+ weak)**
*RHCE*01.20.01*	*RHCE*ceVS.01*	c.733C>G	RH:4 (c+ partial)
			RH:5 (e+ partial)
			RH:10,20 (V+VS+)
			RH:31 **(hr^B+ very weak to neg)**
*RHCE*01.20.02 .01*	*RHCE*ceVS.02 .01*	c.48G>C	RH:4 (c+ partial)
		c.733C>G	RH:5 (e+ partial)
			RH:10,20 (V+VS+)
			RH:–31 **(hr^B–)**
*RHCE*01.20.03*	*RHCE*ceVS.03*	c.48G>C	RH:4 **(c+ partial)**
		c.733C>G	RH:5 (e+ partial)
		c.1006G>T	RH:–10,20 (V–VS+)
			RH:–31 (hr^B–)
*RHCE*01.20.05*	*RHCE*ceVS.05*	c.733C>G	RH:5 (e+ partial)
		c.1006G>T	RH:–10,20 (V–VS+)
			RH:–31 (hr^B–)
*RHCE*01.02.01*	*RHCE*ceTI*	c.48G>C	RH:4 **(c+ weak, partial)**
		c.1025C>T	RH:5 (e+ weak, partial)
*RHCE*01.03*	*RHCE*ce.03*	c.1025C>T	RH:5 (e+ partial)

N/A, not applicable.

RBC unit from a donor carrying *RHCE*ceEK/RHCE*ceMO* would be assigned Tier 3 for a patient with *RHCE*ceEK/RHCE*ceEK*. Both patient and donor RBCs would be predicted to lack hr^S and have a similar phenotype, but the donor red cells express an Rhce molecule with one amino acid (p.223Phe) not expressed by the patient.

The term "probable RH allele" is used to emphasize that the alleles are assigned based on medium-resolution genotyping and therefore carry less than 100% certainty. A case example of how a limitation in the coverage of a genotyping panel impacts selection of donors based on RH allele information involves a patient for which IDCore XT (Grifols) assigns a result of RHCE*ce[733G]/RHCE*ce[733G]. However, when the sample is genotyped using another platform that includes the *RHCE* c.48G>C SNV, the patient was found to be *RHCE*ce48C*,*733G/RHCE*ce733G*. Though the phenotype is the same in this case, this information is useful when performing RH allele matching, and resulted in additional donors being classified as Tier 1 or Tier 2 for this patient.

What's missing on panels: uncommon but consequential variants

Medium-resolution RH genotyping panels do not rule in or out uncommon variants and this limitation should be noted in testing reports and understood by testing laboratory leadership and hospital clients. A case example is an African American patient whose red cells typed e+ E+. The immunohematology reference lab identified a possible anti-hr^B. The commercial *RHCE* genotyping panel identified

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Table 5 Common *ACKR1* (*FY*) single nucleotide variants, allele assignments and phenotypes

FY c.67t/c	*FY c.125G/A*	*FY c.265C/T*	Allele assignment	Predicted phenotype	Comment
t/t	G/A	C/C	*FY*01/FY*02*	Fy(a+b+)	–
c/c	A/A	C/C	*FY*02N.01/FY*02N.01*	Fy(a–b–)	–
t/c	G/A	C/C	*FY*01/FY*02N.01*	Fy(a+b–)	Likely allele assignment in most populations
t/c	G/A	C/C	*FY*01N.01/FY*02*	Fy(a–b+)	Unlikely but possible allele assignment
t/t	G/A	C/T	*FY*01/FY*02W.01*	Fy(a+b+^{w})	–
t/t	G/A	C/T	*FY*01W.01/FY*02*	Fy(a+^{w}b)	Unlikely but possible allele assignment

no variants. Based on this result, the patient would be predicted to carry *RHCE*cE* and *RHCE*ce* alleles. A PCR-restriction fragment length polymorphism (PCR-RFLP) was performed to interrogate an SNV at *RHCE* c.254C>G (rs57992529) and found the patient to be heterozygous. With this additional information, the allele assignment is *RHCE*cE/RHCE*ce01.06.01* and the predicted phenotype is C– c+ E+ partial e+ RH:–31 (hr^{B}–) (24). According to the ALFA project and listed in NCBI (25) this SNV has a global minor allele frequency (MAF) of 0.075 while the MAF in African Americans is 0.2%.

Another gap is found in the commonly used *RHD* genotyping panels that do not interrogate sufficient SNVs to differentiate some of the DAR family of alleles from Weak D 4.0. Cases of anti-D in a D+ patient where the antibody shows properties of an allo-antibody and/or the patient is of African descent may also benefit from *RHD* genotyping. Determination of a partial D phenotype is especially critical in patients who are likely to be multiply transfused, such as those with sickle cell disease.

Inferences due to linkage disequilibrium

The analysis software of a commercial genotyping platform that includes *RHCE* markers uses linkage disequilibrium between *RHCE* and *RHD* to provide interpretation that a specimen may carry the (C)ces haplotype. The (C)ces or r's haplotype is made up of the *RHD*03N.01 (RHD*DIIIa-CE(4-7)-D*) linked to the *RHCE*01.20.03* allele carrying c.48C, c.733G and c.1006T. This haplotype is important due to the loss of D antigen expression and the expression of an altered C antigen by the *RHD*03N.01* allele as well as the loss of RH:31 (hr^{B}) and RH:34 (Hr^{B}) antigens. However, the accuracy of the prediction is not 100% since the *RHCE*01.20.03* allele is also linked to the *RHD*03.01 (RHD*DIIIa*) allele that encodes a partial D phenotype. Therefore, this information is best used to identify samples for which additional molecular testing is warranted. In a D+ patient carrying *RHCE*01.20.03*, *RHD* genotyping would be recommended to rule out co-inheritance with *RHD*03.01*, a partial D phenotype and risk of allo-anti-D. In a D– patient carrying *RHCE*01.20.03*, *RHD* genotyping would be recommended to rule out co-inheritance with *RHD*03N.01* and expression of an altered C antigen that could put the patient at risk of allo-anti-C.

Phase assumptions

Currently, prediction of RBC antigen expression primarily utilizes low resolution SNV detection. When multiple SNVs are detected on the same gene, phase is imputed. Commercial genotyping kits utilize software algorithms to predict phenotypes. These algorithms use allele frequency information to make the most likely prediction. Probably the most classic example of this phenomenon is found in the FY blood group system (26), where most commercial assays include not only the c.125G>A SNV that predicts Fy^{a} and Fy^{b} expression, but also the SNV for the GATA box variant found in the promoter region. The *FY* c.-67t>c variant destroys the binding site for the GATA-1 transcription factor that is essential for expression of the *FY* antigens in red cells (27). This variant is common in individuals of African descent and is primarily found on the *FY*02* allele that encodes Fy^{b}; this allele is *FY*02N.01*. However, it has been found on the *FY*01* allele in other populations including in Papua New Guinea where it has been shown to impact Fy^{a} expression (28); that allele is *FY*01N.01*. In samples found to be compound heterozygous for *FY* c.-67t/c and c.125G/A, though it would be possible for the GATA mutation to be found on the allele encoding Fy^{a} and therefore silence Fy^{a}, it is unlikely in most populations (see *Table 5*). Another example in the FY system of phase assumptions in genotyping panel software involves the other common variant in the FY system, Fy^{x}. While the *FY*

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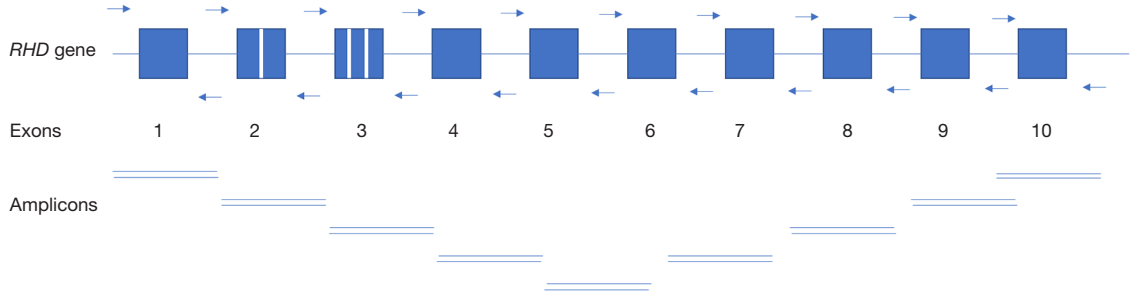

Figure 1 Schematic of the *RHD* gene with boxes representing the 10 exons and lines the intervening sequences. The first step in most SNV genotyping methods is amplification of a fragment or amplicon that contains the variant(s). Representative SNVs are depicted as white vertical lines in exons 2 and 3. This typically involves use of gene-specific primers that are located 5' (right pointing arrows) and 3' (left pointing arrows) of the area of interest. The amplicons are depicted as parallel lines under each exon. When a primer fails to bind and extend, due to one or more SNVs in the primer region, the amplicon is not generated resulting in "allele dropout". Allele dropout can result in a false negative predicted phenotype. SNV, single nucleotide variant.

c.265C>T SNV is typically inherited on the *FY*B* allele, it has been found on the *FY*A* allele (29). Since phase of SNVs is imputed on most genotyping panels, it is important for laboratories performing RBC genotyping using commercial kits to understand their population as well as what algorithms are used by the software to make allele assignments and predict phenotypes.

Equivocal allele assignments due to limitations of SNV genotyping in the context of gene conversion

Medium-resolution *RHD* genotyping, especially in individuals of African descent, can result in equivocal allele assignments due to inability to determine if exons in one allele failed to generate an amplicon due to presence of a hybrid allele. There are many *RHD* alleles where a portion of the gene, including one or several exons, have been replaced with *RHCE* sequences due to a gene conversion event. Since the genotyping panel uses gene-specific amplification of each exon in which SNVs on the panel are detected (*Figure 1*), this can result in "allele dropout". The most common example is the inability to determine if a sample carries the *RHD*03.01 (RHD*DIIIa)* allele in homozygosity (*Figure 2A*) or hemizygosity (*Figure 2B*) or if it is a compound heterozygous for *RHD*03.01* and the *RHD*03N.01 (RHD*DIIIa-CE(4-7)-D)* (*Figure 2C*). In the case of this allele combination, exons 4–7 are amplified only from the *RHD*03.01* allele since exons 4–7 of *RHD*03N.01* are derived from *RHCE* and the primer pairs fail to amplify a product.

Differentiating the allele combination in *Figure 2B,2C* cannot be deduced based on SNV typing alone since both *RHD*03.01* and *RHD*03N.01* carry *RHD* c.186G>T, c.410C>T, c.455A>C SNVs. The impact on phenotype is significant since *RHD*03.01* encodes a partial D phenotype and expression of the low-prevalence antigen DAK while *RHD*03N.01* does not encode the D antigen but instead encodes an altered C antigen. Individuals carrying this allele type C+ but are at risk of making allo-anti-C. While the individuals with *RHD* allele combinations shown in *Figure 2A,2B* would express a partial D phenotype, the individual with *RHD* allele combinations shown in *Figure 2C* would express a partial D phenotype as well as an altered C antigen. If serologic typing for C antigen is available or can be performed, the serologic information can be paired with the genotyping results to assign the alleles and predicted phenotype more accurately.

Equivocal allele assignments due to compound heterozygosity

Medium-resolution genotyping can result in detection of several SNVs such that more than one allele combination is possible. In *RHCE*, this is common in samples found to be compound heterozygotes for c.48G/C and c.733C/G. These SNVs are known to exist by themselves, together and also with additional SNVs (see *Tables 3,4*). *Table 6* shows the two possible allele assignments in this scenario. In the scenario shown at the top of *Table 6*, when the SNVs are inherited *in cis* (on the same allele), the other allele matches the reference. Though the RBCs would be predicted to type V+ and VS+, the patient is not predicted to express partial or variant antigens in the absence of normal

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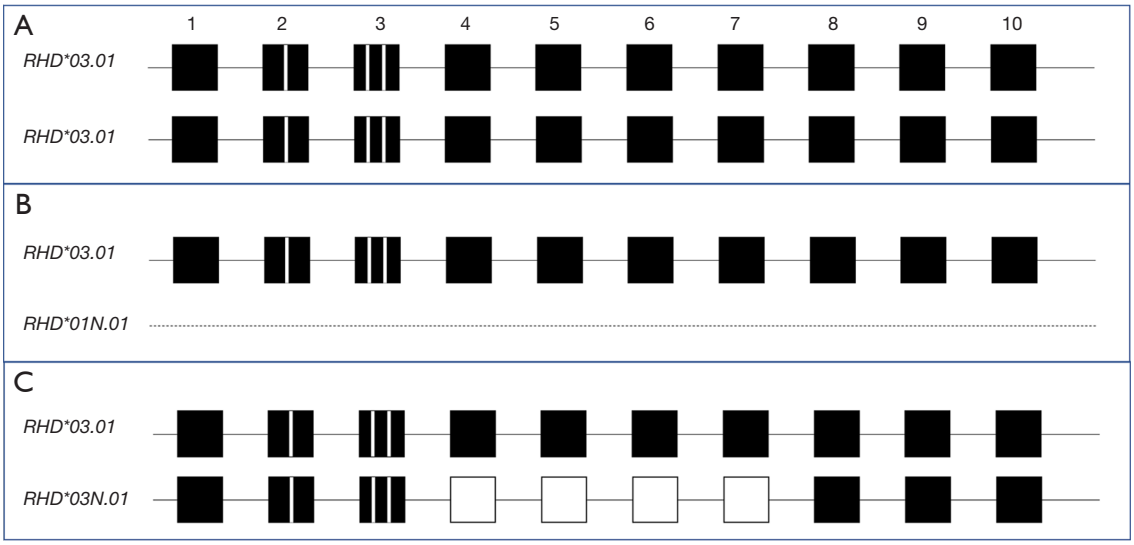

Figure 2 Schematics of the *RHD* gene are shown for three scenarios that can yield the same genotype results if a SNV panel is used. Since these panels cannot determine zygosity and since there are exon replacements, there are often three possible outcomes. The example shown is common when testing subjects of African descent where SNV panels cannot differentiate samples carrying the *RHD*03.01 (RHD*DIIIa*) allele in homozygosity (A) or hemizygosity (B) or if it is a compound heterozygous for *RHD*03.01* and the *RHD*03N.01 (RHD*DIIIa-CE(4-7)-D)* (C). In the case of this allele combination, exons 4–7 are amplified only from the *RHD*03.01* allele since exons 4–7 of *RHD*03N.01* are derived from *RHCE* and the primer pairs fail to amplify a product. SNV, single nucleotide variant.

Table 6 Compound heterozygosity for *RHCE* c.48G/C and c.733C/G, two allele combinations are possible

Possible *RHCE* allele combinations	Predicted phenotype	Risk of alloimmunization
*RHCE*01/RHCE*01.20.02.01 (RHCE*ce/RHCE*ce48C,733G)*	RH:4 (c+)	None
	RH:5 (e+)	
	RH:10,20 (V+VS+)	
	RH:31 (hrB+)	
*RHCE*01.01/RHCE*01.20.01 (RHCE*ce48C/RHCE*ce733G)*	RH:4 (c+)	Allo-anti-f (RH6)
	RH:5 (e+ weak)	Some reports of anti-e-like (possible anti-e^{VAR})
	RH:10,20 (V+VS+)	
	RH:31 (hrB+)	

antigens. In the scenario shown at the bottom of *Table 6*, when the SNVs are inherited *in trans* (on separate alleles), the sample does not carry a reference allele expressing normal antigens. In this case, the individual may be at risk of allo-anti-f since they do not express a normal f antigen. Though standard SNV genotyping panels cannot determine the phase in this scenario, long-range PCR with a sequence-specific primers can be used to determine *cis* or *trans* configuration. Additionally, if total RNA can be isolated from erythrocytes, cDNA PCR can be performed to amplify the *RHCE* transcripts which can be cloned into plasmid vectors such that Sanger sequencing of plasmid DNA isolated from individual bacterial colonies can characterize each allele separately (30). This is depicted in *Figure 3* in which *Figure 3A* depicts the *RHCE* allele with the SNVs *in trans* and *Figure 3B* the alleles with the SNVs *in cis*. *Figure 3C* shows the cDNA amplicons from the scenario where the SNVs are *in trans* and *Figure 3D* the

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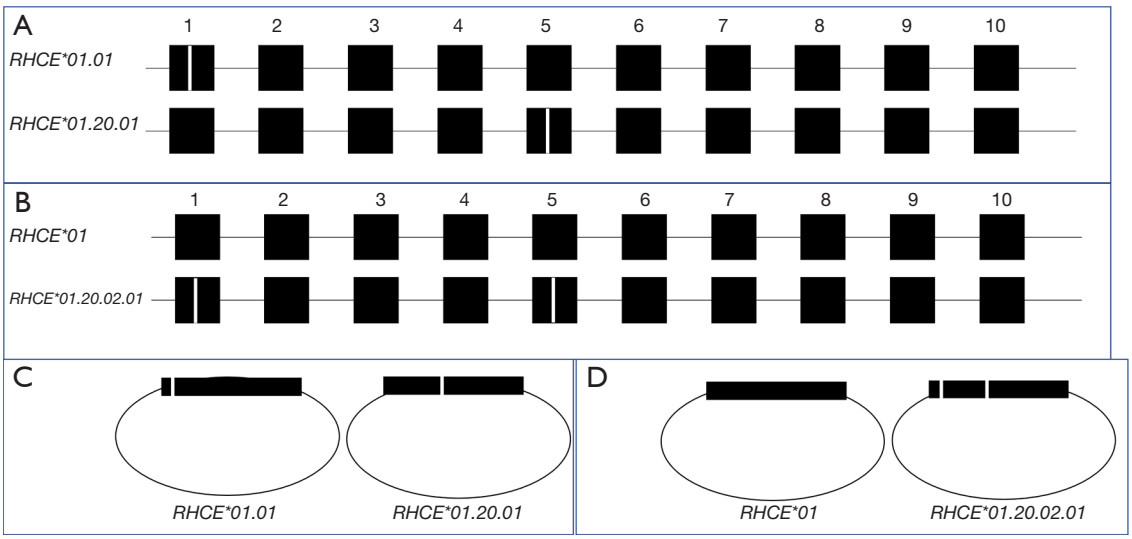

Figure 3 Schematics of the *RHCE* gene are shown to illustrate phase of SNVs with allele names shown on the left. (A) Depicts the *RHCE* allele with two SNVs (c.48G>C and c.733C>G) common in subjects of African descent *in trans*. (B) Depicts the alleles with the SNVs *in cis*. If cDNA is generated from these two different scenarios, and amplicons are cloned into plasmid vectors, the plasmids can be sequenced with vector-based primers. (C) Depicts the amplicons from the scenario where the SNVs are *in trans* such that both Rhce molecules are variant as depicted in (A). (D) Depicts the amplicons from the scenario where the SNVs are *in cis* such that one Rhce molecule is conventional (left) and one is variant (right) as depicted in (B). SNV, single nucleotide variant.

cDNA amplicons from the scenario where the SNVs are *in cis*. This case illustrates how the amplification and cloning of RH cDNAs followed by Sanger sequencing can be used to "solve" the phase of a sample carrying multiple SNVs in compound heterozygosity.

Conclusions

This narrative review describes the limitations of commercially available red cell genotyping panels. The author acknowledges that the cataloguing of the panels was based on experience and internet searches and may be incomplete. In addition, the limitations of genotyping panels were not exhaustive, but instead focused on limitations that may not be well appreciated by clinicians who received genotyping reports or laboratory managers who select and implement the panels in their facilities.

It is important for those performing molecular testing and those utilizing the results of such testing to understand the limitations of current RH genotyping panels, and RBC genotyping panels more generally. However, these limitations should not dissuade clinicians and immunohematology reference laboratory and blood bank staff from utilizing this technology. Serologic methods used to directly test for antigens have limitations as well. Those limitations include lack of reagents, discrepancies due to weak antigens and/or epitope loss in partial antigens especially RhD (31) as well as mixed field reactivity after recent transfusion or in the context of autoantibodies (32).

Though there are limitations to our current understanding of RH genetics and the implications of the inheriting some RH variants, as well as demonstrated efficacy of Rh immune prophylaxis in partial D women, there is much evidence to show that RH genotyping is beneficial in identifying risk of alloimmunization and can also be used to personalized donor unit selection. Currently, low- and medium-resolution testing allows cost-effective and timely resolution of the majority (but not all) cases of serologic weak D phenotypes and typing discrepancies, thus allowing the resulting information to be used for patient management. In complex cases, high resolution methods may be needed that require engaging a molecular reference laboratory that offers such services.

In the future, high-resolution testing is certain to become common place for red cell genotyping including RH genotyping. Although even that technology is not free from challenges with interpreting genetic variation (33,34), molecular methods and software algorithms are improving (35) such that discussions of incorrect phenotype

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predictions are likely to become a thing of the past. Until that time, it is important for laboratorians performing or ordering RH genotyping as well as clinicians ordering or interpreting RH genotyping to understand the limitations of commercial panels and the value of a molecular reference laboratory with expertise in this area, especially in cases where high-resolution testing is warranted (36).

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Footnote

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