

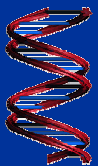
Molecular Testing in Transfusion Medicine



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Medicine



Molecular Blood Group
and Platelet Testing Laboratory

Age Of Molecular Diagnostics

- **Development of DNA-based clinical assays**
 - Result of Genomics revolution
 - polymerase chain reaction (PCR)
 - Amplify region of gene of interest, generate millions of copies for analysis or sequencing
- **Revolutionizing laboratory testing**
 - Disease Diagnosis
 - Molecular Pathology
 - Microbiology
 - Transfusion Medicine

DNA-based Assays

- Why applicable to blood bank ?
 - Blood group antigens are inherited - genes have been identified and cloned
 - Similar to disease gene markers-many result from single nucleotide polymorphisms (SNPs)
 - Identified by using PCR (polymerase chain reaction) gene amplification methods
 - **Can do some things serology cannot**
 - **In some situations is superior to serology**

Blood group antigen typing



Agglutination
Phenotype



DNA
Genotype

Power - in a combination of both methodologies
Future “typing” = phenot***ype*** and genot***ype***

Serology cannot.....

- Determine paternal *RHD* zygosity - one copy or two?
Mother has anti-D: if father homozygote – all offspring at risk for HDN
if father heterozygote (hemizygote) may be unaffected
- Type fetus - from amniocytes or maternal plasma
- Type multiply transfused patients
Alternative: Cell separation – less dense reticulocytes harvested for typing
- Type RBCs with +direct antiglobulin test (DAT)
Alternative: Remove bound immunoglobulin with AET or chloroquine

Serology cannot.....

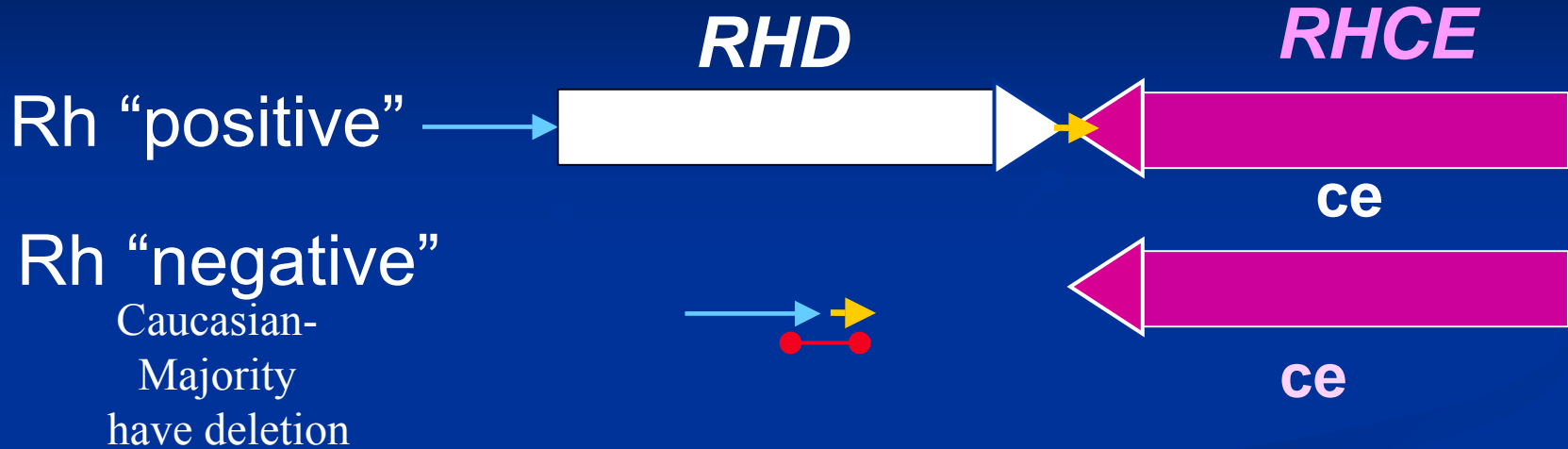
- **Screen donor units** for antigens for which no commercial reagents
 - Dombrock^{a/b}, -Js^a, -Kp^a, -Co^a, -Yt^a-, etc.
- **Detect altered D antigen** (weak D or partial D)
 - Donor - typing discrepancies – may be FDA reportable
 - females - child-bearing age to determine risk of anti-D
- **Resolve antibody identification**
 - is it autoantibody or alloantibody ?
- **Identify patients with complex Rh antigens/antibodies**
 - screen for compatible donor units
 - patients with sickle cell disease

Goal

- Examples of how DNA-based testing is currently utilized
- How expands determination of compatibility in the Rh system
 - RhD altered (weak D and partial D)
 - RhCE (C, c, E, e) altered in sickle cell patients
- Expansion of applications with high-throughput platforms
 - Donor center
 - Transfusion service – patient care

Paternal *RHD* zygosity

one copy (hemizygote) or two (homozygote)?



PCR- assay for presence of deletion region

Positive = father is hemizygous

Possibility that baby could be D negative

Negative = father is homozygous

All children will be D positive

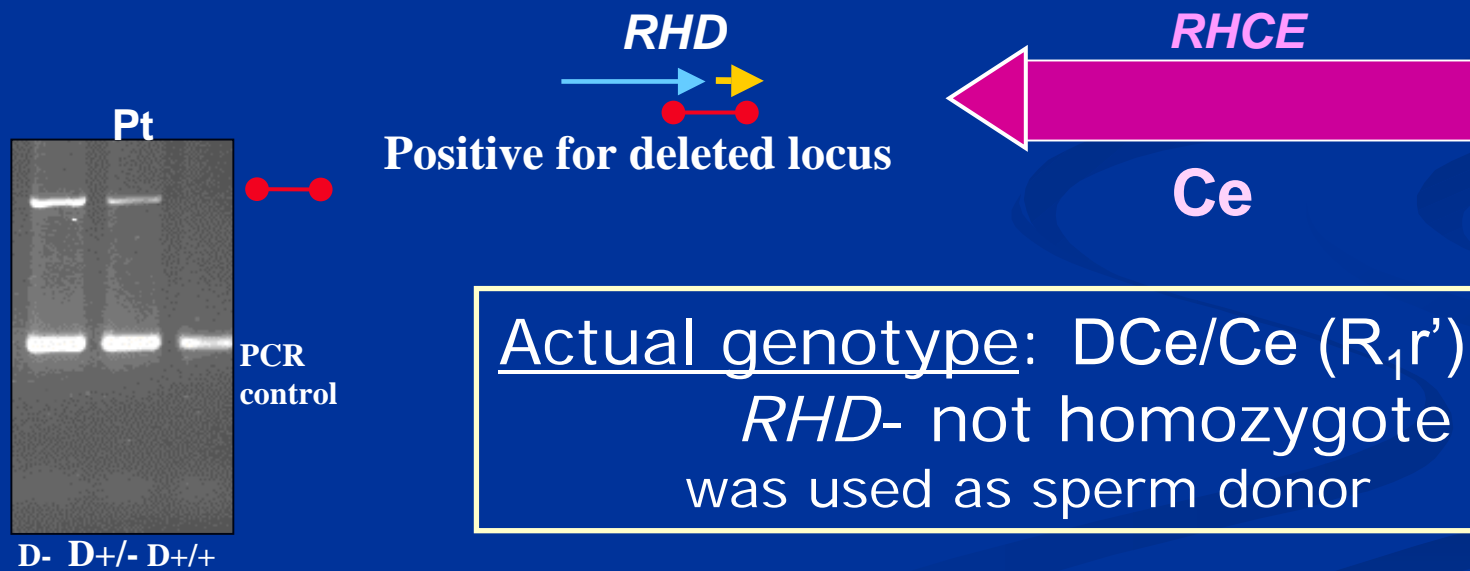
Important for monitoring the pregnancy with anti-D

Case : Paternal Zygoty

HISTORY: Mother with anti-D titer:256; undergoing in vitro fertilization

SEROLOGY: Husband, Caucasian, Rh phenotype = D+, C+/c-, E-/e+
"Probable" genotype: DCe/DCe (R_1R_1) - homozygous D
All offspring will be effected

RH GENOTYPING – to confirm predicted *RHD* zygoty



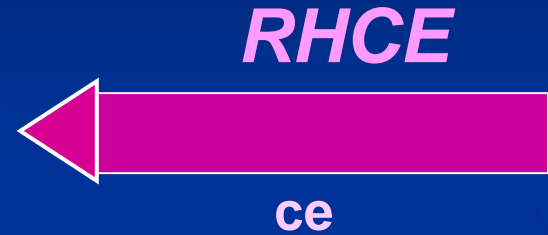
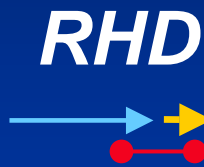
Human Reproduction: Preimplantation genetic diagnosis in the management of severe rhesus alloimmunization: first unaffected pregnancy: Case Report. Seeho, SKM et al. 2005

Paternal *RHD* zygosity

considerations for testing in other ethnic groups

Rh “negative”

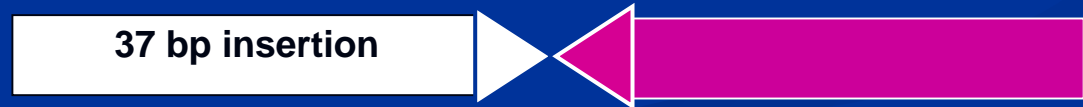
Deletion - in most ethnic groups



Rh “negative”

African or
Hispanic groups

24%



Others



Information on ethnic background important for accurate zygosity testing

Determination of fetal D type

Father - *RHD* Hemizygous

1. **Amniocentesis** - isolate fetal DNA for *RHD* genotyping

2. **Cell-free fetal DNA in maternal plasma** (Lo et al, Lancet 1997)
• from 5-6th week pregnancy – amount increases throughout term
disappears within hours after delivery

Potential for non-invasive genetic testing

Challenges

- very small amount of fetal DNA/maternal DNA (need sensitive real-time PCR)
- proper controls for fetal DNA – Y chromosome, paternal markers
- intellectual property rights purchased by Sequenome

Netherlands:

To avoid unnecessary administration of anti-partum Rh immune globulin.
approximately 20% of Rh negative women carrying Rh negative fetus

- limited resource
- not risk-free

Type multiply transfused patients

HISTORY: Caucasian female, myelodysplastic syndrome receiving multiple transfusions past 9-10 months

SEROLOGIC WORKUP: A Positive, 1+ DAT
 warm autoantibody in serum and eluate
stronger reactivity with c and E positive cells
 possible anti-c? –E?

ANTIGEN TYPINGS: Retic harvest

	D	C	E	c	e	M	N	S	s	Fya	Fyb	Jka	Jkb
<u>Serologic Phenotype</u>	1+ mf	2+/4 + mf	w/1+ mf	2+/4 + mf		4+	4+ mf	2+ mf	3+	1+ mf	1+ mf	2+ mf	2+ ^s
<u>Molecular</u>	DD	C+	E+	c+	e+	NT	NT	S+	s+	neg	pos	neg	pos

RH Genotype: C+, E+ c+, e+ ; (CDe/cDE)

Fy(a-b+), Jk(a-b+), Ss

Screen donor units when reagents are not available

HISTORY: 66 year-old Female, Heart bypass surgery
Anti-E; received 6 E- units on 9/15
9/28 serum “brown” -anemia (Hgb 6)- suspect DHTR

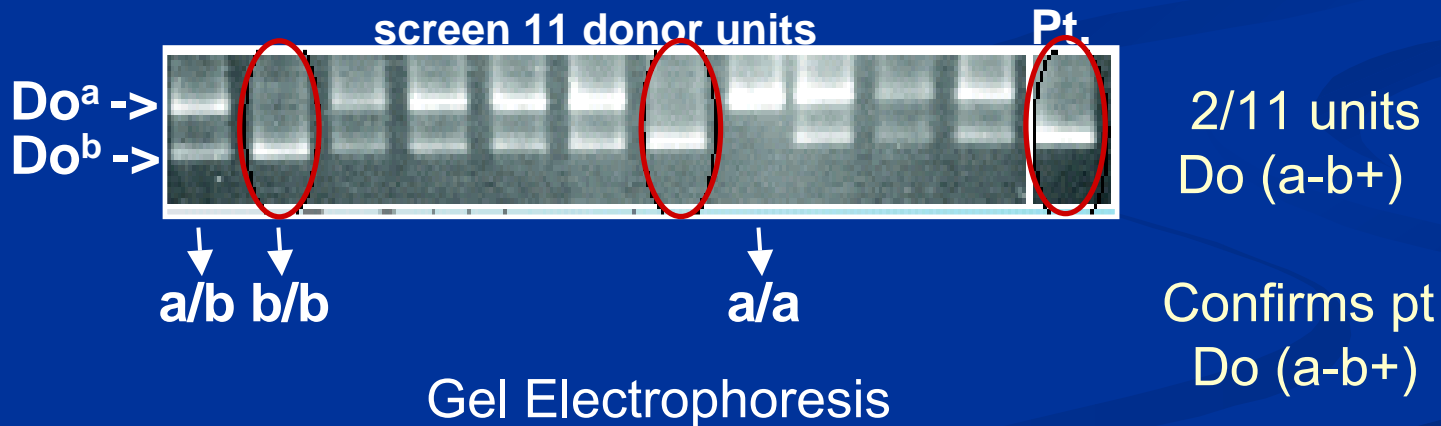
SEROLOGIC WORKUP: O Positive
anti-E; new anti-Do^a (Dombrock a)

PROBLEM: - Antibodies often weak; present with other specificities

- Disappear over time; screening with patients sera unreliable
- Dombrock antibodies can cause transfusion reactions
- Commercial typing reagents are not available

Screen donor units when reagents are not available

- APPROACH: DNA-** Isolate from WBC's (non-leukoreduced donor samples)
- PCR Amplify Dombrock gene segment
 - Do^a and Do^b differ at nucleotide 793G>A
 - Restriction enzyme *BseRI* - cuts Do^b allele
 - does not cut Do^a allele



Example of PCR-RFLP - Restriction fragment length polymorphism Assay

Challenges - Molecular Testing

- Used in transfusion medicine ~ 10 years
- Primarily in a few Reference Laboratories
- Manual methods - **are labor intensive**
- Methodology not familiar to current technologist
 - DNA/PCR methods now part of most biology curriculums
- Laboratory equipment and testing environment
 - Similar to HLA/NAT laboratories
 - Must avoid cross-sample contamination (pre and post PCR areas)

Challenges - Molecular Testing

- **ABO and Rh are more difficult**

- Not associated with a single polymorphism (SNP)
- Many regions of gene must be sampled

Example current testing for:

- ABO - PCR-RFLP with gel separation and amplification and gene sequencing of exon 6 and exon 7 (to detect group O's and subgroups)
- RH - 10-14 different PCR reactions with gel separation and amplification and sequencing of exon 3 and 5, or all 10 exons, OR Rh-RNA isolation amplification and sequencing (to detect variants)

- Automated test platform key
- Development of microarray and DNA chip technology

ABO system

- Group O - any mutation in A or B transferase that result in non-functional enzyme
 - - 61 different alleles
 - 2 O alleles - common in all populations
 - 13 O alleles- common in Blacks (greatest number)
- Group A and B (numerous subgroups)
 - A - 47 A alleles
 - A1 or A2 common in all populations
 - many weaker subgroups
 - B - 29 B alleles
 - B1 common
 - other weak subgroups
 - cis AB - 5 alleles
 - B(A) - 5 alleles
- Problem- still discovering new alleles
- For ABO routine testing -serology superior
- Molecular testing useful for
 - ABO discrepancies
 - Typing cell lines
 - Confirming subgroup for organ transplant

Rh Antigens- defined by serology

Numerical	Symbol
Rh1	D
Rh2	C
Rh3	E
Rh4	c
Rh5	e
Rh6	ce or f
Rh7	Ce
Rh8	C ^w
Rh9	C ^x
Rh10	V
Rh11	E ^w
Rh12	G
Rh17	Hr _o
Rh18	Hr
Rh19	hr ^s

Numerical	Symbol
Rh20	VS
Rh21	C ^G
Rh22	CE
Rh23	D ^w
Rh26	c-like
Rh27	cE
Rh28	hr ^H
Rh29	“total”
Rh30	Go ^a
Rh31	hr ^B
Rh32	
Rh33	
Rh34	Hr ^B
Rh35	
Rh36	Be ^a

Numerical	Symbol
Rh37	Evans
Rh39	
Rh40	Tar
Rh41	
Rh42	
Rh43	Crawford
Rh44	Nou
Rh45	Riv
Rh46	Sec
Rh47	Dav
Rh48	JAL
Rh49	STEM
Rh50	FPTT
Rh51	MAR
Rh52	BARC

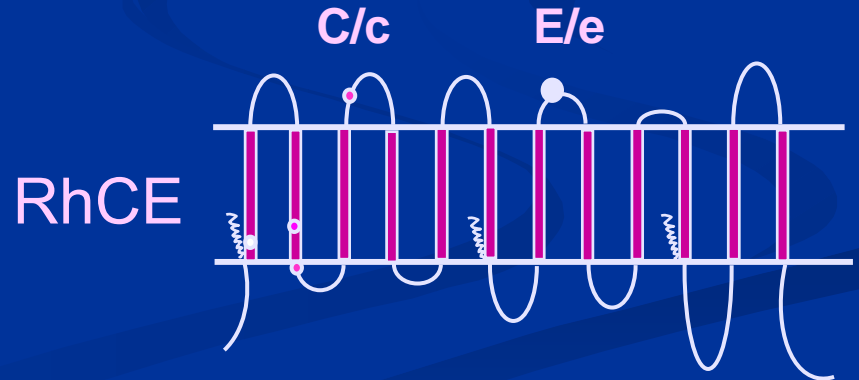
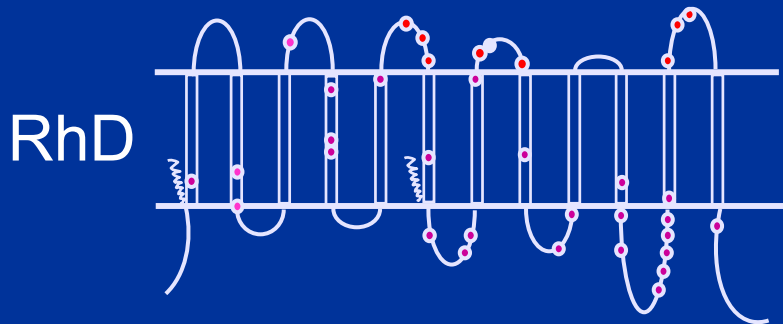
RH genes

- > 120 different *RHD* alleles
- > 60 different *RHCE* alleles

Problem: Only have reagents for detection of 5 principal antigens
 Others - are immunogenic - are not well-defined – “auto”
 Complex system - multitude of additional antigenic epitopes

RH Blood Group Locus

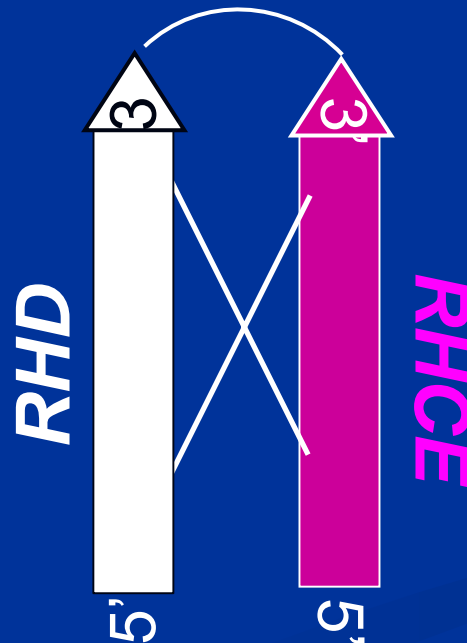
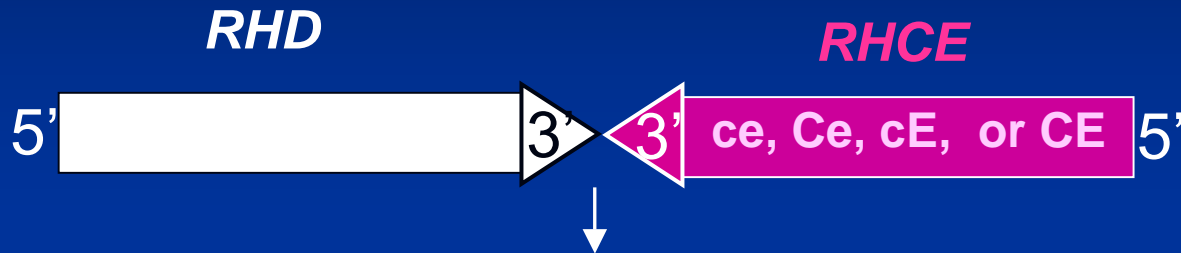
2 Genes-Duplication



32-35 amino acid differences
Explains why D is so immunogenic

RH Blood Group Locus

Genes close on chromosome 1
3' ends together



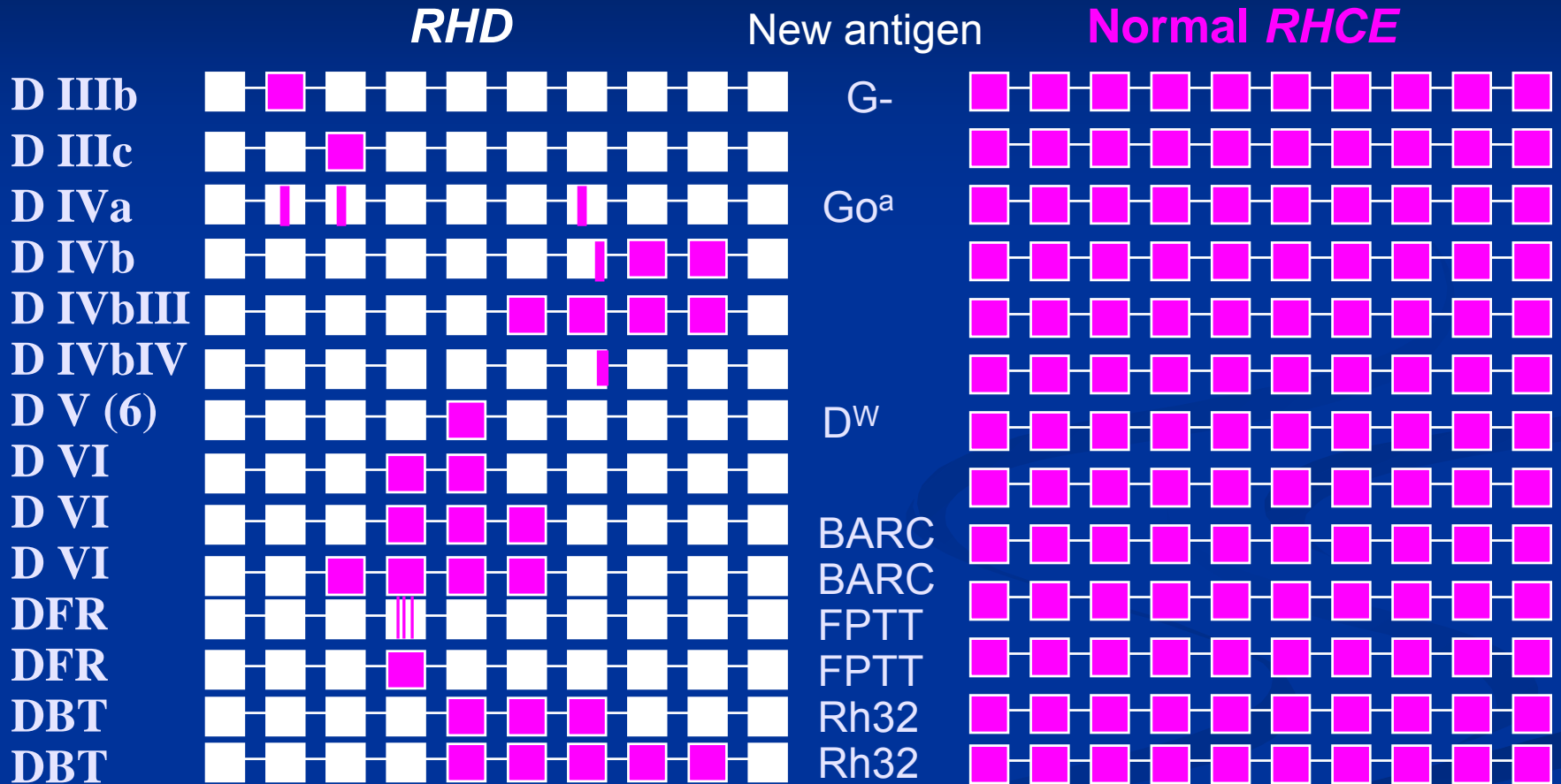
- hair-pin loop structure
- gene exchanges

Gene conversion

- Donor is not changed
- New hybrid genes and proteins
 - part of RhD into RhCE
 - part of RhCE into RhD

Example of hybrid genes: Partial D's

■ *RHD* replaced with ■ *RHCE*



DVI- Most frequent partial D in Caucasians (detected in weak D test)

DIIIa- Most frequent partial D in Blacks (react 3+ IS)

Why is D typing sometimes problematic ?

In U.S.- Large number of variables in serologic testing

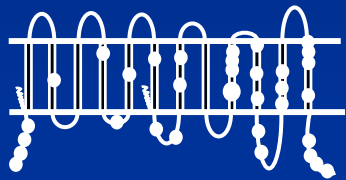
1. **Multiple Methods** – tube test, Solid phase, Gel, automated analyzers
Some perform AHG test for weak D, others do not
2. **Different Reagents** – Contain different clones
Can react differently with partial D or weak D antigens
FDA - only reactivity with DIV, DVa, and DVI need be specified
3. **Variability in expression of RhD protein** (~120 different genes = variations)
All due to changes at the DNA level from “conventional” sequence

Weak D	-	57	different	mutations
Partial D	~	45	“	“
D _{el}	~	8	“	“
“others”	~	18		

Variation in expression of RhD

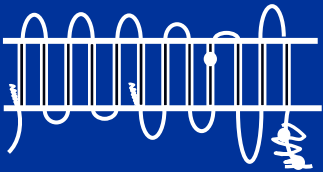
D-negative - 15-17% European Ancestry
3-7% African, 0.5-1% Asians

D-Positive - Majority “conventional”



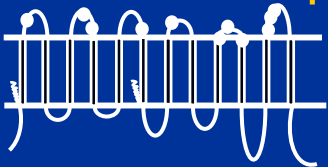
Weak D - require indirect antiglobulin test for detection

Single point mutations-affect QUANTITY- usually do not make anti-D
54 different weak D types (Type -1,-2,-3 = ~ 90%)



D_{el} -

type as D negative even by IAT; adsorb and elute anti-D
Point mutations affect insertion-Asians



Partial D - type as D-positive, but make anti-D

Point mutations that alter D epitopes (~11 different)
MOST are due to hybrid genes (~40 different)
Parts of *RHD* replaced by parts of *RHCE*

D epitopes expressed on Rhce proteins – false positive D typing

D^{HAR} (3+); Crawford (3+); ceRT and ceSL (+w)

Shortcomings of serologic D typing

Rh negative donors

Goal: label donor RBCs with any amount of D as “Rh positive”

Problem: Weak D - some are missed - even with IAT testing

those with low antigen expression (type 2, 5, 9, 10, 12, 15, 17, 18, 33)

D_{el} – all are typed as D negative (more prevalent in Asians)

Can stimulate anti-D in D negative patients

Females - child-bearing potential

Goal: to detect those at risk for anti-D

Strength of serologic typing cannot reliably distinguish

Problem: Weak D – generally not at risk for anti-D (there are exceptions)

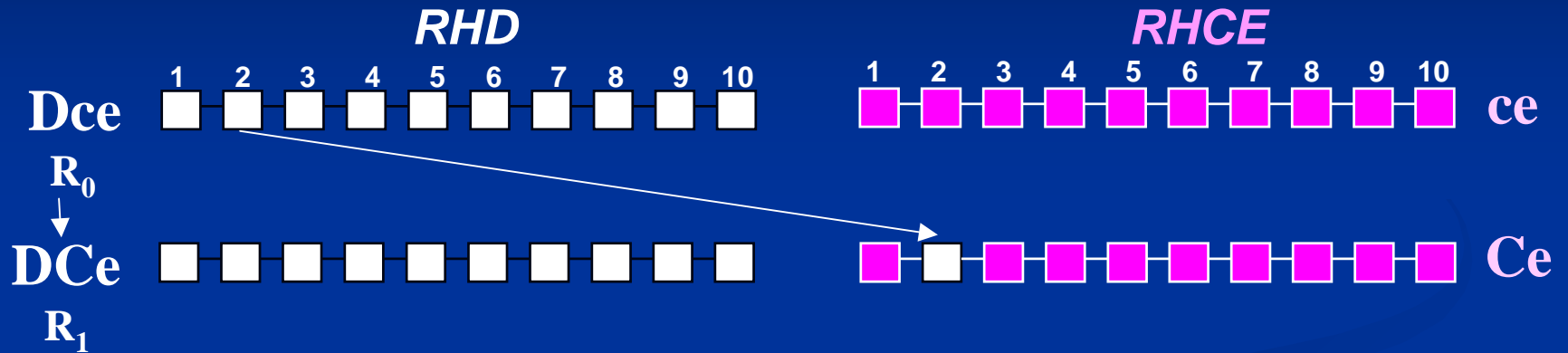
Partial D - type as D positive, are at risk for anti-D

Partial D - Females better served treated as D negative for transfusion and as candidates for Rh immune globulin

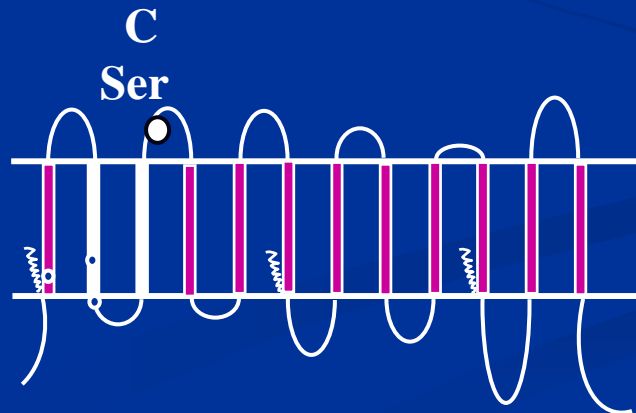
- Consider D negative as recipients - D positive as blood donors

Hybrid *RHCE* alleles

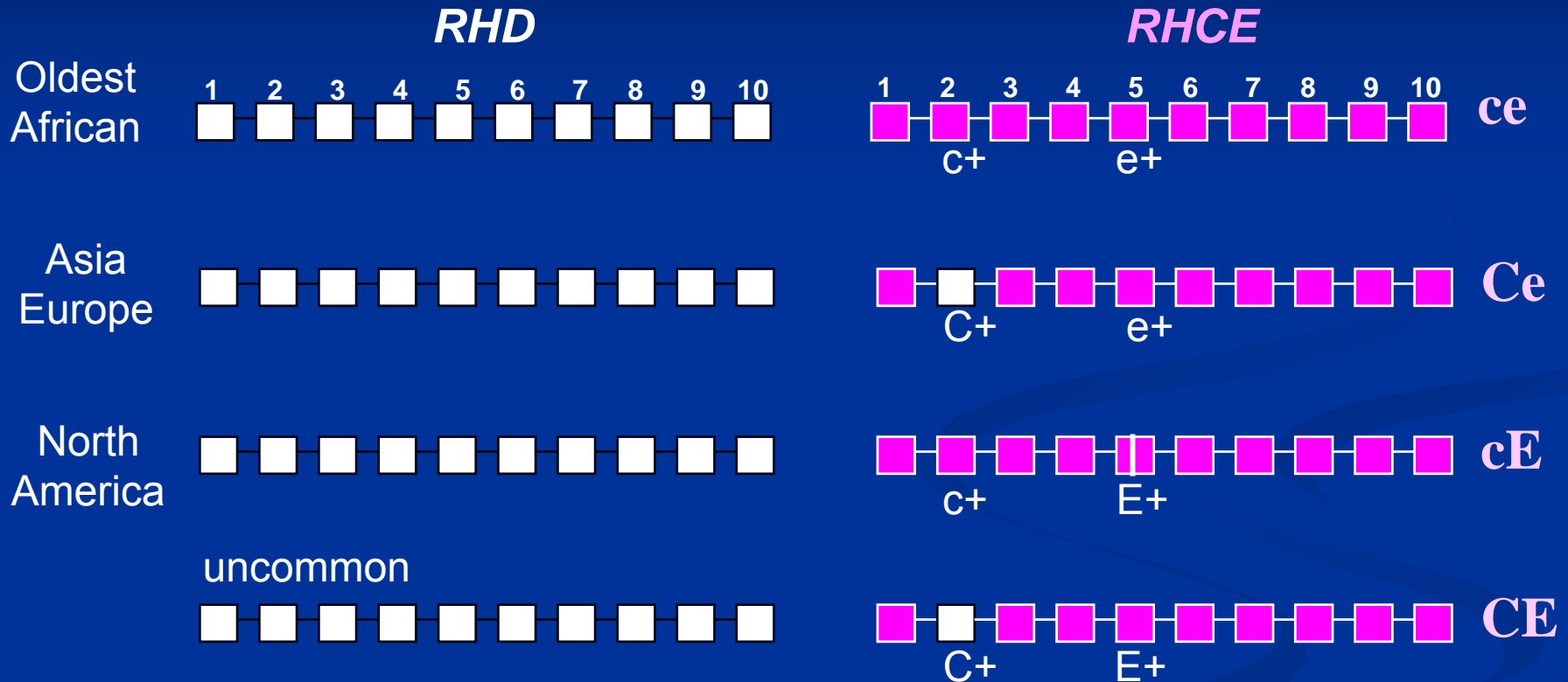
■ *RHCE* replaced with ■ *RHD*



The very common *RHCE**Ce gene results from replacement of *RHCE**ce by exon 2 region of *RHD*



“Conventional” or Common *RHCE* alleles



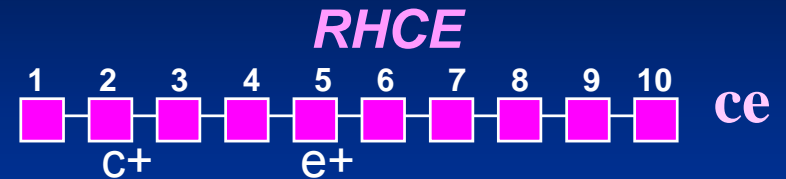
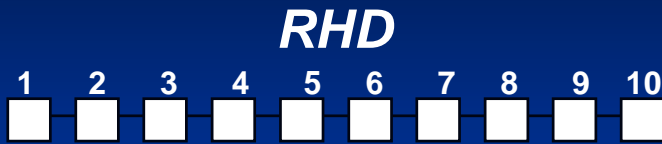
Serologic reagents detect expression of these

D, C, c, E, e

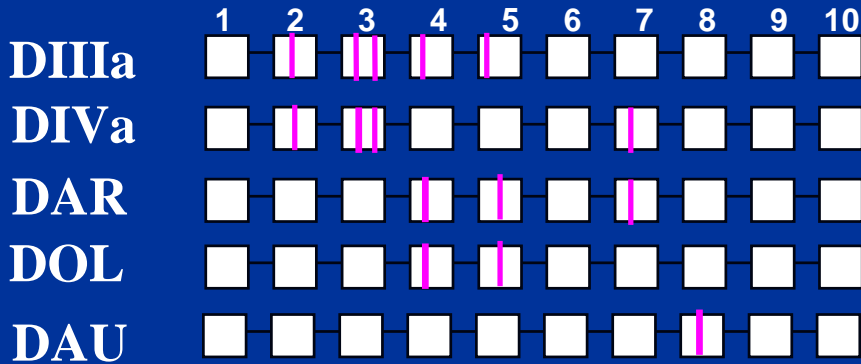
Examples of Altered *RHCE* alleles

Prevalent in individuals with African ancestry

Oldest African

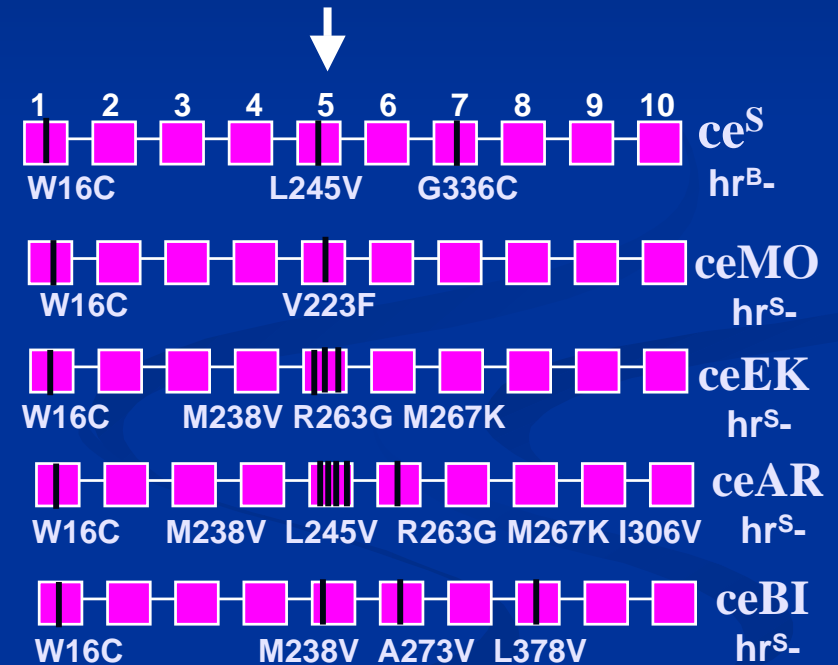


Almost always inherited with



Partial D

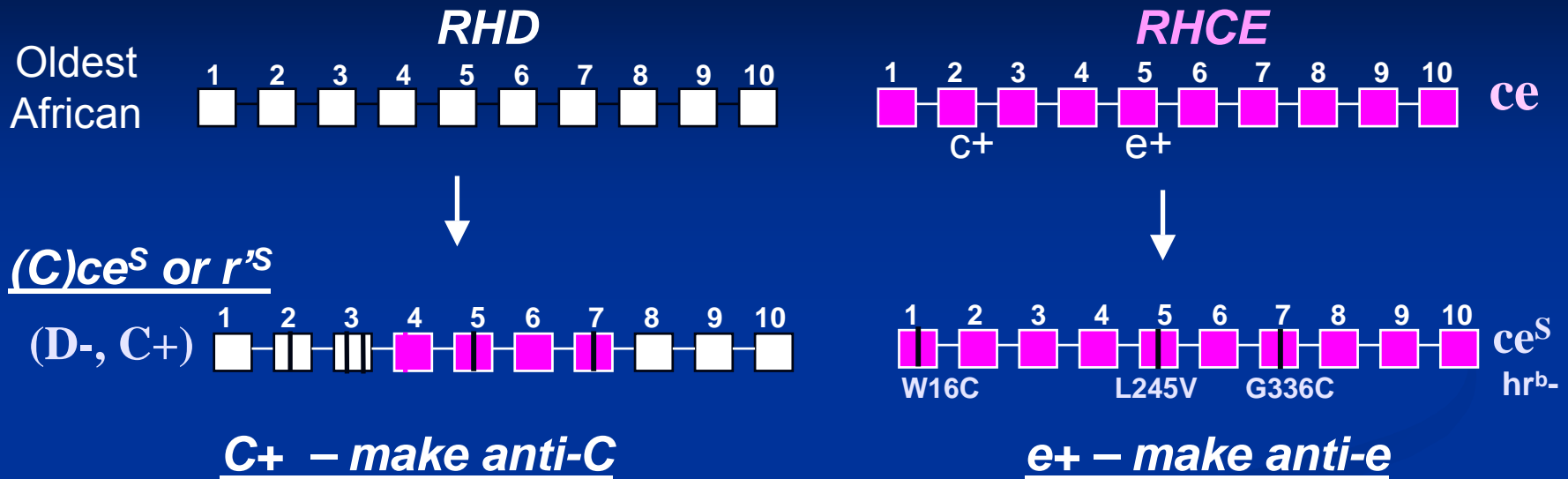
- patients type D positive
- make anti-D



Altered or partial e

- patients type e positive
- make anti-e
- often called “auto-e”

Hybrid *RHD* with altered *RHCE*



Prevalence of haplotype: ~ 8-22% African-Americans

Analyzed *RH* genes in 46 SCD patients with antibodies to high prevalence Rh antigens

- 75% had r^S haplotype

Problem: No serologic reagents to discriminate these

- Prevalent in pts with SCD - have caused transfusion fatalities
- hr^S and hr^B only serve as broad terms for complex specificities
- Are not all compatible with each other
- Can induce additional incompatibility

Questions - Molecular Testing

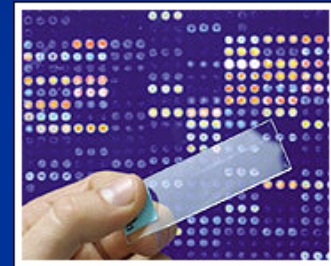
Automation is expanding potential use

- How will be integrated into transfusion medicine practice ?
 - Would it replace some or eventually all serologic typing ?
 - How would expanded use improve practice of transfusion medicine?

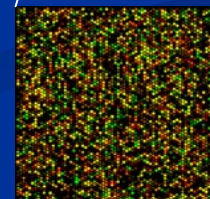
High-throughput Testing

- Automated Microchip or Beadchip Technology
 - Multiplex PCR – amplify numerous gene targets in one tube
 - Product of PCR reaction is hybridized to target oligonucleotides on glass slides or on colored beads

DNA Microchip

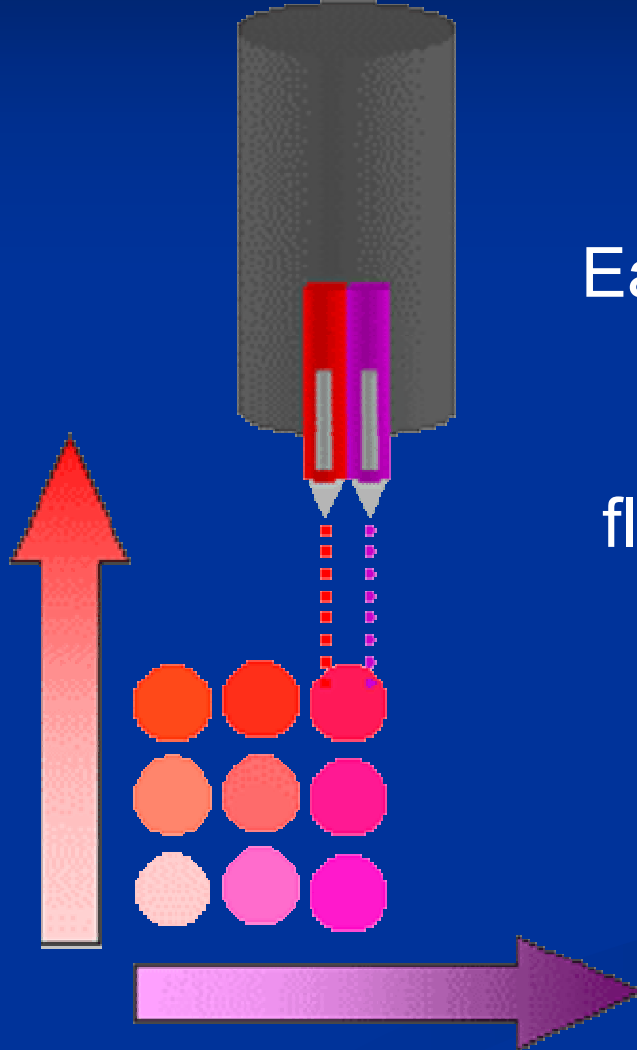


Beadchip



Bead assays

Each Bead Is A Precise
Blend of Two Colors

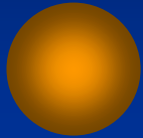


Each unique microsphere
is color-coded using a
blend of different
fluorescent intensities of
two dyes.

$N = 120+$

EVOLVING BEAD ASSAY FORMATS

FIRST GENERATION → **SECOND GENERATION** → **THIRD GENERATION**



**SINGLE BEAD ASSAYS
(ABBOTT DIAGNOSTICS)**
Late 1970's

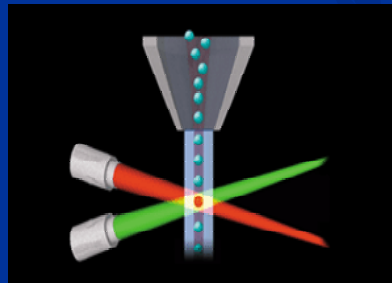
**MULTIPLEX BEAD
96-well plate ASSAYS**



One sample/well
 ≤ 100 assays/well
9,600 assays/plate

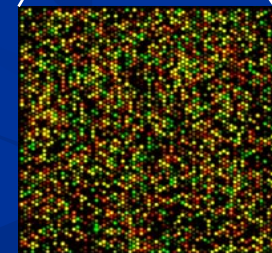
**ONE OR MORE
WASH STEPS**

Luminex
System
HLA



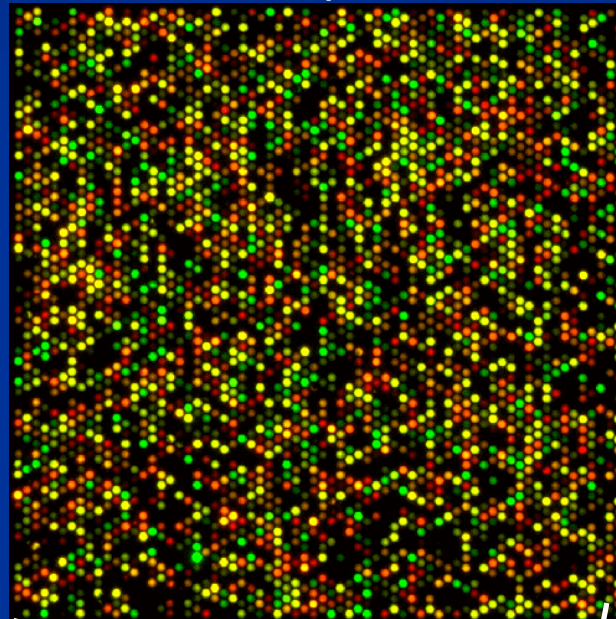
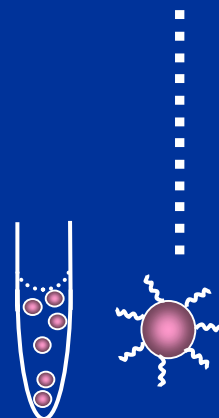
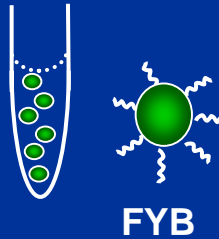
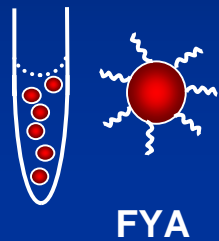
Bead color identifies assay
Green laser identifies signal
Flow cytometer

**MULTIPLEX
BEAD CHIP FORMAT**



Example: HEA BeadChip™

20-30 beads
each probe



Steps:

1. Isolate DNA
2. PCR amplification of targets
3. BeadChip hybridization and extension reaction
4. Wash/read

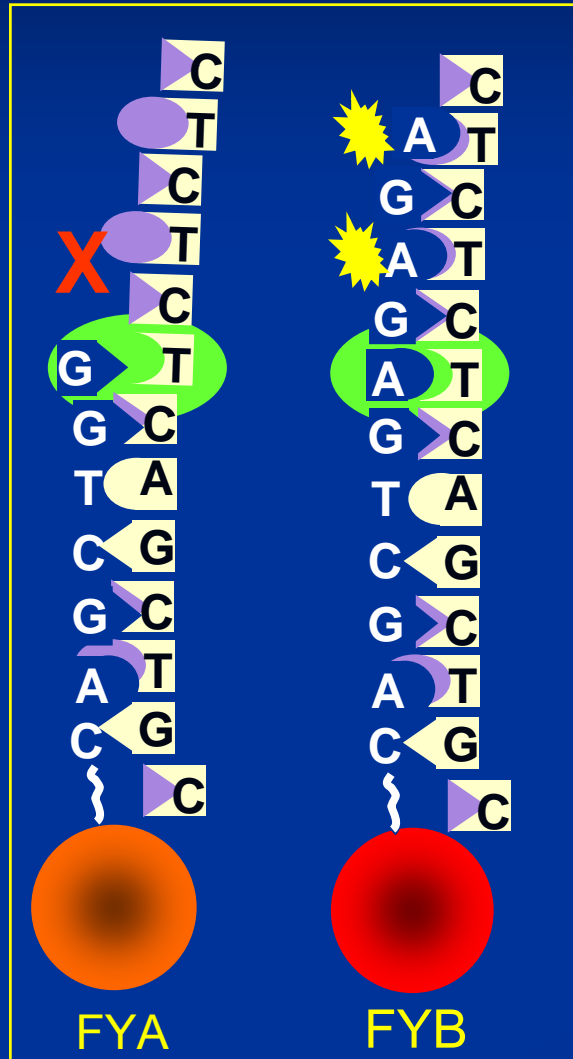
C/c, E/e, K/k,
Jk^{a/b}; Fy^{a/b}; MNSs, Do^{a/b} Hy/Jo
Co^{a/b}; Di^{a/b}; LW^{a/b}; Js^{a/b}; Lu^{a/b}; Sc1/2

24 antigens in 10 blood group
systems

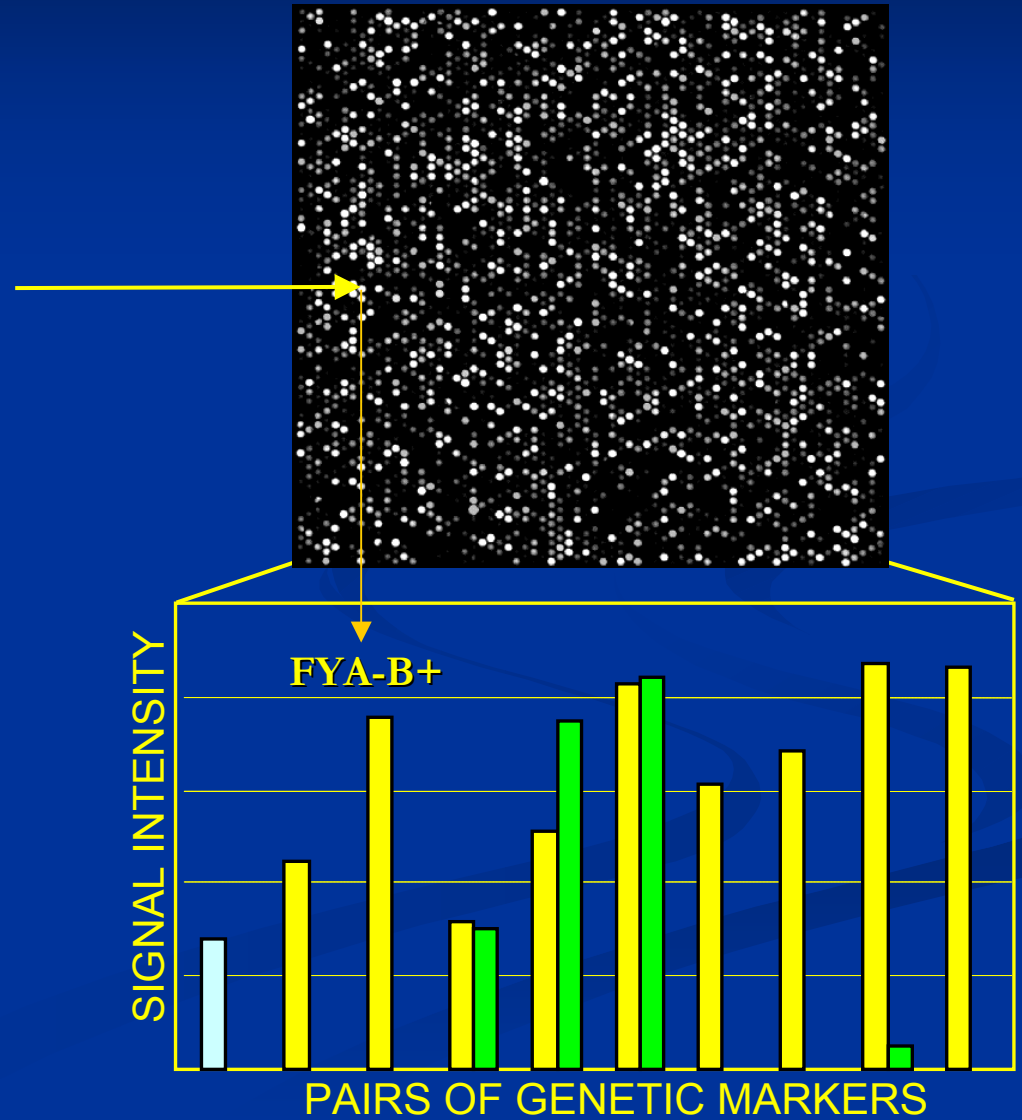
Not ABO, RhD

DETECTION OF BLOOD GROUP POLYMORPHISMS – ELONGATION ASSAY

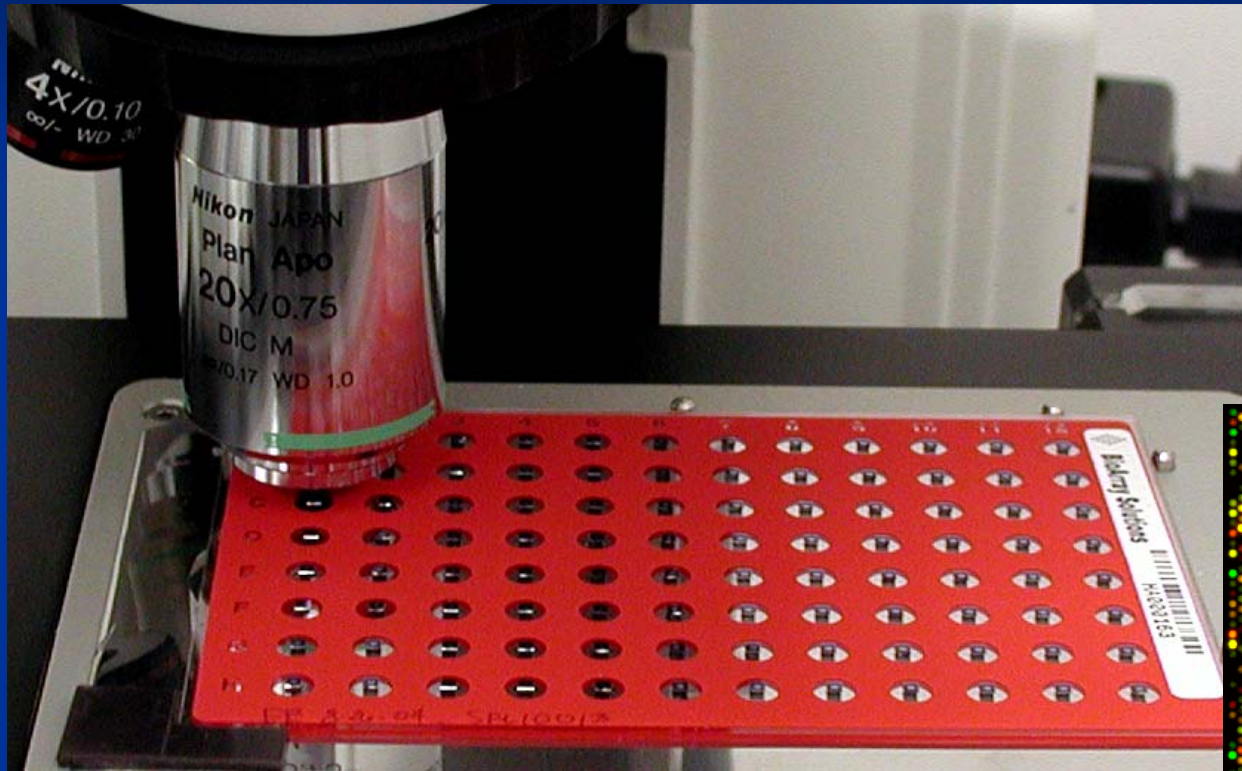
ON-BEAD ELONGATION



ASSAY IMAGE

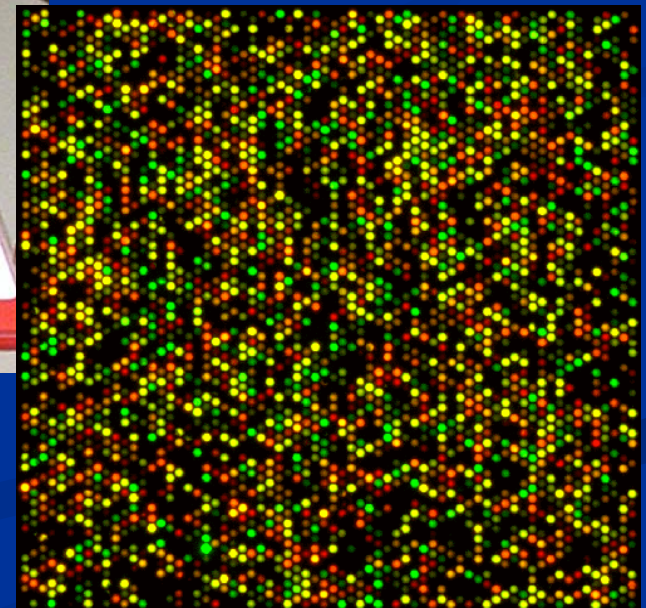


AUTOMATED IMAGE ACQUISITION



BARCODE SCANNING
“SNAPSHOT” ACQUISITION

DECODING IMAGE



ASSAY IMAGE

Questions - Molecular Testing

Would it replace serologic typing ?

Considerations:

- Not (yet) FDA approved
- Need to show equivalent (or improved) accuracy
- Not direct detection of antigen on RBC
 - Gives “prediction” of antigen type
 - False positive if gene is silenced by a mutation
- Test turnaround time impacts application (~5-8 hours)
- **ABO** – “prediction” of antigen expression not adequate
- **RhD** – serology appropriate for vast majority
- Does not do antibody screening

Not “good-bye to agglutination”

Applications – Donor Centers

1. Screening - for antigen-negative donor units

increase number of rare donor products

- Confirmation of negatives (when antibodies available)

Will provide the opportunity to validate genotyping and detect exceptions in different populations

2. Confirm Rh negative (D-) donor status

- Eliminate those weak D (type 2) and D_{el} RBCs not detected by serology from the D- donor pool

■ Challenges with current platform

- **Upstream - Need automated DNA extraction for high-throughput**
- **Downstream- Need automated data management system**
- **Frequency of testing - once would be goal**

Applications - Patients

Genotyping for numerous blood group antigens would allow possibility of extended matching of patients and donors to reduce or eliminate alloimmunization

- **Historically ~2-3% of patients become alloimmunized**
 - Higher in chronic transfused (32%)
 - Higher in minorities undergoing chronic transfusion (35-60%)
- **Risks of alloimmunization**
 - Significant delay in transfusion
 - Increase cost of products and workups
- **Additional Risks**
 - Warm Autoantibody production
 - Delayed or hemolytic transfusion reaction

Applications - Patients

1. Type multiply transfused patients

2. Genotype-matching to reduce/prevent alloimmunization

- Patients with sickle cell disease
 - C, E, K negative provided in many markets
 - Potential to include Duffy, Kidd, and complex Rh
- Patients facing chronic transfusion
- Patients with warm autoantibodies
 - Potential decrease number of complex allo and auto adsorptions
- Female children and women of child bearing age
 - Avoid anti-K and anti-c (done in Europe)
 - Kell – 10% potentially exposed
 - Anti-Kell - 1/100 pregnancies; 40% K+ babies have anemia
 - c – 18% potentially exposed
 - Anti-c associated with 32 fetal deaths in England and Wales (1977-1990)



MEDICINE

Blood-Matching Goes Genetic

Hoping to prevent adverse transfusion reactions and save lives European researchers are lobbying to replace serology-based blood typing with matching based on DNA tests.....

Serologic typing



Shortcomings

- Need fresh sample - intact RBCs
 - Not contaminated with donor RBCs
- Limits to sensitivity & specificity of reagent antibodies
 - **False negative**
 - weak Fy^b , weak Jk^a
 - Some weak D, all D_{el}
 - Cannot distinguish partial/weak D
 - **False positive**
 - Jk^a , N, D^{Har} , Crawford
- No reagents for some
 - $Do^{a/b}$, Js^a , Kp^a , Yt^a etc
 - Altered D,C,e (patients with SCD)
- Labor intensive - expensive

Strengths

- Fast
- Easy
- Reliable
- Familiar

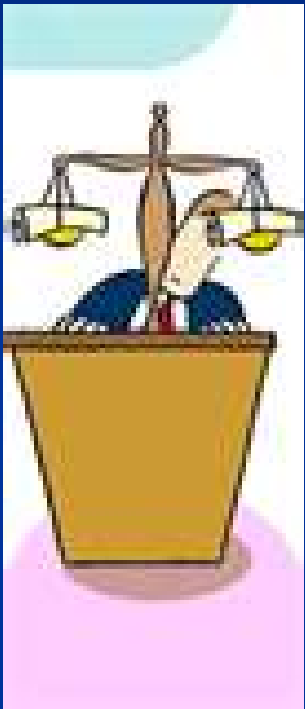
Molecular typing

Shortcomings

- Not detecting RBC expression
 - “Predicted” antigen phenotype
 - New mutations possible (family or population) that silence gene
 - False positive
- Assay design critical
 - Target nucleotide (s) must be 100% associated
 - False negative
- Test turnaround time
 - 5-8 hours

Strengths

- Cells from any tissue source
 - Including aged samples
- Can predict a phenotype for any blood group if the gene polymorphism known
- Amenable to high-throughput
- Less expensive
 - Reagents & Labor



Blood group antigen typing



Agglutination
Phenotype



DNA
Genotype

Power - in a combination of both methodologies
Future “typing” = phenot***ype*** and genot***ype***

DNA-based Assays

- Why applicable to transfusion medicine ?
 - Focus on pharmacogenomic testing
 - “personalized medicine”
 - tailor drug therapy and/or treatment to individual
 - based on genetic markers (metabolism rates, etc.)
 - Blood – drug
 - real potential for personalized therapy