Serology and molecular testing to resolve complex Rh cases

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New York Blood Center

Transfusion Science Course
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RH Blood Group System

- After ABO, the most clinically significant blood group system
- Currently 55 reported antigens
- 5 common (major) antigens
  - D, C, E, c, and e; C/c and E/e are antithetical
  - Make 8 haplotypes:
    - DCe(R_1) Ce(r′)
    - DcE(R_2) cE(r″)
    - Dce(R_0) ce(r)
    - DCE(R_Z) CE(r^Y)

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Rh system (ISBT 004): antigens RH8 to RH62

ISBT # / Name	Preva-lence	ISBT # / Name	Preva-lence	ISBT # / Name	Preva-lence	ISBT # / Name	Preva-lence
RH8 / C^W	Low	RH26 / c-like	Poly	RH39 / Rh39	High	RH51 / MAR	High
RH9 / C^X	Low	RH27 / cE	Poly	RH40 / Tar	Low	RH52 / BARC	Low
RH10 / V	Low	RH28 / hr^H	Low	RH41 / Rh41	Poly	RH53 / JAHK	Low
RH11 / E^W	Low	RH29 / Rh29	High	RH42 / Rh42	Low	RH54 / DAK	Low
RH12 / G	Poly	RH30 / Go^a	Low	RH43 / Crawford	Low	RH55 / LOCR	Low
RH17 / Hr_o	High	RH31 / hr^B	High	RH44 / Nou	High	RH56 / CENR	High
RH18 / Hr	High	RH32 / Rh32	Low	RH45 / Riv	Low	RH57 / CEST	High
RH19 / hr^S	High	RH33 / Rh33	Low	RH46 / Sec	High	RH58 / CELO	High
RH20 / VS	Low	RH34 / Hr^B	High	RH47 / Dav	High	RH59 / CEAG	High
RH21 / C^G	Poly	RH35 / Rh35	Low	RH48 / JAL	Low	RH60 / PARG	Low
RH22 / CE	Low	RH36 / Be^a	Low	RH49 / STEM	Low	RH61 / CEVF	High
RH23 / D^W	Low	RH37 / Evans	Low	RH50 / FPTT	Low	RH62 / CEWA	High

Missing numbers caused by antigens becoming obsolete

For updates see:
www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology

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What makes Rh complex?

- The terminology !
- Numerous antigens, phenotypes and alleles
- One phenotype can arise from several genotypes
- Autoantibodies often have Rh specificity
- Difficulty in determining auto- versus allo-antibody when the patient is transfused
- Partial antigens where individuals can make an alloantibody to an antigen expressed on their RBCs
- Weak antigen expression

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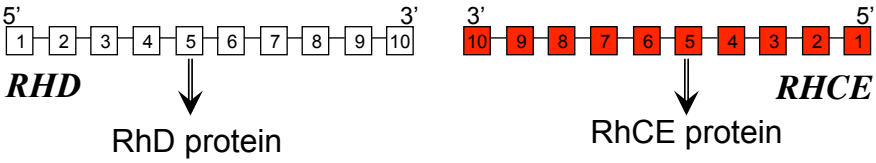
RH genes on chromosome 1 at 1p36.11

- Comprised of 2 genes (as P. Tippett predicted in1986)
  - *RHD* and *RHCE*, each consisting of 10 exons
  - Gene duplication; *RHCE*ce* is the ancestral gene
  - Genes are homologous (>95% identity at nucleotide and thus at the amino acid level)



- A third ancestral homologous gene (*RHAG*), on chromosome 6, encodes the Rh-associated glycoprotein (RhAG)
  - RhAG is essential for Rh antigen expression

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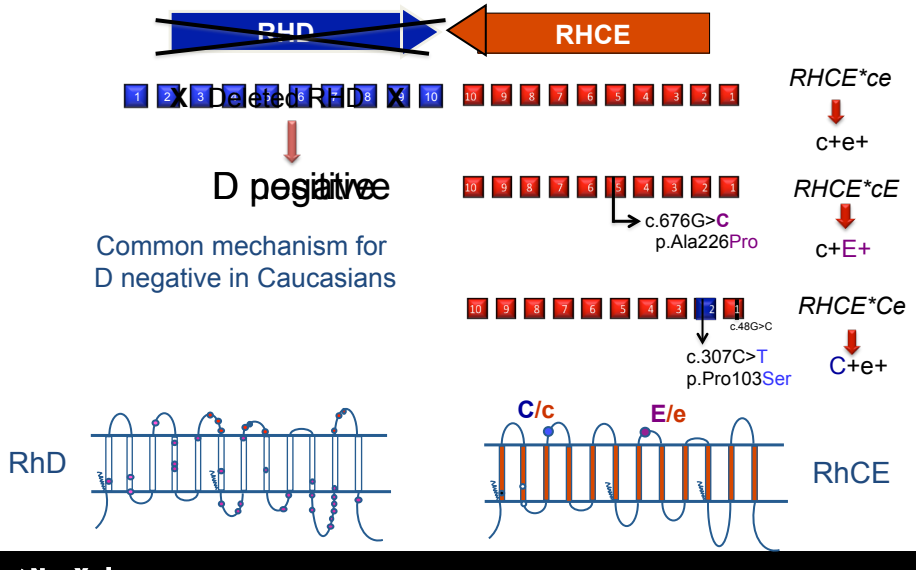
RH locus



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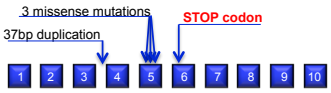
Common ways to be D negative

- Deletion of the RHD gene

-No D protein
-D negative

- Inactive RHD pseudogene (Dψ) – common in Blacks



-Premature stop codon
-No D protein
-D negative

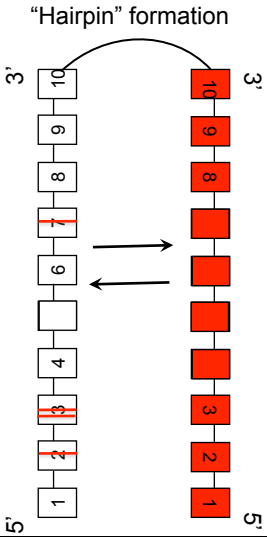
- Hybrid allele
  - Specifically Hybrid *RHD*DIIIa-CE(4-7)-D*

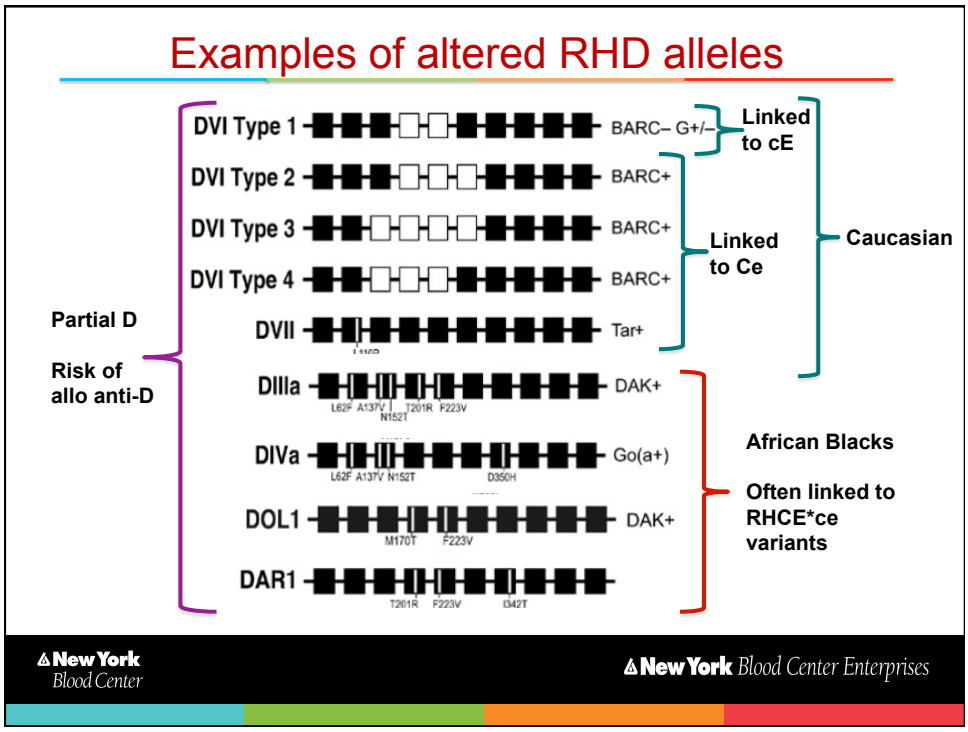
[C+(partial)]

-Protein made does not express D antigen
-D negative

Why so many Rh antigens and phenotypes?

- Point mutations
- Gene conversion:
  - Portions of *RHCE* into *RHD*
  - Portions of *RHD* into *RHCE*
  - No reciprocal crossover
- *RHD*: >495 alleles
  - Weak D, Partial D, Del
- *RHCE*: >110 alleles
  - ce, including hr^B–, hr^S –
  - Ce
  - cE
  - CE




Examples of altered RHD alleles
DVI Type 1
BARC– G+/–
DVI Type 2
BARC+
DVI Type 3
BARC+
DVI Type 4
BARC+
DVII
Tar+
DIIIa
DAK+
DIVa
Go(a+)
DOL1
DAK+
DAR1
Linked to cE
Linked to Ce
Caucasian
Partial D
Risk of allo anti-D
African Blacks
Often linked to RHCE*ce variants
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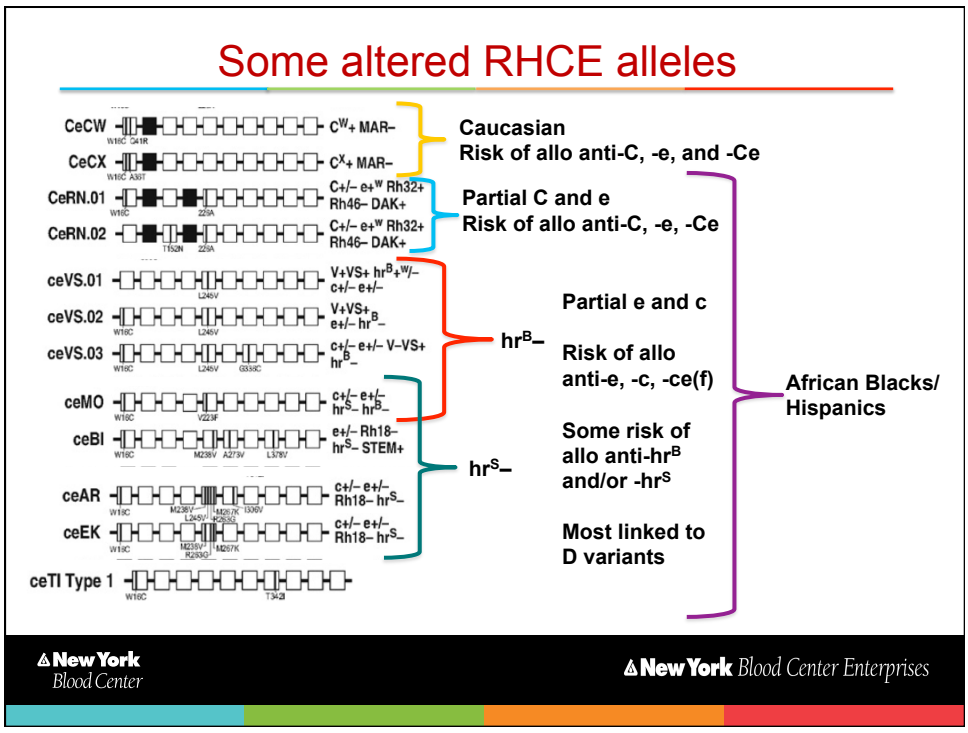
Some altered RHCE alleles
CeCW
CeCX
CeRN.01
CeRN.02
ceVS.01
ceVS.02
ceVS.03
ceMO
ceBI
ceAR
ceEK
ceTI Type 1
Caucasian
Risk of allo anti-C, -e, and -Ce
Partial C and e
Risk of allo anti-C, -e, -Ce
hrB–
hrS–
Partial e and c
Risk of allo anti-e, -c, -ce(f)
Some risk of allo anti-hrB and/or -hrS
Most linked to D variants
African Blacks/ Hispanics
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Variation of D antigen expression

- More than 500 different RHD alleles in populations

Three Primary Categories (beyond conventional D)

- Weak D
  - changes decrease antigen expression level
  - definition: react weaker than expected (≤2+ tube OR require IAT)
  - Not at risk for anti-D (rare exceptions, but no HDFN or HTR)
    - » weak D types 1, 2, 3 most common
- Partial D
  - changes alter the epitopes or epitopes are missing
  - may react 3+ : not serologically distinguishable **BUT** some partial D type weakly D+
  - At risk for clinically significant anti-D
- D_{el}
  - Type D-negative even by IAT; adsorb and elute anti-D
  - Severe defects - point mutations affect insertion - Asians
  - Identify in donor situation (if possible)

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Variation of D antigen expression

- More than 500 different RHD alleles in populations

Three Primary Categories (beyond conventional D)

- Weak D
  - changes decrease antigen expression level
  - definition: react weaker than
  - Not at risk for
- Partial D
  - changes alter the epitopes or epitopes
  - may react 3+ : not serologically distinguishable **BUT** some partial D type weakly
  - significant anti-D
- D_{el}
  - Type D-negative even by IAT; adsorb and elute anti-D
  - Severe defects - point mutations affect insertion - Asians
  - Identify in donor situation (if possible)

Cannot be distinguished by routine serologic D typing

Can be distinguished by DNA analysis

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How to interpret weak D antigen expression?

- Manufacturer instructions & cautions vary
- Examples from FDA licensed anti-D:
  - "Reactions less than 2+ should be evaluated since they may be false positive"
  - "Agglutination <1+ at IS should be tested using alternative reagent by IAT prior to final determination"
  - "Patients should not be classified as D+ on basis of a weak reaction with a single anti-D"
  - "If a clear positive not obtained it is safer to classify the patient as D−"

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Doubts with D

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Two patients referred for D resolution

RBCs	Patient 1		Patient 2	
Anti-D	IS	IAT	IS	IAT
Reagent 1	Micro+	4+	$1+^{W}$	3+
Reagent 2	$1+^{W}$	4+	1+	2+
Reagent 3	$1+^{W}$	4+	1+	2+
Reagent 4	$1+^{W}$	3+	1+	3+

Both still weak with similar reactivity

Points to consider for interpretation of results as D+ or D−

- Result entry into computer system and patient records
- Transfusion recommendation/RhIG administration
- Gender and age of the patient
- Perform DNA testing

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Testing options for *RHD* (and *RHCE*)

SNP arrays:

- Common red cell antigens, including few *RHCE* variant markers
- *RHD* – targets many hybrid alleles and alleles associated with weak and partial D
- *RHCE* – targets many variant c, e, C, and E alleles

Lab developed assays (LDTs):

- Multiplex PCR: *RHD* exons 4 and 7, inactive *RHDψ,* and C/c
- *RHD* zygosity
- *RHD* exon 8 for 1136C>T (DAU alleles)
- *RHCE* exon 2 for 254C>G (*RHCE*ce254G*), exon 4 (*RHCE*CeRN*), and exon 6 (silenced *RHCE*cE*).
- Presence/absence of exons and Gene sequencing

RNA-based versus DNA based testing

- Detect new, rare, or hybrid alleles or link changes to specific allele

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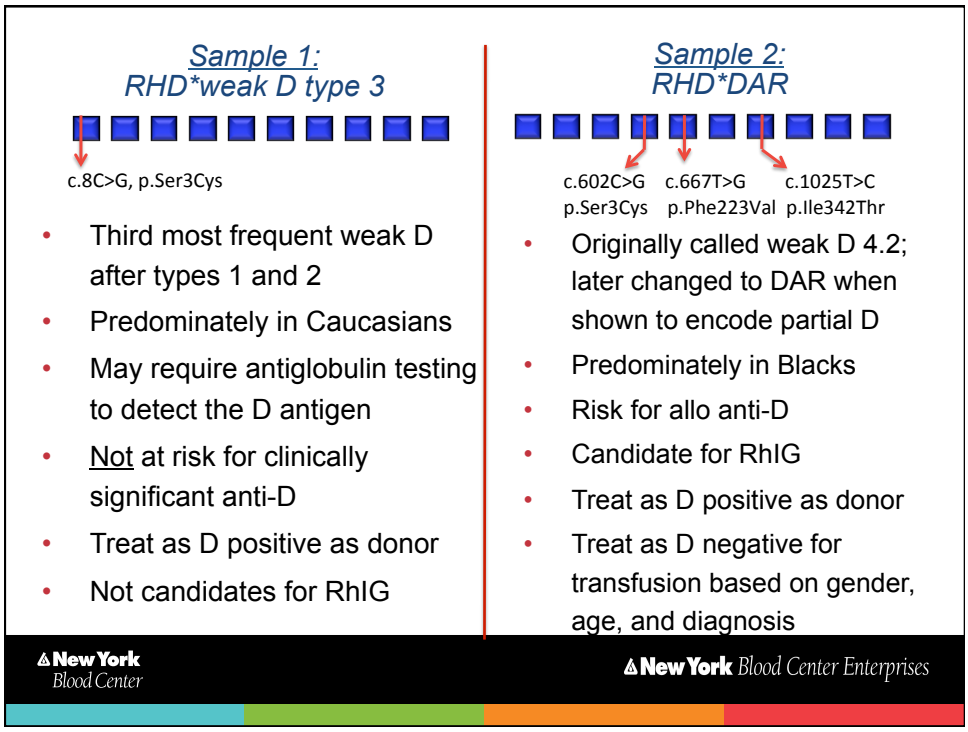

Case history

- 61 year old Black female
- RBCs typed strongly D+ (4+ with automation)
- Anti-D was identified in her plasma
  - 3 to 4+ by IgG gel

➢ Sample was submitted for DNA testing for *RHD* variants

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Initial DNA results

- Multiplex assay:
  - *RHD* exons 4 and 7 absent
  - *RHCE*C/c*
- *RHD* SNP array: LS (low signal)
  - Low signal for all markers
  - interpreted as "Deleted D"
- *RHD* exons 2, 8, and 10 also absent by LDTs
  - ➢ Results consistent with absence of the RHD gene
  - ➢ **BUT**…serology indicated RBCs type strongly D+

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Additional serology testing performed

- RBCs with multiple anti-D

Anti-D	Proband
Immucor Series 4	4+
Immucor Series 5	4+
ALBAclone alpha	4+
ALBAclone blend	4+
Gamma-clone	3+
Ortho BioClone	2+^{s}

Moderate to strongly reactive with all anti-D

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ALBAclone partial D kit

Kit ID	Anti-D cell line	Wk D type 1 & 2	DII & DNU	DII	DIV	DV	DCS	DVI	DVII	DOL	DFR	DMH	DAR	DAR-E	DHK & DAU4	DBT	R_o^{Har}	Proband
A	LHM76/58	+	+	+	+	+/0	+	0	+	+	+	+	+	0	0	0	(+)/0	**0**
B	LHM76/59	+	+	+	0	+	+	+	+	+	+	+	+	+	+	0	0	**0**
C	LHM174/102	(+)/0	+	+	0	0	+	0	+	0	0	+	0	0	0	0	0	**0**
D	LHM50/2B	+	+	+	+	+	+	0	+	+	+	+	+	+	+	0	0	**0**
E	LHM169/81	+	+	+	0	0	+	0	+	+	+	+	0	0	0	0	0	**0**
F	ESD1	+	+	+	0	+	+	+	+	+	+	+	+	+	+	0	0	**0**
G	LHM76/55	+	+	+	0	+	+	+	+	+	+	+	+	+	+	0	0	**0**
H	LHM77/64	+	0	+	0	+	+	+	+	+	+	+	+	+	+/0	0	0	**0**
I	LHM70/45	(+)/0	+	+	0	0	0	0	+	0	0	0	0	0	0	0	0	**0**
J	LHM59/19	+	+	+	+	+	+	0	0	0	0	(+)	0	(+)	+	+	0	**2+**
K	LHM169/80	+	+	+	+	+	+	0	+	+	+	+	+	+	0	0	0	**0**
L	LHM57/17	+	+	+	+	+	**0**	**0**	+	+	**0**	+	+	**0**	**0**	+	**0**	**2+**

- Patient RBCs reacted with 2 clones, J and L
- Pattern consistent with partial D, DBT phenotype

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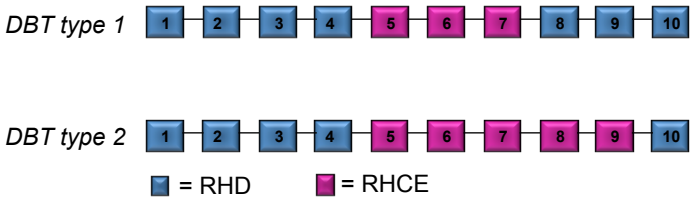
DBT alleles



- Sample does not have *RHD*DBT*
- No RHD gene was detected

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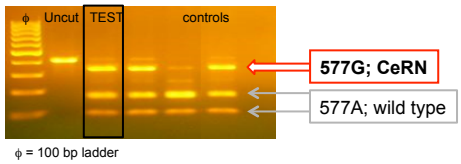
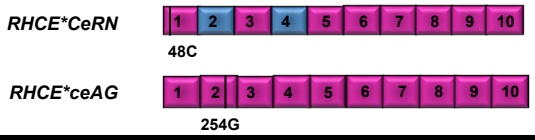
Additional DNA and RNA testing and results

- *RHCE* BeadChip: Predicted C+E–c+e+
  - No variants detected
  - Ruled out Crawford (*RHCE*ceCF*), DHar (*RHCE*ceHAR*), *RHCE*ceSL, RHCE*ceRT*
- LDT PCR-RFLP testing: positive (heterozygous) for *RHCE*CeRN*



- AS-PCR: positive (heterozygous) for c.254C>G associated with *RHCE*ceAG*
- RhCE-cDNA results:



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*RHCE*CeRN*

- In 1996, Rouillac *et al* (*Blood* 87:4853-4861) identified the molecular basis of R^N
  - RHCE hybrid allele with exon 4 of *RHCE*Ce* replaced with that of *RHD*
- R^N (previously R_N) haplotype
  - *RHD in cis* with *RHCE*CeRN*
  - associated with weakened and partial C and e antigen expression
  - low-prevalence antigens Rh32 and DAK
    - lacks the high-prevalence antigen Rh46 when homozygous
  - some reports found elevated expression of D antigen
- First indication that *RHCE*CeRN* encodes for D antigen expression in the absence of a RHD gene as evident by the strong reactivity of the RBCs with commercial anti-D.
- D reactivity encoded by *RHCE*CeRN* masked due to frequent linkage to *RHD*.

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E discrepancy

- Caucasian female blood donor, Group O+
- 10 prior donations
- On 2 donations RBCs typed D+C+**E−**c+e+
- Unit labeled and shipped as E−
- Re-typing by the hospital indicated **E+**

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Testing with multiple anti-E reagents

- E+ with four sources
  - Strongly reactive with two

	Gamma clone (GAMA402)	SeraClone (MS60/12)	ALBA clone (DEM1)	In-house polyclonal
Donor RBCs	4+	4+	$2+^{mf}$	very weak

- E− with four sources

	Ortho BioClone (C2)	Immucor polyclonal	Immucor Series I (MS12)	In-house polyclonal
Donor RBCs	0	0	0	0

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DNA results

- RHCE SNP array results:
  - Negative for *RH*E*
  - Genotype: *RHCE*Ce* and *RHCE*ce*
  - Predicted phenotype C+**E–**c+e+
- Manual PCR-RFLP for E/e:
  - c.676G/G, predicted **E–**e+
- *RHCE* exon 5 sequencing:
  - c.676G/G, predicted E-e+
  - Novel c.674C>G (p.Ser225Cys)

*Is the change present on the RHCE*Ce or RHCE*ce?*

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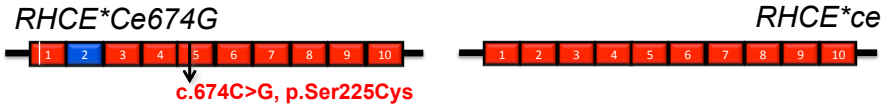
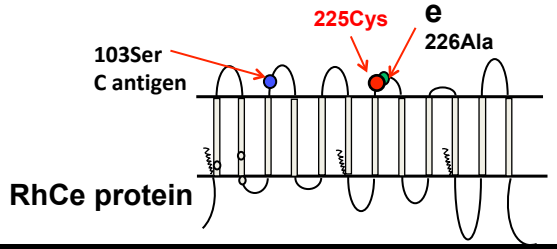
RNA / RhCE-cDNA analysis

➢ SNP-specific primer sequencing linked c.674C>G to *RHCE*Ce*



➢ Must alter the protein configuration creating an E epitope?

225Cys in Ce protein = reactivity with some anti-E



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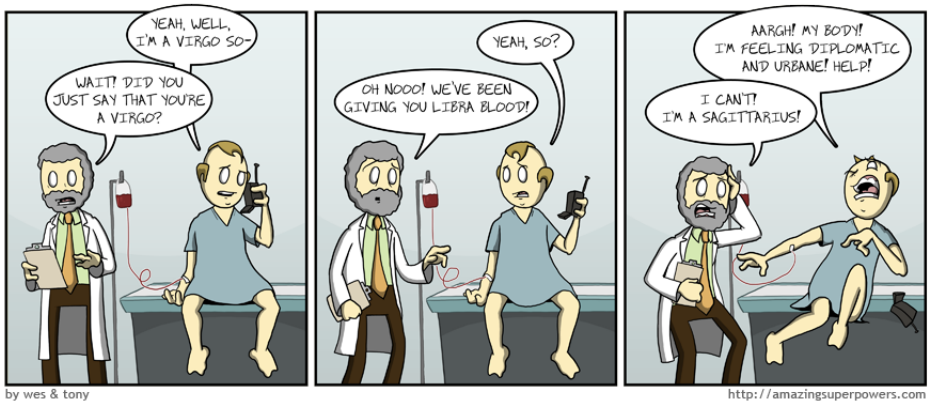
R(H)ight match



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Case History

- 74 year old African-American female
- Anemia; Hct of 18%
- 19 days earlier received 1 unit of RBCs
- Hospital had identified anti-E, -K, and -Jk^b
- Request for 2 units of RBCs
- Urgent!

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Serology results

Patient's plasma:

- Anti-E, -K and -Jk^{b} confirmed
- However, all E– K– Jk(b–) RBCs reacted
- Autocontrol was negative
- Antibody to a high prevalence antigen suspected

Patient's RBCs:

- D+ C+ E– c+ e+; S–; K–; Fy(a–b–); Jk(b–)
- Typing with antibodies to high prevalence antigens showed them to be Js(b–) and hr^{B}–

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Serology continued

- Anti-Js^{b} identified in the patient's plasma
- Patient urgently needed blood
- Tested RBCs from 4 "phenotype matched" donors with patient's plasma
  - Js(b–), D+C+E–c+e+, K–, S–, Fy(a–b–), Jk(b–)

	Rh-hr					MNSs				Kell				Duffy		Kidd		IAT
	D	C	E	c	e	M	N	S	s	K	k	Js^{a}	Js^{b}	Fy^{a}	Fy^{b}	Jk^{a}	Jk^{b}	PEG
Donor 1	+	+	0	0	+	0	+	0	+	0	+	+	0	+	0	+	0	3+^{S}
Donor 2	+	+	0	+	+	+	0	0	+	0	+	+	0	0	0	+	0	3+^{S}
Donor 3	+	+	0	+	+	+	+	0	+	0	+	+	0	0	0	+	0	3+^{S}
Donor 4	+	+	0	+	+	+	0	0	+	0	+	+	0	0	+	+	0	3+
Auto	3+	2+**	0	4+	Nt	4+	4+	0	4+	0	4+		0	0	0	3+	0	0

** some free RBCs seen with anti-C
But the patient was transfused a month ago

All were 3+ incompatible!

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Case study continued

- Anti-C identified
- C– E–, K–, S–, Fy(a–b–), Jk(b–), and Js(b–) RBCs were compatible

BUT…

- Patient's RBCs had been typed as C+
  - 2+ with GammaClone anti-C

Sample was reflexed to DNA testing

- Performed initial DNA SNP array

Blood Group	Antigen	Result	Comments
Rh	c	+	
	C	(+)*	
	e	+	
	E	0	
	V	0	
	VS	+	
Kell	K	0	
	k	+	
	Kpa	0	
	Kpb	+	
	Jsa	+	
	Jsb	0	
Kidd	Jka	+	
	Jkb	0	
Duffy	Fya	0	
	Fyb	(0)*	
MNS	M	0	
	N	+	
	S	0	
	s	+	
	U	+	
	Dob	+	
	Hy	+	
	Joa	+	
Landsteiner-Wiener	LWa	+	
	LWb	0	
Scianna	Sc1	+	
	Sc2	0	

Confirms Js(b–)

But what is the C result?

NOTE: Not all platforms are created equal.
Depending on the array platform, the C result may not be in question

DNA Results

- Array result:

c	+
C	(+)*
e	+
E	0

→ Possible r'S

C+ (partial) OR C–

- Array predicts (+)* as it targets 2 markers on the RHCE*ceS allele.
  - Allele commonly linked to hybrid RHD allele and could be C+.

Hybrid *RHD*DIIIa-CE(4-7)-D*

↓

C+ (partial)

↓

C–

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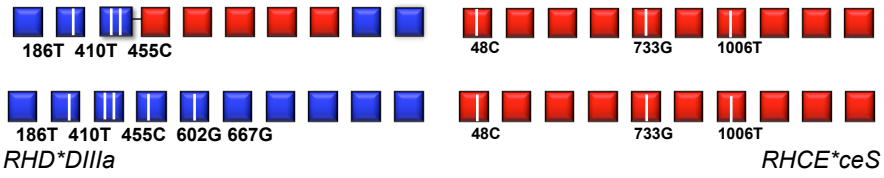
RH results

- RHD genotyping was performed

Hybrid *RHD*DIIIa-CE(4-7)-D*



*RHD*DIIIa* *RHCE*ceS*

- Results summary:
  - Hybrid *RHD*DIIIa-CE(4-7)-D*: partial C; confirms allo anti-C
  - *RHD*DIIIa*: **partial D** phenotype and at risk for allo anti-D
  - Homozygous *RHCE*ceS*: **partial c and e** (and f), VS+V–, hr^B–, and at risk for allo anti-e, -c, -f, and/or -hr^B

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Transfusion

- The anti-C made by the patient is an alloantibody
- RBCs that are C– should be used for transfusion
- If the patient later made an anti-hr^{B} and needed rare blood, RH genotype matched units may be needed

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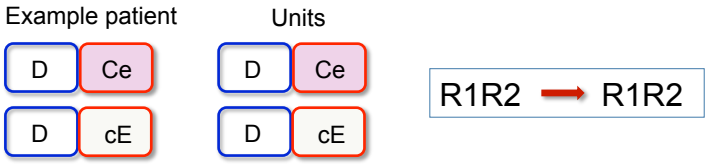
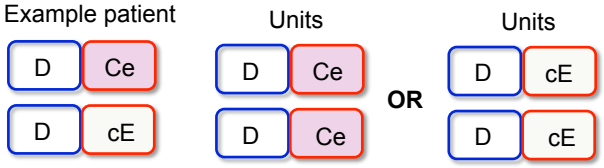
RH genotype matching

Exact match (no mismatch)



Haplotype match (no mismatch) R1R1 or R2R2 → R1R2



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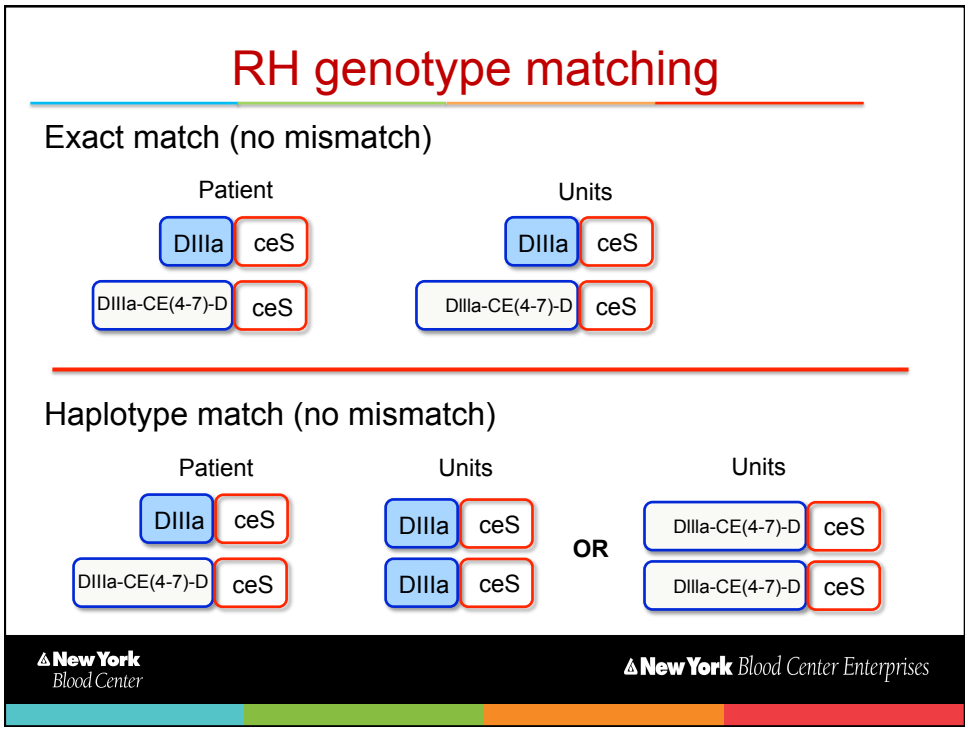
RH genotype matching
Exact match (no mismatch)
Patient
DIIIa ceS
DIIIa-CE(4-7)-D ceS
Units
DIIIa ceS
DIIIa-CE(4-7)-D ceS
Haplotype match (no mismatch)
Patient
DIIIa ceS
DIIIa-CE(4-7)-D ceS
Units
DIIIa ceS
DIIIa ceS
OR
Units
DIIIa-CE(4-7)-D ceS
DIIIa-CE(4-7)-D ceS
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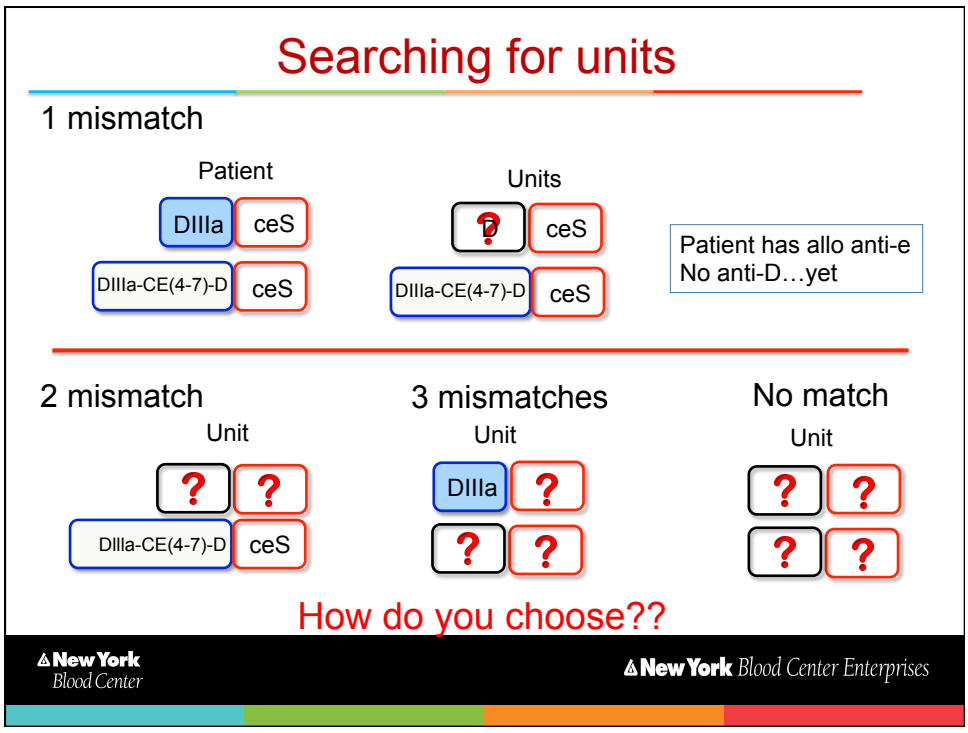
Searching for units
1 mismatch
Patient
DIIIa ceS
DIIIa-CE(4-7)-D ceS
Units
? ceS
DIIIa-CE(4-7)-D ceS
Patient has allo anti-e
No anti-D…yet
2 mismatch
Unit
? ?
DIIIa-CE(4-7)-D ceS
3 mismatches
Unit
DIIIa ?
? ?
No match
Unit
? ?
? ?
How do you choose??
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Next steps

- Look more closely at the RH genotypes of the units
  - Are alleles similar?
  - Do they have similar mutations/changes?
  - Could you predict if some alleles would be more compatible?
- Compare predicted phenotypes of the different alleles
- Test and crossmatch potential units with patient's plasma

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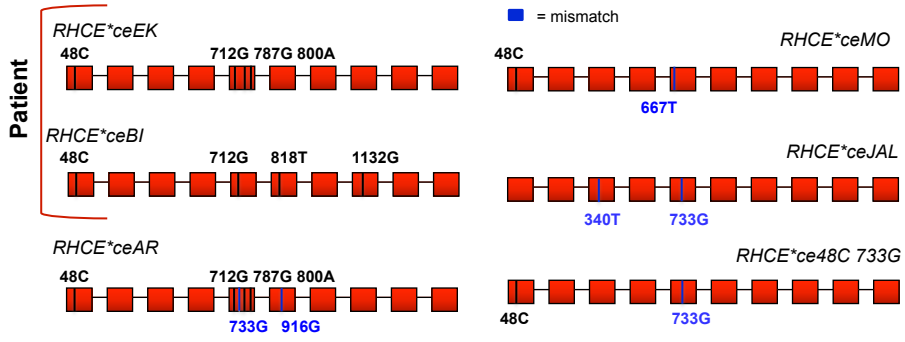
Example: patient with anti-hr^S in her plasma

Patient: *ceEK / ceBI*

- Compatible with 3/7 hr^S– phenotype cells

  Compatible: *ceAR / ceEK* and *ceAR / ceBI*

  Incompatible: *ceMO / ceMO* *ce48C 733G / ceJAL* *ceEK / ceJAL*



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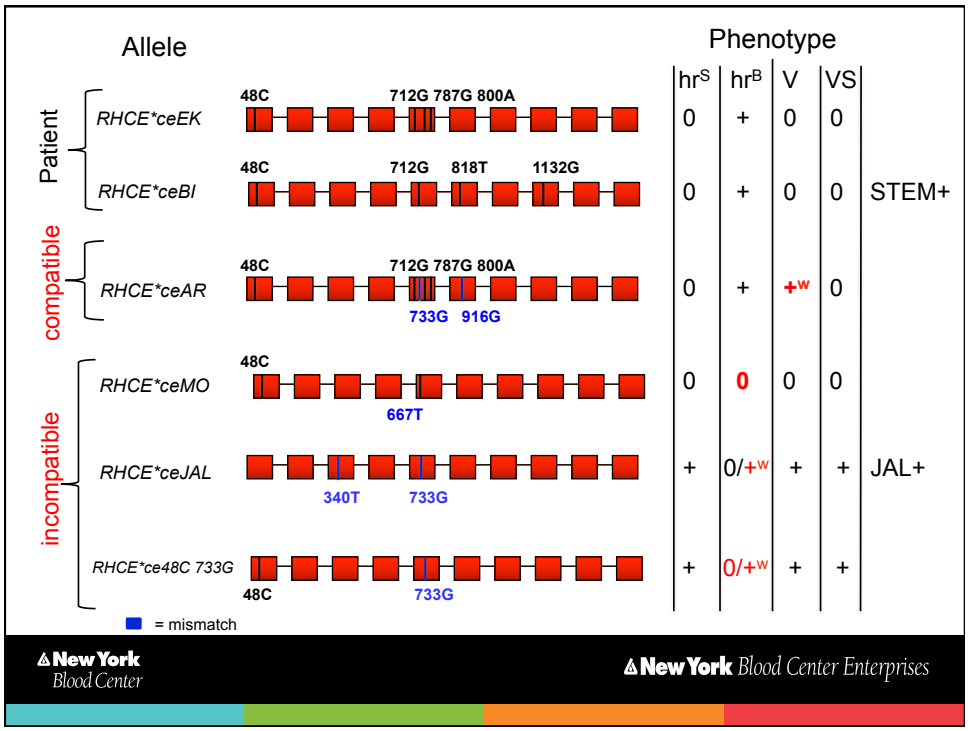

RH genotyping of donors

- Commercially RHD and RHCE genotyping kits are not inexpensive
- Need for better electronic data management of donor genotypes
- Potential strategies:
  - identify rare Rh donors with initial screen using red cell SNP arrays with markers present for common hr^B– and/or some hr^S–
  - choose donors on ABO type, antigen negative profile, frequency of donations, and/or ethnicity
  - develop in-house screening platforms for rare donors based on population needs
- Goal to have haplotype homozygous donors (ie. r'^S/r'^S)

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Serology and DNA: better together

Serologic testing:

- Reveals the presence, absence or weakening of Rh antigens
- Reveals the presence of antibodies

DNA analysis:

- Reveals molecular basis of a phenotype and detects variants and potential for allo antibody production
- Helps to focus investigation
- Allows more precise matching of donor and patient

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