

## Molecular Testing for *RHD* on patients with serologic weak D phenotype

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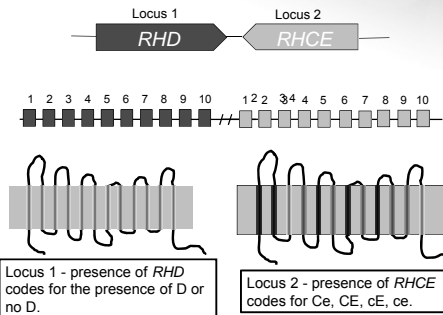
### Objectives

- Discuss the benefits of using a molecular approach in testing patients with a serologic weak D phenotype.
- List the methods used to perform *RHD* genotyping.
- Describe an approach for phasing in *RHD* genotyping for transfusion medicine practice.

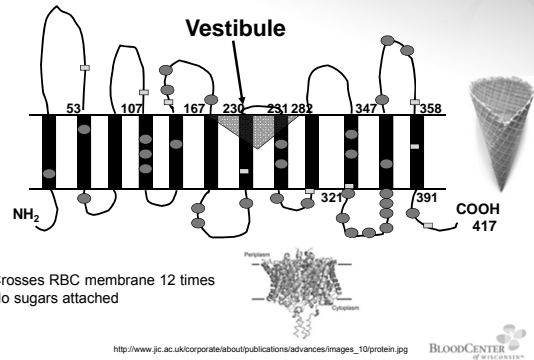


### *RH* Genes – Rh Positive

Chromosome 1



### RhD Protein



### RhD Negative

- Deletion of *RHD*
- Inactivating mutations of *RHD*
  - *RHD* $\psi$  in African backgrounds
- Hybrid *RHD-CE-D* in African backgrounds



### Terminology Guidelines for *RH* Alleles

- D positive - *RHD* conventional sequence is assigned *RHD*\*01
- D negative phenotypes (*RHD* null) designated with N and the background allele on which the null mutation has occurred
  - Common Rh negative - *RHD*\*01N.01
  - 37 bp insertion - *RHD*\*01N.02

<http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/>



### Terminology Guidelines for *RH* Alleles

- Alleles encoding partial D are given numbers associated with the phenotype category or classification
  - Alleles encoding partial DVI are numbered as *RHD\*06*.
  - DVI types encoded by different alleles numbered sequentially as *RHD\*06.01*, *RHD\*06.02*

<http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/>



### Terminology Guidelines for *RH* Alleles

- Weak D alleles are designated by *RHD\*01W.01* (type 1) OR numbers begin with 100
- Weak D phenotypes described with anti-D in numerous cases relative to their prevalence, the terminology “weak partial D” is used to reflect both weak D RBC typing and risk for anti-D production

<http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/>



### Serologic Weak D Phenotype Definition

Sandler SG, et al Transfusion 2015 Mar;55(3):680-9

- Anti-D reagent giving no or weak ( $\leq 2+$ ) reactivity in initial testing, but agglutinating moderately or strongly with antihuman globulin

Identified by weak reactivity or by discordant typing results



### Weakened Expression of D 2 Categories

- *Not at risk of making anti-D*
- *At risk of making anti-D*



## WEAK D

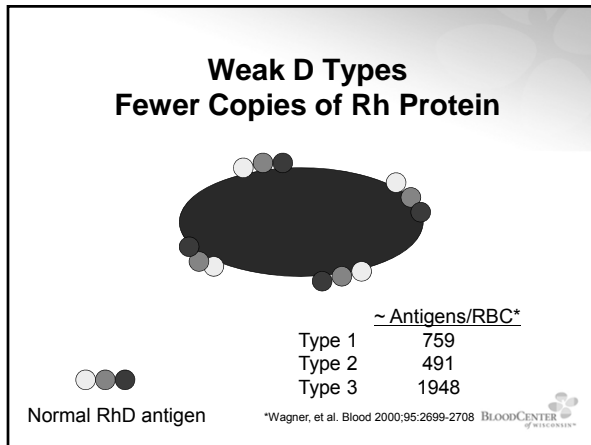
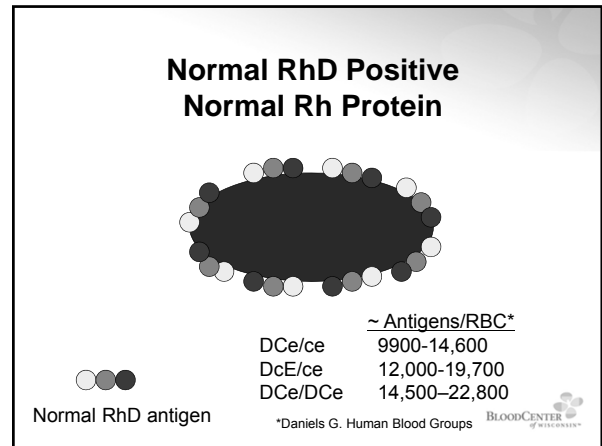
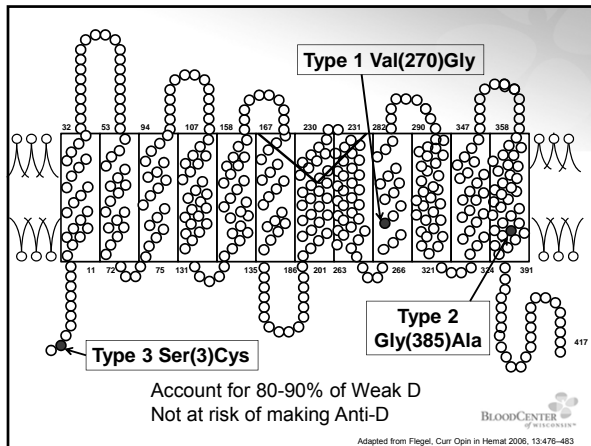
***Not at Risk of Making Anti-D***



### Weak Expression of D *Not at Risk of Making Anti-D*

- Weak D “Types”: amino acid change(s)
  - Usually single change
  - Types 1, 2, 3 most common in Caucasians



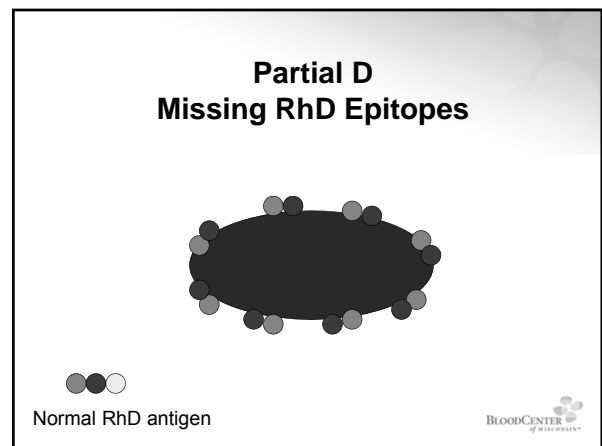


## WEAK EXPRESSION OF D

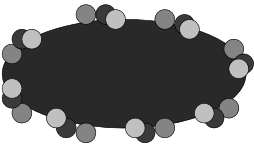
*At Risk of Making Anti-D*

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- ### Weak Expression of D *At Risk of Making Anti-D*
- Partial Ds: hybrid *RHD* alleles
  - Weak partial D
  - DEL: detected by adsorption/elution
  - D epitopes on *RHCE* gene
- BLOODCENTER of WISCONSIN



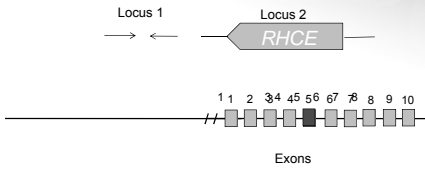
### Partial D Altered RHD Epitopes



Normal RHD antigen



### D Epitope on *RHCE* Gene - *ceHAR*




No D antigens                      ce antigens

*ceHAR* results from one *RHD* exon inserted into the *RHCE* gene.

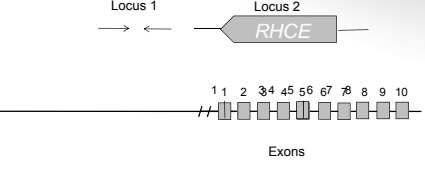
	IS
Anti-D	3+*

See AABB Tech Manual, 18<sup>th</sup> ed. 2014 for reactivity with different Anti-D

\*Often weaker in IAT/AHG



### D Epitope on *RHce* Gene - *ceCF*




No D antigens                      ce antigens

*ceCF* results from 3 nucleotide changes, 48G>C, 697C>G, 733C>G in *RHce* gene.

GammaClone	IS
Anti-D	3+*

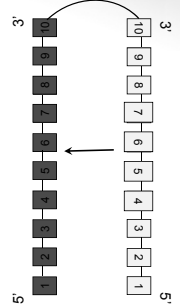

\*Often weaker in IAT/AHG

See AABB Tech Manual, 18<sup>th</sup> ed. 2014 for reactivity with different Anti-D



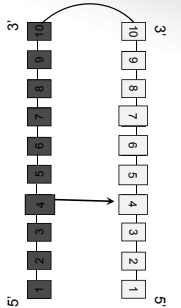

### Gene Conversion

- Portions of *RHCE* into *RHD*

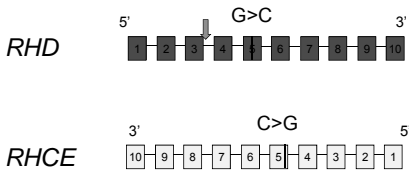



### Gene Conversion


- Portions of *RHD* into *RHCE*

### Missense, Nonsense, Frameshift & Splice Site Mutations




Missense – amino acid change  
 Nonsense or Frameshift – prevent expression  
 ↓ Splice site – no or reduced expression



### RH Gene Diversity

- **RHD: > 200 alleles** causing weak expression of RhD antigen
  - Weak D “Types” - >135 alterations
  - Partial D - >100 alterations
  - DEL - >40 alterations


Frequency of Serologic Weak D phenotypes – estimated 2.9% among mixed population in USA



### Commentary

#### It's time to phase-in *RHD* genotyping for patients with a serologic weak D phenotype

Sandler SG, Flegel WA, Westhoff CM, Denomme GA, Delaney M, Keller MA, Johnson ST, Louis Katz, Queenan JT, Vassallo RR, Simon CD  
Transfusion 2015 Mar;55(3):680-9





<http://www.aabb.org/advocacy/statements/Pages/statement150722.aspx>



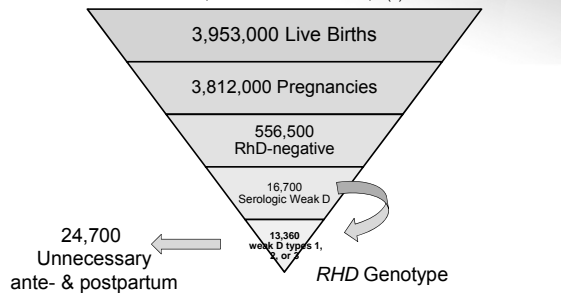
### Recommendation of the Work Group

- “*RHD* genotyping is recommended whenever a weak D phenotype is detected by routine Rh blood typing of pregnant women and other females of childbearing potential.”
- Strong Recommendation: based on high-quality evidence from observational studies (1A)




### Unnecessary RhIG Injections in USA

Sandler SG, et al Transfusion 2015 Mar;55(3):680-9




3,953,000 Live Births
3,812,000 Pregnancies
556,500 RhD-negative
16,700 Serologic Weak D
13,360 weak D types 1, 2, or 3
24,700 Unnecessary ante- & postpartum RhIG

*RHD* Genotype




### Reasons to Resolve Weak Expression Pregnancy

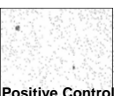
- Avoid giving RhIG to women who do not need it (Rh status is confirmed for historical discrepancies)
- Resolve early in pregnancy to eliminate false-positive rosette tests




Weak D+ Mom



Negative Control



Positive Control



**Protocols for High-Risk Pregnancies**  
An Evidence-Based Approach  
SIXTH EDITION  
JOHN T. QUEENAN  
CATHERINE Y. SPONG  
CHARLES J. LOCKWOOD  
WILEY Blackwell

p. 360

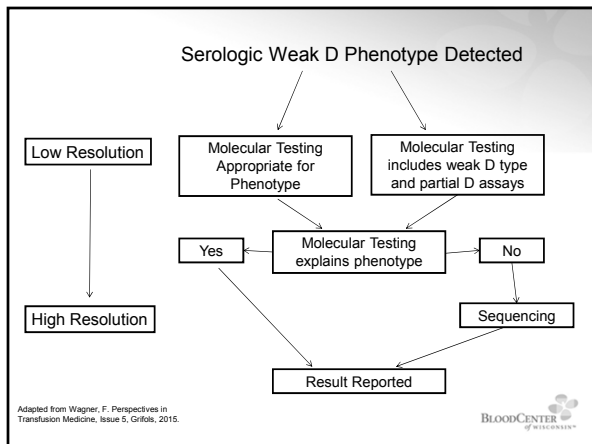
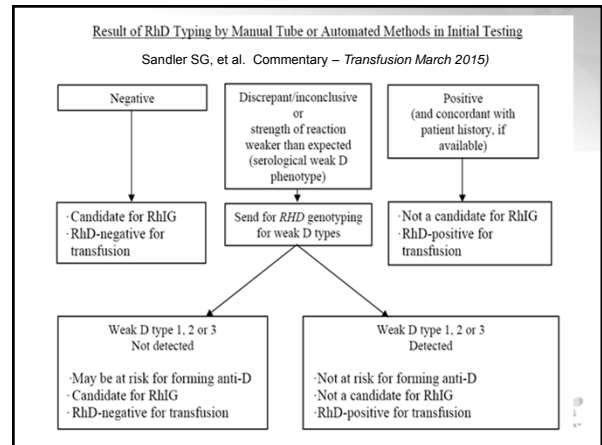
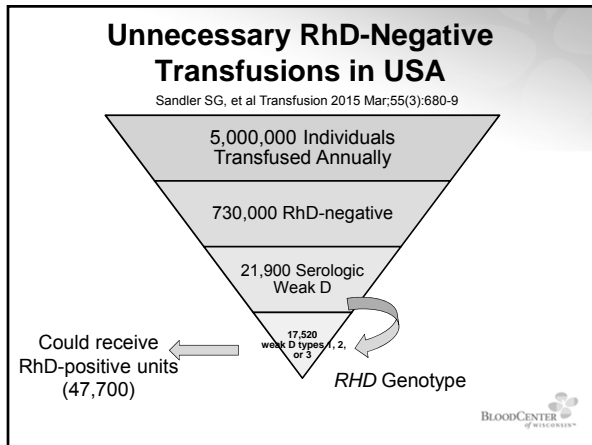
“Testing for “weak D”, formerly “Du” antigen is being recommended in a new approach due to new genotyping capabilities and information. ...They can be genotyped and if their genotype is type 1, 2 or 3, they may be managed as RhD-positive...”

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### Additional Notes

- CPT code 81403 for *RHD* genotyping (Tier 2 Molecular pathology procedure, Level 4)
- Reimbursement rates for the Tier 2 code established
- **ACOG** updating its Practice Bulletin!!

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### Weak and partial D management in patients

ISBT

WA Flegel, ST Johnson, S Nance - USA representatives  
ISBT Working Party on Immunohematology

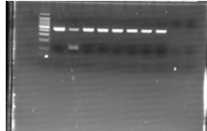
#### Which techniques do you use for *RHD* genotyping?

- PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)
- PCR with (Allele or) Sequence Specific Primers (PCR-SSP)
- Bead Technology (*RHD* BeadChip)
- BioChip Technology (*RHD* BloodGen)
- Sanger Sequencing of genomic DNA and cDNA
- Exon Scanning Assays

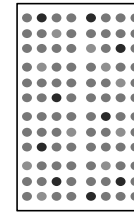
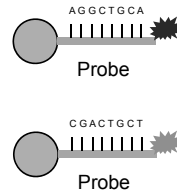
International Society of Blood Transfusion

### PCR with Allele-Specific Primers

- Isolate DNA
- Allele-specific amplification by PCR
  - Target weak & partial D variants
- Amplified products visualized on gel electrophoresis



### Bead Chip Arrays



### DNA Sequencing

- Gold standard for mutation detection
- Determines precise order of nucleotides
- Any method or technology used to determine order of the four bases—adenine, guanine, cytosine, and thymine—in DNA strand
  - Next Generation sequencers simplify sequencing of genomes (introns & exons)
- Analyze many genes at one time



### RHD Reference Sequence

ex1 (148 bp)	ex2 (187 bp)	ex3 (151 bp)	ex4 (148 bp)	ex5 (167 bp)
48	1 1 1 2 2 2 3 3 3 3 3 3 3 3 4 4 4 5 5 5 5 5 5 5 6 6 6 6 6 7 7 7 7 8 8 8			
G	A G G G T T A T T T A C A A T A G T T G A C C C T C G G G C G A F G			
				(A)
ex6 (138 bp)	ex7 (134 bp)	ex8 (80 bp)	ex9 (70 bp)	ex10 3'UT (24 bp) R
9 9 9 9 9 9 9 9 9 9 9 1				
1 3 4 5 6 7 7 8 8 8 9 9 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 2 2				
6 2 1 7 7 4 9 5 6 9 2 8 0 2 4 5 5 5 6 6 6 3 3 7 7 9 5 7				
G A G G C G A G G A A G G T G C G A G C G G C C T T A F G				

[http://www.ncbi.nlm.nih.gov/projects/gv/mhc/xslogi.cgi?cmd=bgmut/systems\\_info&system=rh](http://www.ncbi.nlm.nih.gov/projects/gv/mhc/xslogi.cgi?cmd=bgmut/systems_info&system=rh) BLOODCENTER of WISCONSIN PART OF VERDITT

### Identifying When to Perform RHD Genotyping

- **AABB/CAP Work Group Recommendations**
  - Anti-D reagent giving no or weak ( $\leq 2+$ ) reactivity in initial testing, but agglutinating moderately or strongly with antihuman globulin
  - Discordant result with history or between reagents



### Identifying When to Perform RHD Genotyping

- **Two Method Strategy** (Luo et. al, *Blood Transfus DOI* 10.20450/2017.0274-16)
  - Gel Test
    - Monoclonal Blend Anti-D
  - Test Tube
    - Monoclonal/Polyclonal Blend Anti-D



### Monoclonal Reagent Types

- Blend of monoclonal & polyclonal antibodies
- Blend of two or more monoclonal antibodies, each secreted by a different cell line
- IgG or IgM, or combination of IgG + IgM
- Why?
  - D antigen has >30 different epitopes
  - Variant D antigens



### FDA Approved Reagent Anti-D - Tubes

Reagent	Anti-D	
	IgM	IgG
Gamma-Clone	GAMA401	F8D8
Immucor-4	MS201	MS26
Immucor-5	TH28	MS26
Ortho Bioclone Tube	MAD2	Human polyclonal
Bio-Rad Seraclone - Blend	BS232	BS221 H41 11B7
Bio-Rad Seraclone - 226	BS226	
Quotient (Alba) - Alpha	LDM1	
Quotient (Alba) - Beta	LDM3	
Quotient (Alba) - Delta*	LDM1 ESD1-M	
Quotient (Alba) - Blend	LDM3	EDS1

\*Not for patient testing, detects DVI at IS

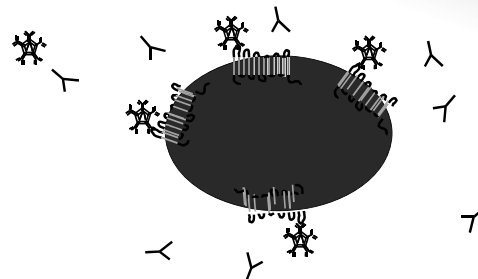


### FDA Approved Reagent Anti-D - Other Methods

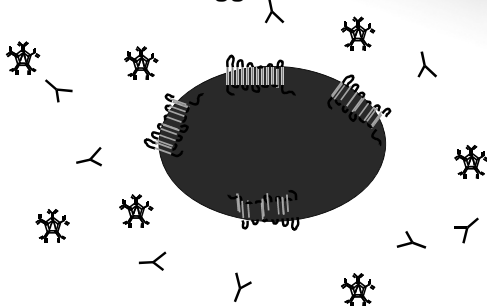
Anti-D	Method	Anti-D	
		IgM	IgG
Immucor - Series 4	Galileo Echo®/Neo®	MS201	MS26
Immucor- Series 5	Galileo Echo®/Neo®	TH28	MS26
Ortho	Gel/Provue®	MS201	
PK1	PK7200®/PK7300®	P3X61	
PK2	PK7200®/PK7300®	HM10	
Blend	PK7200®/PK7300®	P3X61 P3X21223B10	P3X290 P3X35
Bio-Rad 226	Tango®	BS226	
Bio-Rad 232	Tango®	BS232	
SolidScreen II Blend (weak D testing)	Tango®		H411B7 BS221
Grifols	DG Gel/Erytra®	P3x61	



### Monoclonal IgM/IgG ANTI-D



### Monoclonal IgM/IgG ANTI-D #1 Direct Agglutination - IS



### Two Method Strategy Criteria for RHD Genotyping

Luo et. al, *Blood Transfus* DOI 10.20450/2017.0274-16

1. Gel at least 2+ or greater then tube test result
2.  $\leq 2+$  with both methods or  $\leq 2+$  with gel only or  $\leq 1+$  with test tubes only
3. Anti-D detected





### Two Method Strategy Results

Luo et. al, *Blood Transfus* DOI 10.20450/2017.0274-16

- 50 cases
  - 14 retrospective
  - 36 prospective
- 32 women & 18 men
- 27 African American, 16 Hispanic, 6 Caucasian, 1 unknown



### Two Method Strategy RHD Genotyping Results

Luo et. al, *Blood Transfus* DOI 10.20450/2017.0274-16

- D variants identified in 49 cases
  - 39 partial D (*RHD\*<sup>+</sup>DAR, DAU4, DAU5*)
  - 10 weak D
- Identified more partial Ds
- Predictive Value – 98%



### What is this donor's RhD type?

- 27 y/o Caucasian donor typed O Rh Negative on 1<sup>st</sup> donation
- On 2<sup>nd</sup> donation PK7300® results Rh type as NTD
- Sample is sent to IRL for discrepancy resolution



### What is this donor's RhD type? IRL Results

Anti-D	IgM	IgG	IS	IAT
Gamma-Clone™	GAMA401	F8D8	0	W+
Immucor-5	TH28	MS26	0	0
Ortho Bioclone™	MAD2	Human	0	0
Biotest (Bio-Rad) Blend	BS232	BS221 H41 11B7	0	W+

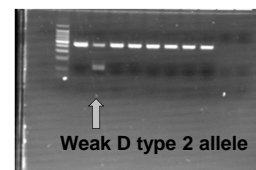


### What is this donor's RhD type? IRL Results

Anti-D	IgM	IgG	IS	IAT
Gamma-Clone™	GAMA401	F8D8	0	W+
Immucor-5	TH28	MS26	0	0
Ortho Bioclone™	MAD2	Human	0	0
Biotest (Bio-Rad) Blend	BS232	BS221 H41 11B7	0	W+



### What is this donor's RhD type? BAGene® DNA-SSP Kit Weak D Results



### What is this donor's RhD type?

RBCs Tested	Anti-C	Anti-E	Anti-c	Anti-e
Donor	3+	3+	3+	3+
Pos Ctl.	3+	3+	3+	3+
Neg Ctl.	0	0	0	0

Rh Phenotype – D+C+E+c+e+  
 Probable Rh Genotype – DCE/dcE

Most Probable - **DcE/dcE**



### What is this donor's RhD type?

- Weak D Type 2 Allele & *RHCE\*Ce* likely *in trans*
- Further decreasing RhD expression

Blood Donor – Rh Positive

Prenatal Patient – Rh Positive

Transfusion Recipient – Rh Negative



### Objectives

- Discuss the benefits of using a molecular approach in testing patients with a serologic weak D phenotype.
- List the methods used to perform *RHD* genotyping.
- Describe an approach for phasing in *RHD* genotyping for transfusion medicine practice.



## Thank You!!

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