Resolving Typing Discrepancies Using All the Tools in the Toolbox

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Conflicts of Interest

The presenters have no conflicts to declare.

Resolving Typing Discrepancies using All the Tools in the Toolbox

- Effective use of test methods
- Experience of the technologist and supervisor
- Critical evaluation of the case history
- Critical evaluation of the serologic and molecular test results

- Cases will include discrepancies between historic and current typings, serologic typings with different reagents or methods, and discrepancies between genotype-predicted phenotype and serologic phenotype.

It's all about D

1997

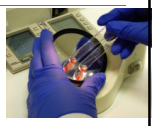
- "Normal" D antigen – gene and protein normal
- "Abnormal" D antigen
  - Weak D: used to be defined as D+ by IAT only**
    - **New Weak D definition in use now
  - Partial D: differences in gene resulting in alterations in antigen expression
    - One very weak partial D:
      - DEL : D antigen detected only by adsorption and elution or molecular methods
  - Increased D: D – –
- *RHD* is an extremely polymorphic gene

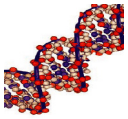
Dr Landsteiner Featured on the 1997 Austrian 1000 Schilling

Anti-D Reagent Information

With current monoclonal reagents:

- Most D+ RBCs react at Immediate Spin
- Many partial D RBCs react at Immediate Spin
  - Partial D status not known until anti-D detected
  - To detect partial D in D+, need molecular testing on D+ patients in the prenatal period or pretranfusion

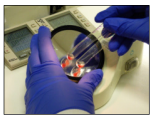


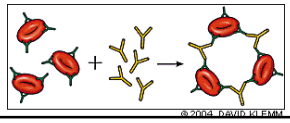


Anti-D Reagent Information

With current monoclonal reagents:

- IgG component in tube test needed to detect D antigens reactive by AHG
- No single monoclonal anti-D reagent detects all D+ antigens
- Polyclonal anti-D tested by AHG phase detects most partial and weak D except Del





Serologic Testing

7 IRL Technician Carla Hands performs antigen testing

Automated Typing Instruments for Patients

ECHO

IH-1000

Erytra

NEO

Ortho Vision

8

Beckman PK7300 for Donor Testing

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Weak D – Serologic Characterization

- Reported to be low D antigen copy number weakly reactive or requiring weak D test (indirect antiglobulin test previously called the D^u Test) Reactivity is varied
  - According to sample (#D sites per RBC)
  - According to method (sensitivity of test)
  - Most are detected by blood bank automation which detects ≥1+ weak D test (AHG phase in tube)
- <1% Caucasian population

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Partial D - Serologic Characterization

- D antigen lacks defined epitope(s)
- Serologic Identification - historic
  - Monoclonal anti-D patterns identify the subtypes
  - Most partial D RBCs react with reagent anti-D at immediate spin phase
  - Most partial D RBCs detected by automated typing instruments
- Many partial D types at risk for alloimmunization to D

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Anti-D Reagents

Polyclonal (high protein)
Monoclonal (low protein)
- IgM monoclonal
- Monoclonal blends
  - IgM monoclonal / IgM monoclonal
  - IgM monoclonal / IgG monoclonal

Monoclonal/Polyclonal blend
- IgM monoclonal / IgG polyclonal

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D Testing – AABB BBTS Standards

- AABB Std 5.8.2 - Donors: If the initial test with anti-D is negative, the blood shall be tested using a method designed to detect weak D
- AABB Std 5.14.2 - Recipients:
  - Rh type shall be determined with anti-D reagent
  - The test for weak D is unnecessary when testing the patient
- AABB Std 5.30.2.(3) - Newborns of D– moms (RhIG candidates): ...weak D testing is required when the test for D is negative

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D Testing Requirements AABB

AABB Std 5.8.2 - **Donors**:
If the initial test with anti-D is negative, the blood shall be tested using a method designed to detect weak D

Blood Bank Automation approved by FDA for donor testing detects donors with weak D test ≥1+ (by parallel AHG tube test)

- ∴ many weak D resulted as D+ in donors currently
- ∴ most partial D resulted as D+ in donors currently
- To detect all partial D, molecular testing all D+ and D- donors needed

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D Testing Requirements AABB

AABB Std 5.14.2 - **Recipients**:
Rh type shall be determined with anti-D reagent. The test for weak D is unnecessary when testing the patient

- Test method selected by facilities may yield final D interpretation differences in patients
  - Some facilities type all patients by automation (detects most weak and partial D RBCs)
  - Some use tube testing with weak D (IAT phase) for all patients (detects more weak and partial D RBCs)
  - Some do not do weak D testing, only immediate spin as required
- Some donors may be D+ as donor, but D neg as patient

NY Times

Mitigation: Education of staff on reasons for discrepancies and when to investigate with molecular testing

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D Interpretations Different if Donor or Patient

- Autologous Donor presents for donation
  - Typed in donor center by Beckman PK7300 as D+
- Patient Pre-surgical type and cross
  - Typed by Transfusion Service on Galileo as D+

  OR
  - Typed by BB in Tube Test as D negative, weak D test not done (not required) – interpretation D neg
- Autologous unit labeled D+, patient is resulted in BB computer as D-
  - Easily resolved with weak D test

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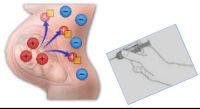
Implications for Weak D Patients

- Selection of anti-D reagent/testing method important (some inserts say <2+ result is D negative)
- Weak D testing not required for transfusion recipients and prenatal patients, but if not done:
  - Weak D patients are resulted as D negative and receive D negative blood they may not require
  - Prenatal patients may be determined to be RhIG candidates and they may not require it



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Implications for Weak D Patients – cont.

Weak D testing not required for transfusion recipients and prenatal patients, but if done:

- Weak D positives are resulted as either:
  - D+ and receive D+ blood OR
  - Weak D and receive D- blood or D+ (facility dependent)
- Prenatal patients are resulted as D+ and are not RhIg candidates (facility dependent)
  - Accepted by ACOG (American College of Obstetricians and Gynecologists)

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Implications for D negative Patients

- Selection of anti-D reagent/testing method important for donors and babies (automation vs. tube test)
- Detection of Weak D < 1+ by automation not required for donors ∴ some weakly reactive D antigens are not detected
- Weak D babies of D negative mothers may not be detected by automation, potential for D alloimmunization of the mother
  - Weak D detection required for babies of D negative mothers
- Both expected to be very rare events, but will happen

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Rh Discrepancies Caused by Variable Reactivity of Partial and Weak D types with Different Serologic Techniques*

- Monoclonal anti-D reagents did not distinguish between partial and weak D Types 1 and 2
- Weak D Types 1 and 2 do not show consistent reactivity with FDA-approved tube test reagents and technology
- Molecular tests that distinguish common partial and Weak D types provide the solution to resolving D antigen discrepancies
- **To limit anti-D alloimmunization, it is recommended that samples yielding an immediate-spin tube test of 1+ agglutination or not more 2+ by gel technology be considered D– for transfusion and Rh immune globulin prophylaxis**

*Gregory A. Denomme, Louann R. Dake, Daniel Vilensky, Lily Ramyar, W. John Judd. *Transfusion* 2008;48:473-478

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Challenges in D Testing Interpretation

- Differences among FDA licensed reagents/methods may cause discrepancies between records:
  - Historical vs. current
  - Donor vs. recipient
  - Between facilities

Mitigation: Educate staff on reasons for discrepancies and when to investigate with molecular testing

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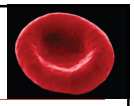
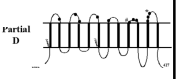
Common Partial D – DVI



- Most common form of partial D in Caucasians, reactivity is like weak D:
  - Not detected at immediate spin
  - Usually detected at antiglobulin phase
  - Generally resulted as D neg as patients and D+ as donors
- But one reagent - Alba Bioscience's anti-D reacts at IS
  - ∴ ideal for testing donors but not for patients
- Anti-D produced by DVI has resulted in fatal HDFN
  - ∴ potential for clinical significance in pregnancy
- Treat as D neg for transfusion and RhIG prophylaxis
- Lack outcome data on RhIg effectiveness in partial D patients with D+ fetus/newborn



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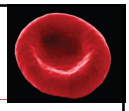
Reagent Reactivity with DVI RBCs



DVI RBCs	Immediate Spin	Antiglobulin Phase
Polyclonal	0	+
Ortho BioClone	0	+
Ortho GEL	NA	0
Gamma-clone blend	0	+
Immucor Series 4	0	+
Immucor Series 5	0	+
Seraclone IgM	0	NA
Seraclone blend	0	+
Erytype S IgM	0	NA
Solidscreen II IgG	NT	+
ALBAclone α & β IgM	0	NT
ALBAcone blend IgM/IgG	0	+
ALBAclone Δ IgM	+	NT

Adapted from Regina Leger

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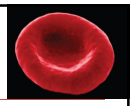
FDA Requirements for DVI



- FDA requirement that monoclonal anti-D not detect partial DVI on immediate spin
- Goal to ensure that females of childbearing years who are of partial DVI type will be typed as D negative
- Donors with partial DVI type will type positive with weak D test
- It is the reason samples from pregnant women do not require weak D testing

*Sandler SG, Flegel WA, Westhoff CM *et al.* It's time to phase in *RHD* genotyping for patients with a serologic weak D phenotype. *Transfusion* 2015 55(3):680-9.

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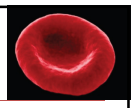
Uncommon Partial D: DEL



- Del RBCs
  - Negative in routine IS and IAT tube tests
  - Negative in automated tests
  - Very low levels of D antigen detected by adsorption/elution or molecular methods only
  - Most frequently found in D– East Asians (~30%), rare in Europeans (different mutations)
  - Del donors type D-negative and when transfused to D– recipients, rare case reports of anti-D formed

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Anti-D Crossreactivity

- RBCs with epitopes of D expressed on RHCE protein react strongly at immediate spin with some anti-D, these samples do not have D protein:
  - DHAR (formerly called RoHAR, better expressed as ceHAR)
    - German ethnicity
  - Crawford
    - African American ethnicity

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Anti-D Crossreactivity - ceHAR

- Hybrid *RHCE-D-CE* gene, no *RHD* gene
- Rare, < 0.01%
- Routine high protein anti-D reagents usually do not detect
- ceHAR RBCs react strongly with some IgM anti-D
- ceHAR individuals can make anti-D
- Blood recipients with hybrid *ceHAR* gene should be given D negative RBCs
- Perhaps a reason to have different anti-D for patients and donors

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Anti-D Crossreactivity - ceHAR

	Immediate Spin
Gamma-clone	+
Immucor Series 4	+
Immucor Series 5	+
Ortho BioClone	0
Ortho Gel (ID-MTS)	+
Seraclone IgM	+
Seraclone blend	+
ALBAclone alpha & beta IgM	+
ALBAclone blend	+
ALBAclone delta IgM	+

28 Adapted from Regina Leger

Anti-D Crossreactivity – Crawford–Rh43

- Low incidence antigen – African American ethnicity (~0.1%)
- Rare allele *RHCE*ceCF* , no RhD protein
- Only a few reported anti-D are reactive:
  - anti-D clone GAMA401
  - Anti-D ALBAclone (may be reactive)
- Pregnant patients and transfusion recipients should be interpreted as D negative and receive RhIG and D negative blood

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Common Partial D Reactivity and Anti-D Crossreactivity – Summary

D type	As a Donor	As a Patient
D^{VI}	D+	D-
ceHAR, Crawford	D- *	D-*
Del	D+**	D-

*Difficult to manage electronically when anti-D typing sera is positive, but RBCs are not really D+

**Ideally, likely does not happen now

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Vox Sang International Forum Genotyping for red blood cell polymorphisms

Do you apply genotyping for RBC polymorphisms for the following:

- *RHD* genotype of the fetus to avoid unnecessary administration of anti-D immunoglobulin G (IgG) to D-negative pregnant women during pregnancy
  - If so: Do you use fetal DNA from the maternal plasma?
- Typing for the *RHD* genotype of the fetus only in cases in which the fetus is at risk for hemolytic disease

31 C. E. van der Schoot *et al.* International Forum, *Vox Sanguinis* 2009;96:67–179

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Vox Sang International Forum Genotyping for red blood cell polymorphisms

Summary:

- The fetal *RHD* genotype is determined in all participating countries if the fetus is at risk of anti-D hemolytic disease
- In the USA and Canada, DNA from amniotic fluid (amniocytes) is still used predominantly
- In the European countries and Brazil, cell-free fetal DNA isolated from maternal plasma is used for genotyping as well as amniotic fluid
- No countries routinely determine the *RHD* genotype of the fetus to prevent unnecessary prenatal administration of anti-D immunoglobulin

32 C. E. van der Schoot *et al.* International Forum, *Vox Sanguinis* 2009;96:67–179

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Algorithm to Select Samples for Genotyping

Current method in facility:

- Two typing for first time patients are performed with two methods
  - Gel and Tube (monoclonal/polyclonal blend at IS)
  - or Gel D

33 Luo X, Keller M, James I, Grant M, L, Massey KS, Czulewicz A, Nance S, Li Y. Strategies to Identify Candidates 1 for D Variants Genotyping (in press) DOI 10.2450/2017.0274-16

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Algorithm to Select Samples for Genotyping

Three criteria used to determine if the sample should be sent for genotyping:

- Discrepancy between 2 methods and Gel reaction strength at least 2+ stronger than tube
- Serologic weak reaction strength <2+ regardless of testing method if both tube test and Gel performed
  - OR <2+ if only Gel test performed
  - Or <1+ if only tube test performed
- Presence of anti-D in D positive patient with no history of RhIg in last 3 months

34 Luo X, Keller M, James I, Grant M, L, Massey KS, Czulewicz A, Nance S, Li Y. Strategies to Identify Candidates 1 for D Variants Genotyping (in press) DOI 10.2450/2017.0274-16

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Algorithm to Select Samples for Genotyping

- A total of 50 patients ranging from newborn to 93 years in age were identified to be genotyped
- Genomic testing confirmed D variants in 49/50 cases
- Positive predictive value (PPV) of 98%
  - 1/50 (2%) was D+ with anti-D with 1 conventional *RHD* allele
    - auto-adsorption not performed
- Identified more partial D+ cases than other approaches
  - Identified 39/50 (78%) partial D+ cases
    - May be due to race/ethnicity of cohort (54% AA, 32% Hispanic)
    - May be due to two test method approach (51% of partial D alleles found based on this criteria)

35 Luo X, Keller M, James I, Grant M, L, Massey KS, Czulewicz A, Nance S, Li Y. Strategies to Identify Candidates 1 for D Variants Genotyping (in press) DOI 10.2450/2017.0274-16

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Summary of Challenges of Serologic Typing

- Serologic methods vary
  - Tube
  - Gel
  - Solid phase
- Serologic reagents vary or are unavailable
  - Monoclonal
  - Polyclonal
  - Blends
  - Patient source
- Simple (e.g. Fy^a/Fy^b) vs complex (e.g. RhD) epitopes
- Antigen variants can be missed (e.g. U variants)
- Expression level can hamper detection (e.g. weak D)
- Cross-reactivity (e.g. ceHAR, ceCF)

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Table 1. Utility of RBC genotyping for donors and patients (with selected references)

Scenario
Predict antigen status when reagents unavailable (e.g., hr^B, hr^S, Jo^a, Hy, U)
Predict antigen status when weak antigens can be missed serologically (e.g., Fy^b, D)
Predict antigen status when antibody-coated RBCs hamper serologic typing
Predict antigen status when recent transfusion hampers serologic typing
Predict antigen status when variant antigen is suspected to be causing typing discrepancy (current vs. historic, reagent 1 vs. reagent 2, method 1 vs. method 2, molecular vs. serologic)
Predict antigen status when variant antigen is suspected based on alloimmunization (e.g., e+ with anti-e)
Identify alleles encoding partial antigens when allele matching of donors and patients may be considered
Efficiently identify antigen-negative status for multiple antigens simultaneously
Predict antigen status in reagent red cells used for antibody screening
Determine impact of unlinked genetic factors on antigen expression [e.g., In(Lu)]
Determine zygosity as it relates to HDFN
Determine zygosity as it relates to reagent red cells used for antibody screening

RBC = red blood cell; N/A = not applicable; HDFN = hemolytic disease of the fetus and newborn.

Anti-CD38 and anti-CD47

IMMUNOHEMATOLOGY, Volume 31, Number 2, 2015

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Molecular Testing Methods Differ in their Resolution

- **Low Resolution**
  - Gel-based methods
    - SSP-PCR for known SNPs
    - PCR-RFLP for known SNPs
  - High melt resolution analysis
- **Medium Resolution**
  - Arrays
    - Immucor BeadChip and Grifols IDCore
    - Taqman
  - MALDI-TOF
- **High Resolution**
  - DNA sequence analysis
    - Exon scanning
    - cDNA analysis
    - *NextGen Sequencing*

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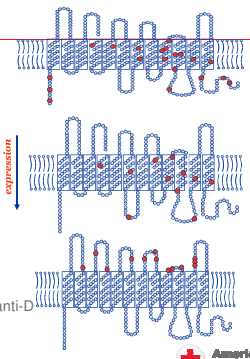
RhD Variants

Weak Ds
-alter quantity of protein, but not the surface D-epitope
-individuals do not usually make anti-D

Del
-Very weak expression of D, type D-
-can stimulate anti-D in D- patients

Partial Ds
-have altered epitopes
-can type strongly D+
-can type weakly +
-often not recognized until they make anti-D



But it's not always that clear cut!

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Diagrams courtesy of Lilian Castilho

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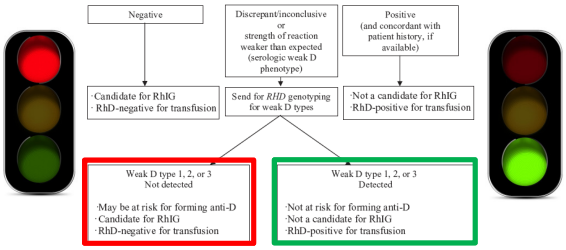
It's Time to Phase In *RHD* Genotyping



*Sandler SG, Flegel WA, Westhoff CM et al. It's time to phase in *RHD* genotyping for patients with a serologic weak D phenotype. Transfusion 2015 55(3):680-9.

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Case 1: Background

- 24 year old Caucasian pregnant female
- Types D negative on ECHO with Immucor Series 4 and 5
- Types D w+ at immediate spin, 2+ at antihuman globulin
- Ordered *RHD* genotyping to resolve the discrepancy and determine candidacy for RhIg

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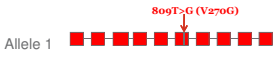
Case 1: *RHD* Genotyping



Allele 1 — ***RHD*01W.01*** **(weak D type 1)** → Express RhD weakly

Allele 2 — ***RHD*01N.01*** → Does not express RhD

Predicted Phenotype: Weak D+

Based on the *RHD* alleles identified, the patient is not at risk for production of allo-anti-D.

Rh Immunoglobulin not indicated for this patient

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Case 2: Background

- 40 year old Hispanic pregnant female
- Typed Group O D negative at outside lab
- Listed as O Positive in hospital records
- Current sample types
  - D negative on ECHO with Immucor Series 4 and 5 anti-D
  - D negative at immediate spin, 2+ at AHG
- Ordered *RHD* genotyping

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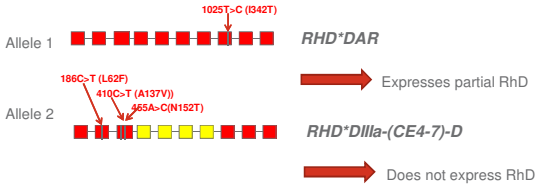
Case 2: *RHD* Genotyping



Predicted Phenotype: Partial D+

The patient is at risk for production of allo-anti-D

Characterization of the *RHCE* genes may be warranted

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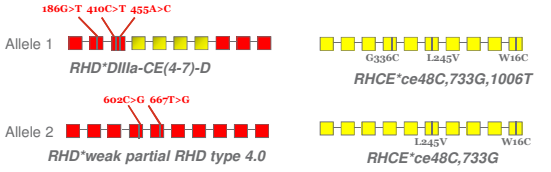
Case 2: *RH* Genotyping



Predicted Phenotype: Partial D+, Altered C+, E- partial c+ partial e+ V+ VS+ hr^B-

The patient may be at risk for production of allo-anti-D and is at risk of allo anti-C, -c, -e, -hr^B

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Case 3: Background

- 28 year old Caucasian female
- Pregnant
- Types D+ at immediate spin (w+ to 3+) with 3 of 4 sources of anti-D
- Typed D- at IS and at AHG with the fourth anti-D
- Requested *RHD* genotyping

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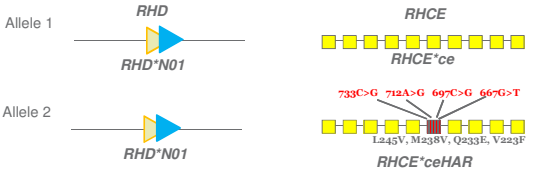
Case 3: *RH* Genotyping



Predicted Phenotype: **D-**, C-, E- c+ e+ DHAR+ FPPT+

The patient is not predicted to express D. They carry the *RHCE*ceHAR* allele

Individuals with a *RHCE*ceHAR* allele express D epitope(s) that type strongly D+ with some monoclonal anti-D reagents

Such individuals should be considered D negative

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Anti-D Cross Reactivity – *RHCE*ceHAR*

Reagent	Reactivity at Immediate Spin
Gamma-clone	+
Immucor Series 4	+
Immucor Series 5	+
Ortho BioClone	0
Ortho gel (ID-MTS)	+
Seraclone IgM	+
Seraclone blend	+
ALBAclone alpha & beta IgM	+
ALBAclone blend	+
ALBAclone delta IgM	+

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Case 4: Background

- 25 year old Hispanic female, pregnant
- D typing discrepancy
  - Patient typed repeatedly D negative with negative Ab screen
  - Post-partum, both patient and infant type D negative at IS and positive (3+) at AHG
- Requested *RHD* variant workup

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Case 4: Routine (targeted) testing

TESTING PERFORMED			RESULT
RHD Variants	Method	Analyte: Nucleotide (Amino Acid)	Nucleotide(s) Detected
w*RHD* BEADCHIP™	*RHD* array	No variant markers detected	

*Array includes 35 markers. Only nucleotides that differ from the consensus sequence are listed

Probable *RHD* Genotype: *RHD*01 / RHD*01N.01*

Predicted RhD phenotype: D+

But this is not consistent with the reported serology!

Higher resolution testing is warranted.

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But Remember Resolution?

- **Low Resolution**
  - Gel-based methods
    - SSP-PCR for known SNPs
    - PCR-RFLP for known SNPs
- **Medium Resolution**
  - Arrays such as BeadChip™
- **High Resolution**
  - DNA sequence analysis
    - Exon scanning
    - cDNA analysis
    - *NextGen Sequencing*

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Case 4: High Resolution Testing

cDNA analysis:

TESTING PERFORMED			RESULT
RHD Variants	Method	Analyte: Nucleotide (Amino Acid)	Nucleotide(s) which differ from consensus
RHD SEQUENCING	cDNA seq	Plasmids	c.28T (10Trp)

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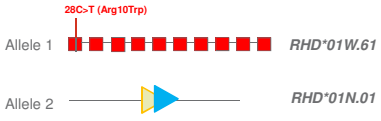
Case 4: Revised Interpretation



Predicted Phenotype: weak D+

The patient was found to carry a variant not interrogated by the targeted *RHD* genotyping performed previously

Due to the lack of information about the risk of alloimmunization in patients carrying this variant, it is recommended that this patient be treated as D negative for purposes of transfusion and a candidate for Rh Immunoglobulin

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Case 5: Background

- 19 year old female blood donor
- Jk^a typing discrepancy

- Donor typed Jk(a-) by serology
- Red cell unit distributed as Jk(a-)
- Hospital reported unit is XM incompatible
- Requested *JK* variant workup

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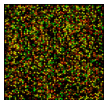
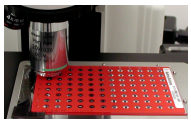
Case 5: Targeted Genotyping

- Genomic DNA extracted from pilot tube
- DNA tested using PreciseType HEA Molecular BeadChip
  - Predicted donor to type Jk(a+b+)





Kidd	Jka	+
	Jkb	+

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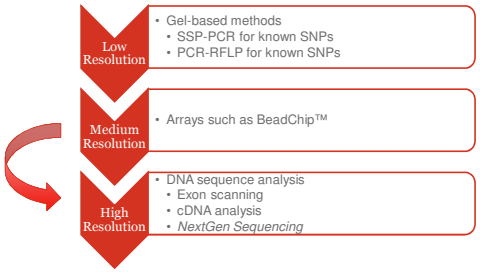
But Remember Resolution?



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Case 5: High Resolution Genotyping

- Exon Scanning of coding exons

Gene or Region	Method	Analyte	Result: Variants detected	Interpretation: Predicted Amino Acid
JK	gDNA seq*	Exon 4	130G/A	E44K
		Exon 5	None	N/A
		Exon 6	None	N/A
		Exon 7	588A/G	silent
		Exon 8	None	N/A
		Exon 9	838G/A	D280N
		Exon 10	None	N/A
		Exon 11	None	N/A

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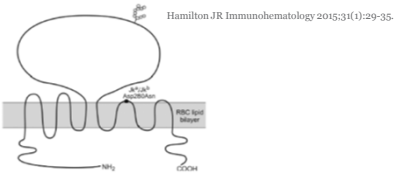
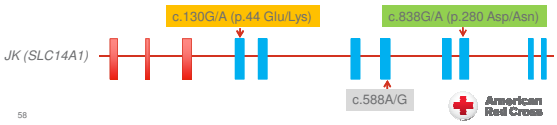
Case 5: High Resolution Genotyping





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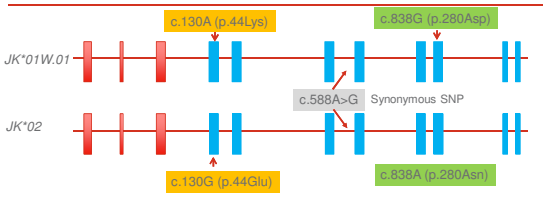
Case 5: Assigning Alleles and Predicting Phenotypes



Predicted Phenotype: Jk(a+^{w} b+)

Genotyping identified a *JK* variant associated with weak antigen expression and typing discrepancies. The donor record should reflect the predicted phenotype.

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Summary

- RhD variants are categorized as weak, partial and D_{el} and in many cases serology cannot resolve the type and determine alloimmunization risk
- *RHD* genotyping can be used to resolve serologic weak D types not at risk for alloimmunization
- *RHD* genotyping can identify patients with partial D phenotype at risk to form allo-anti-D
- RBC genotyping can resolve typing discrepancies, and may require multiple molecular methods, with varying levels of resolution

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Thank you for your attention!

Questions?

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