

High-throughput genotyping for red cell and platelet blood group antigens by DNA micro arrays

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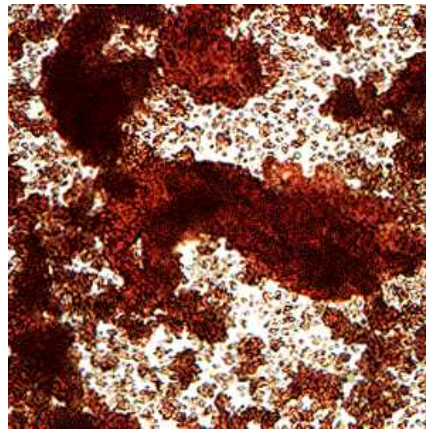
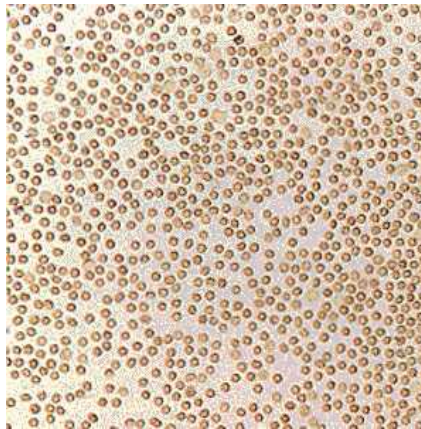


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Discovery of blood groups

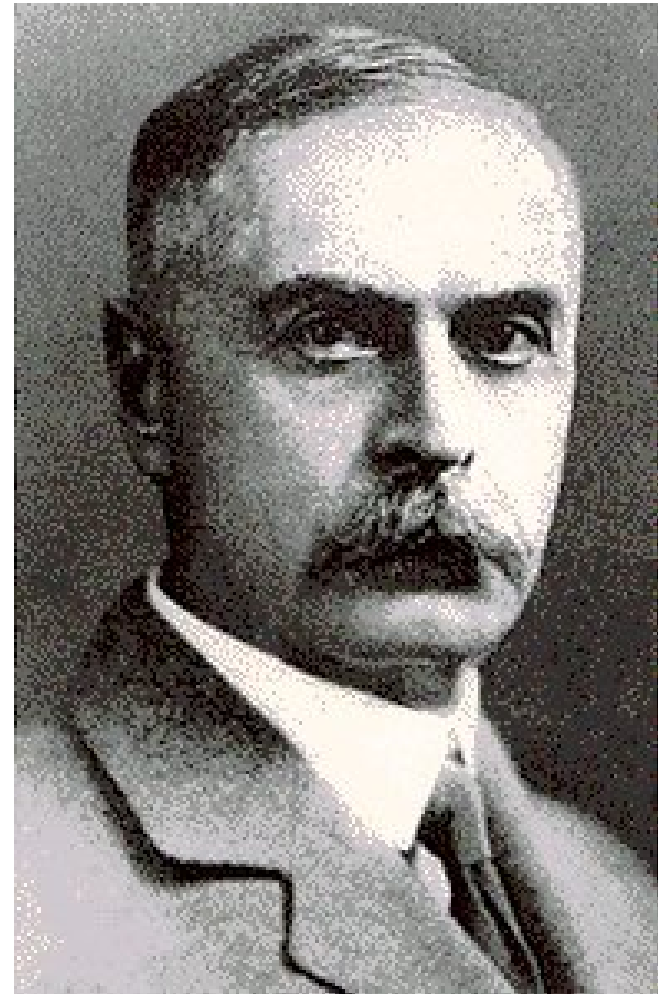
◆ Karl Landsteiner (1868-1943)

Noticed plasma from some individuals agglutinated red cells from others → ABO

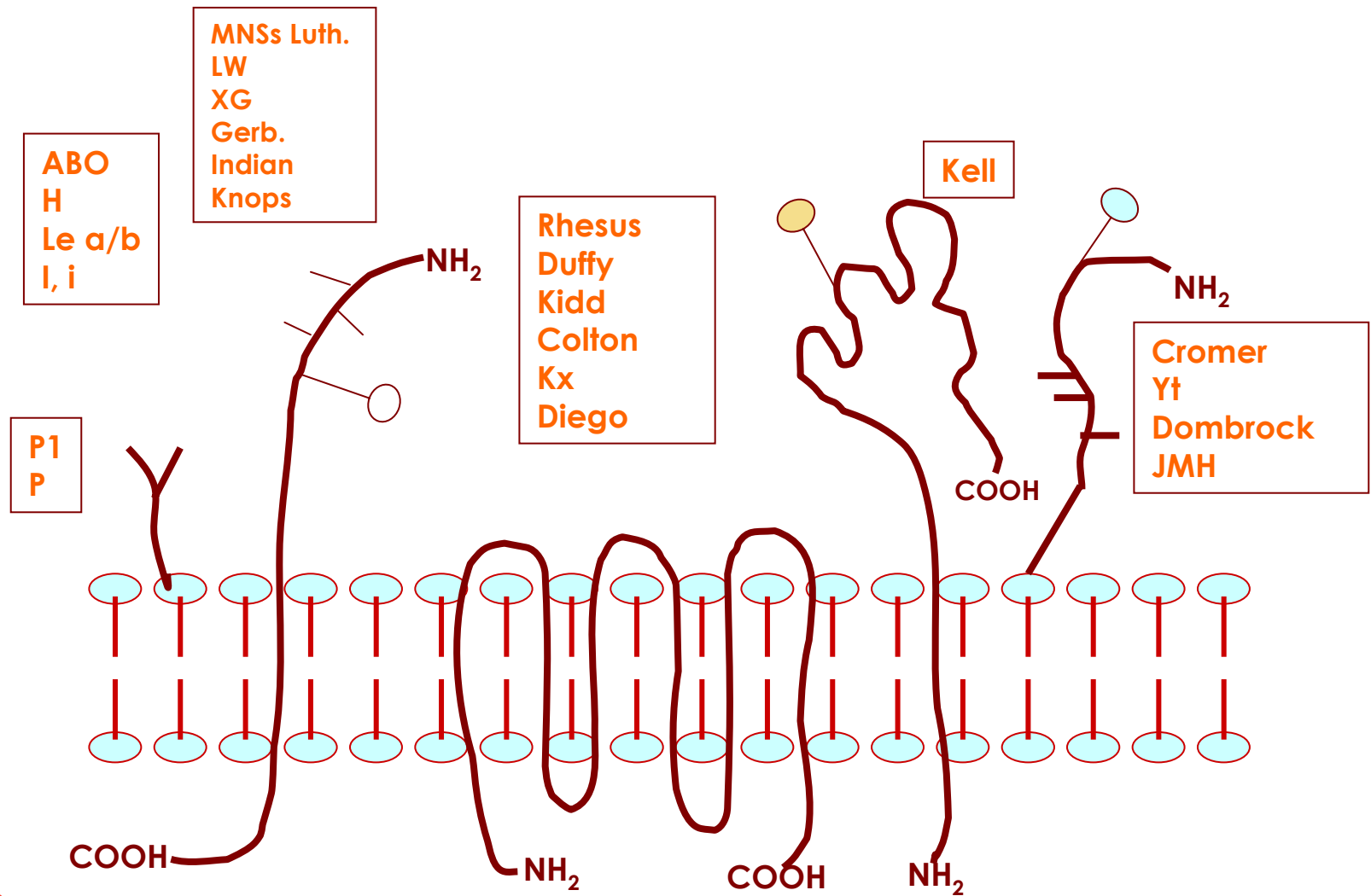


◆ Published in 1901, discovery hardly noticed at first but he received the Nobel price for his discovery in 1930

◆ 45 years later development anti globulin test (Coombs et al); detection of non agglutinating antibodies. Since then science of blood group serology blossomed → now about 270 blood group antigens known, belonging to 29 different blood groups



Blood Group carrying red cell (glyco)proteins/lipids



Why blood group typing?

- ◆ Red cells carry more than 29 blood group systems of which more than 270 different antigens are described
- ◆ Platelets carry 5 defined Human Platelet Antigens (HPA) and about 9 systems with low frequency
- ◆ With blood transfusions almost impossible to give 100% matched blood.
- ◆ Everybody has naturally occurring antibodies against A or B (except blood group AB) and Rhesus D blood group is highly immunogenic



Current blood transfusion policy

- ◆ ABO and Rhesus-D-matched blood
- ◆ Patients are screened for the presence of antibodies
- ◆ If woman < 45 years: ABO, D-matched and K negative
- ◆ If in need of multiple transfusions: ABO, Rhesus CcDEe and K – matched.

Thus: every blood transfusion will lead to an incompatibility for some blood group antigens:

- ◆ Some patients will make alloantibodies (<3%)
 - ◆ Risk of transfusion reactions
-
- ◆ If alloantibodies are formed: antigen-negative blood is needed.

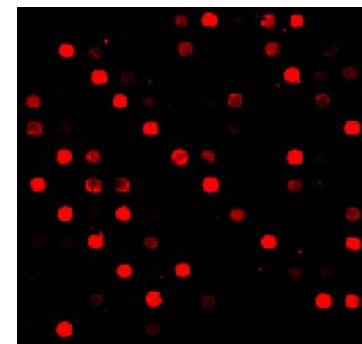
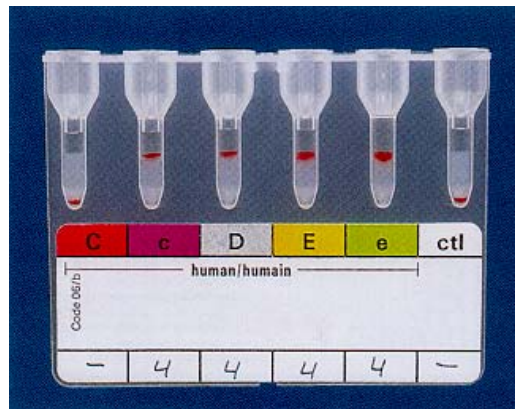
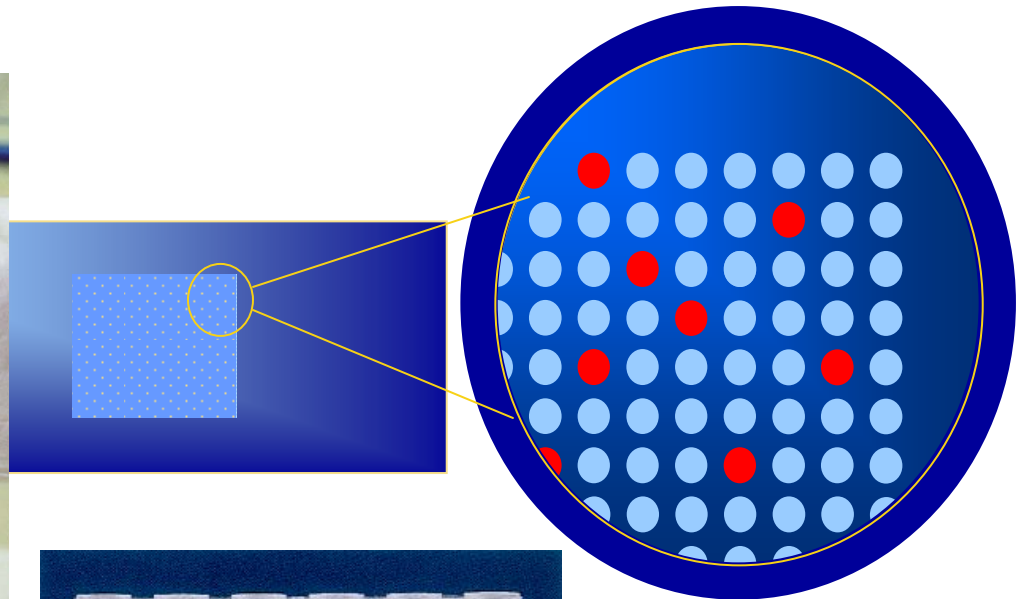


Routine genotyping of blood group antigens

- ◆ Large-scale serological typing of multiple red cell and platelet blood groups is expensive and can be impossible due to the small supplies or absence of reagents (rare blood groups)
- ◆ Complete phenotyping of blood donors too laborious and expensive
- ◆ Genotyping: complete donor database → availability of completely typed blood and blood with rare blood group antigens (antigen frequency < 1:1000) or with rare antigen combinations



Blood Group Typing: change of methods? Potential use of microarrays for genotyping



Genotyping by DNA microarray:

- ◆ Typing of a donor for most clinically relevant red blood cell and platelet antigens in a single test
- ◆ Automation required by blood bank → relatively simple procedure
- ◆ High-throughput → more donor samples per slide
- ◆ Costs → allele-specific hybridisation, PCR and labelling in one multiplex PCR reaction

- ◆ Genotyping is a prediction of the phenotype!
- ◆ Mutations may lead to assay failure (e.g. mismatch of the primers)



Molecular basis of blood group antigens

- ◆ Almost all antigens are the result of single point mutations or Single Nucleotide Polymorphisms (SNPs) in the encoding genes
- ◆ For some systems (e.g. Rh, MNS) gene-variants exist due to gene conversions between homologous genes
- ◆ Different type of null alleles exist:
 - ◆ Gene deletion
 - ◆ Mutation in promoter region
 - ◆ Mutation in coding region



Scheme for genotyping blood group antigens

DNA isolation



PCR amplification of gene fragment carrying the polymorphic site using fluorescent primers



Labeling and fragmentation of PCR products



Purification of PCR products



Hybridisation on array



Measurement of the fluorescence



Interpretation of the signals: typing of the sample



Multiplex PCR

- PCR of gene fragments carrying the SNP of interest
 - Multiple genes/chromosomes
 - Gene fragments as small as possible

• 30 antigens → 30 PCR amplifications



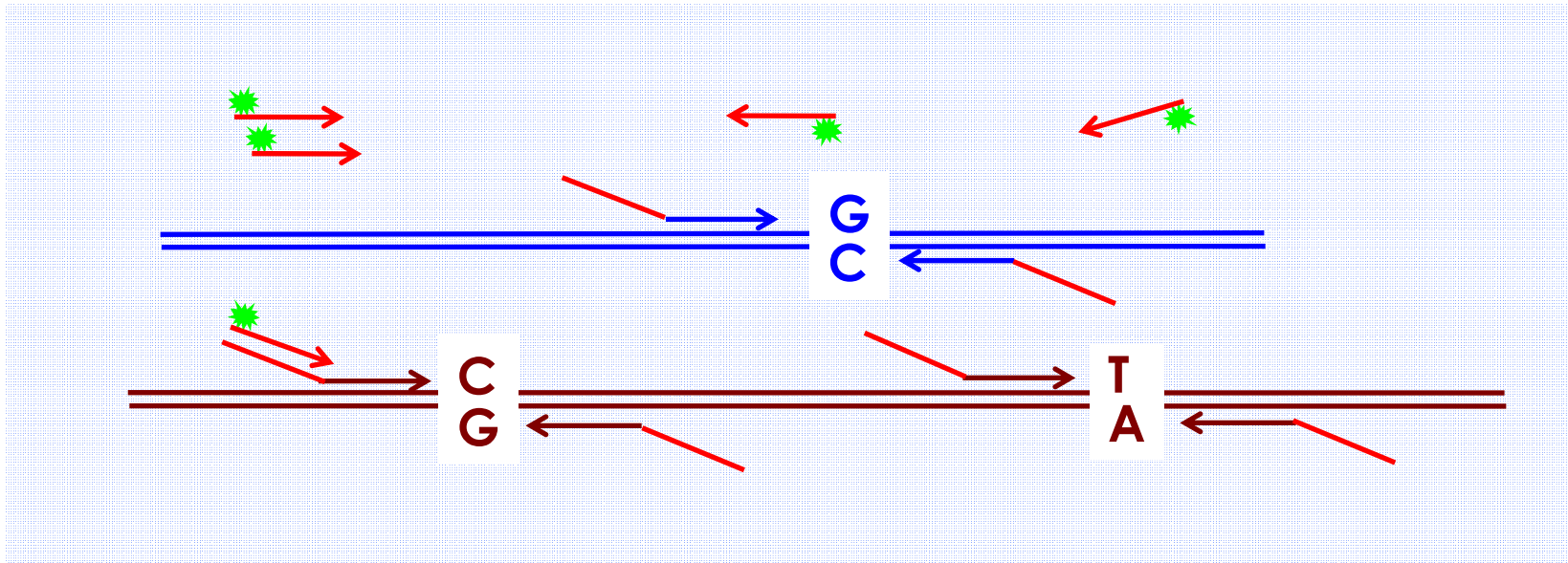
Multiplex PCR;

30 PCR amplifications in 1 tube



Principle of Multiplex PCR with common primers

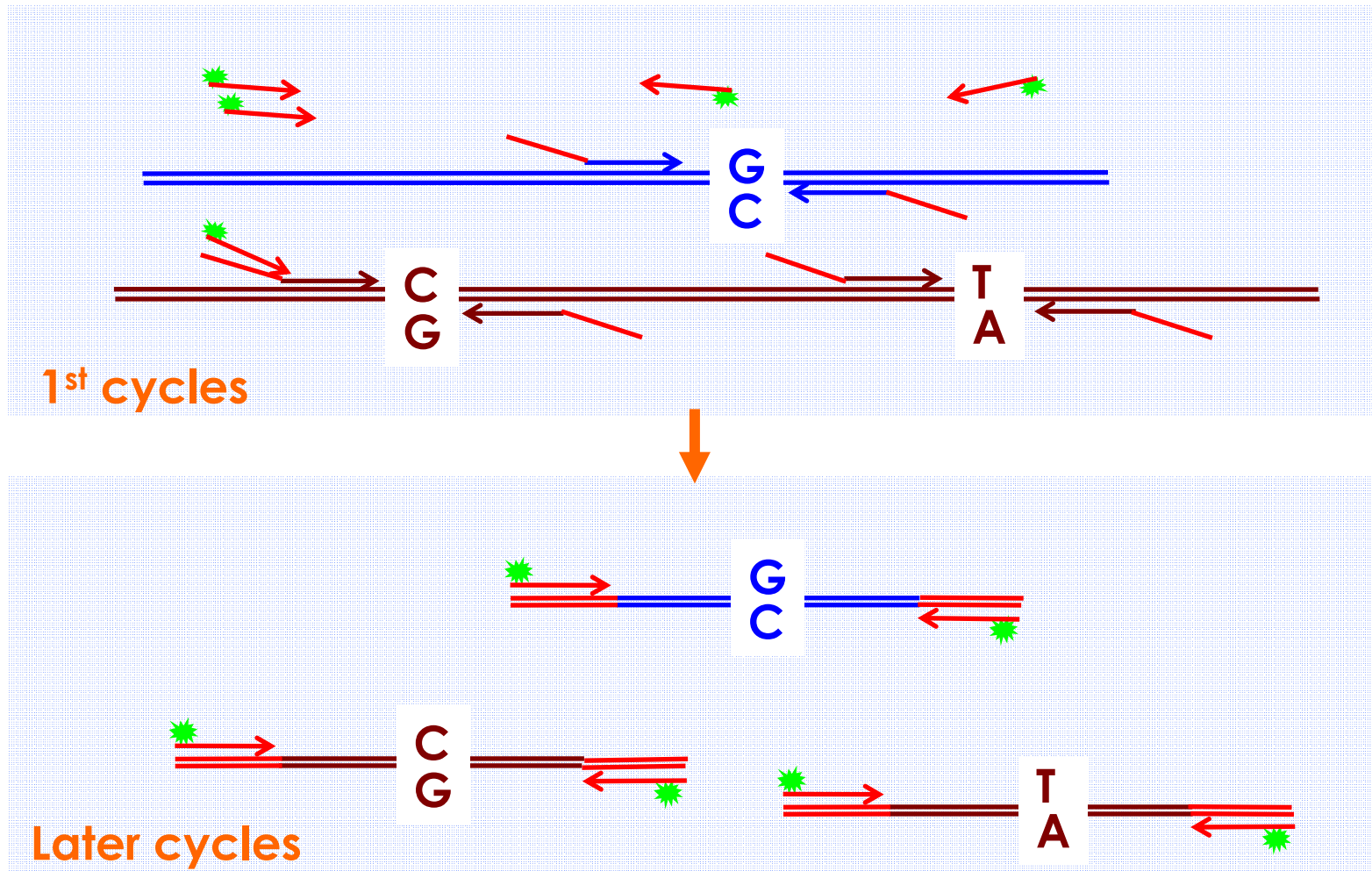
A mixture of gene-specific-tagged primers and labeled “common” primers is added.



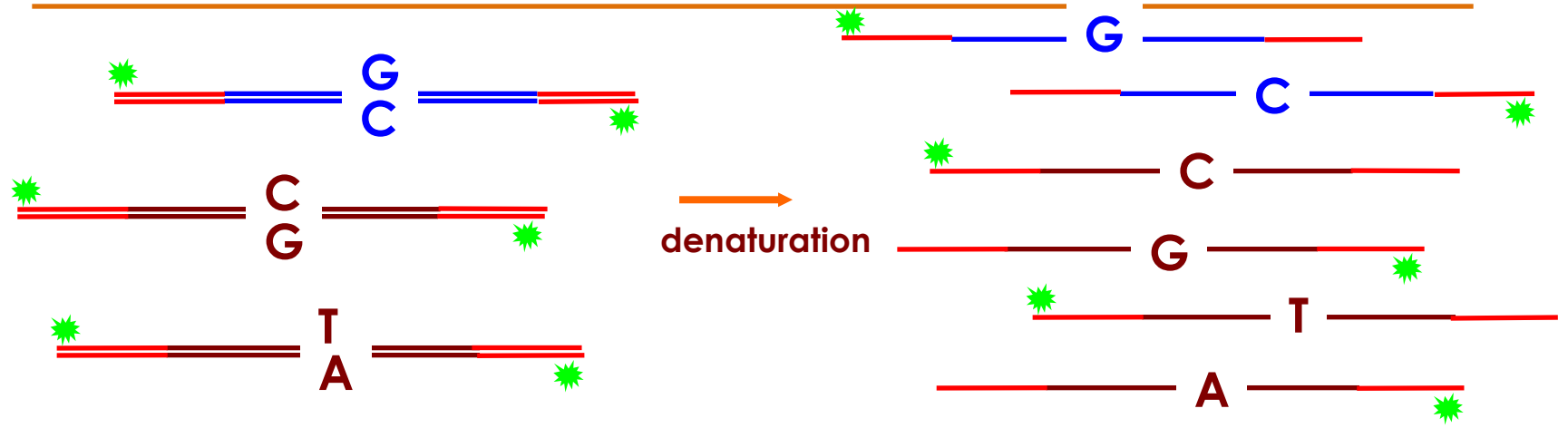
1st cycles: amplification via gene-specific-tagged primers



Principle of Multiplex PCR with common primers



Gene fragments on the array



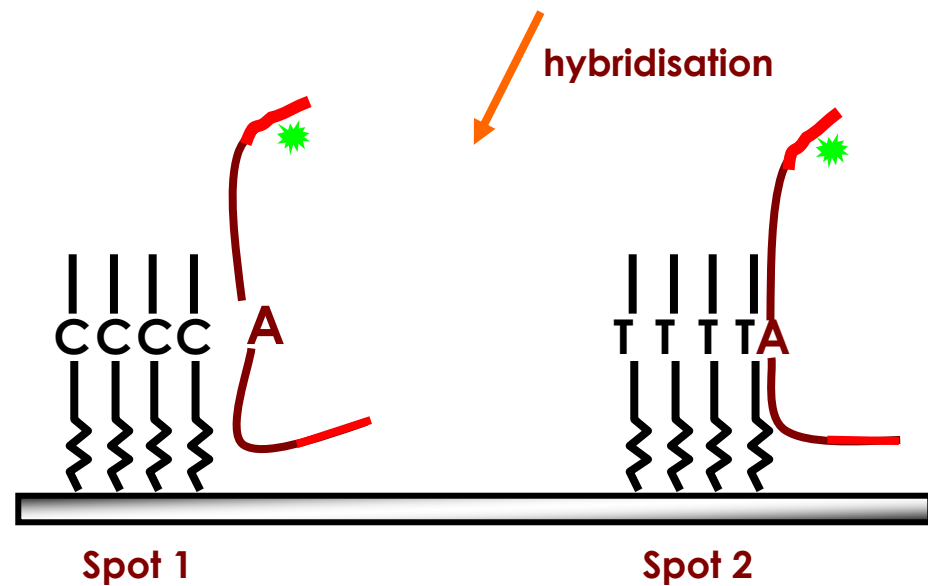
Allele specific oligo hybridization (ASO) →

Differences in thermal stability between mismatched and perfect matched hybrids

Oligo

Spacer

Glass



Multiplex PCR: points of attention

- ◆ PCR product size between 100-200 bp
 - ◆ Not always possible because of SNP position/gen specific nucleotides (e.g. RhD/RhCE)
 - ◆ Our MPX contains fragments between 86-400 bp
- ◆ PCR primers: similar T_m values, specificity (Celera, BLAST <http://www.ncbi.nlm.nih.gov/BLAST>, BLAT <http://genome.ucsc.edu/cgi-bin/hgBlat>)
- ◆ Reduce primer-dimer formation (Shuber; Brownie*):
 - ◆ Use low amount of chimeric and high amount of universal primers
 - ◆ Check chimeric primers for primer duplexing (Oligo 6 primer analysis software, Molecular Biology Insights, Inc., 1998)
 - ◆ Choose primers with $\Delta G > -10$ kcal/mole

* Shuber et al., 1995, Genome Res. 5, 488-493

Brownie et al., 1997, Nucl. Ac. Res. 25, 3235-41



Multiplex PCR: points of attention

◆ Use fluorescent universal primers

◆ Qiagen Multiplex PCR kit

Actual amplification is done by universal primers

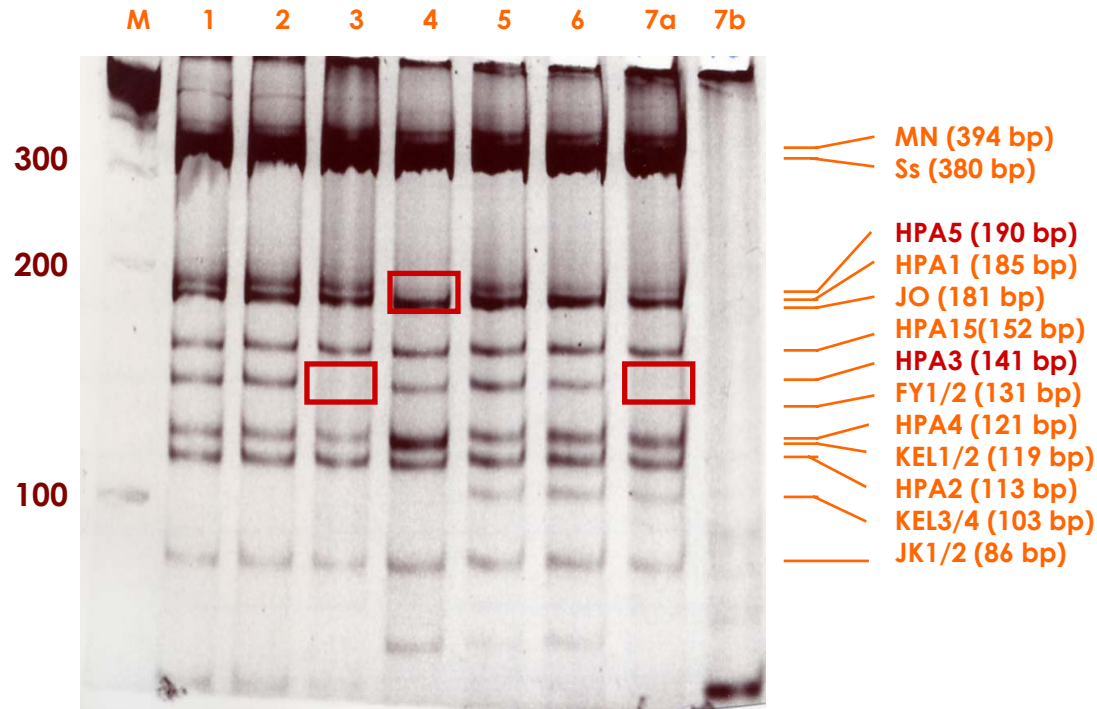
→ Similar yields of PCR product

→ [primer] adjustment hardly



Multiplex PCR

Some PCR primers interfere with other primers



HPA3-left 5'-GCCTGACCACTCCTTTGC-3'

$\begin{array}{cccccccc} | & | & | & | & | & | & | & | \\ \hline & & & & & & & \end{array}$

 3'-GGTGAGGACATTGGACGACC-5' FY1/2-right

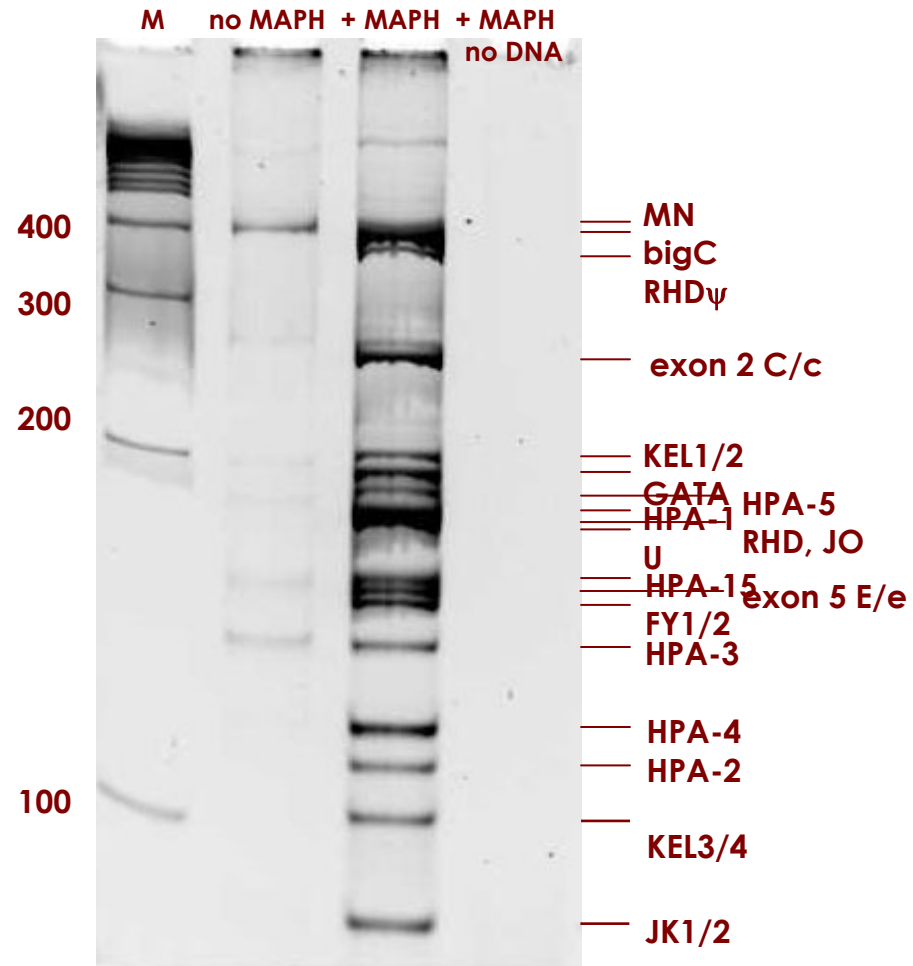
→ Check new primers with oligo6



Multiplex PCR

One simple PCR

15 min 95°C
30 s 94°C } 45 cycles
90 s 57°C }
90 s 72°C }
10 min 72°C



HPA glass array

Design 10 oligo's per allele, sense + antisense

PROBE PICKING RESULTS FOR HPA-1

Tm	gc%	any 3'	sequence	
65.86	68.42	7.00	3.00	tacagccctgcctctggg HPA-1a1
66.92	72.22	5.00	3.00	aggccctgcctctgggct HPA-1a2
67.79	73.68	4.00	1.00	ccctgcctctgggctcacc HPA-1a3
67.35	68.42	4.00	2.00	tgcctctgggctcacctcg HPA-1a4
64.65	68.42	4.00	2.00	ctctgggctcacctcgctg HPA-1a5
64.65	68.42	4.00	1.00	cagcgaggtgagcccagag HPA-1a1 CR
67.35	68.42	4.00	1.00	cgaggtgagcccagaggca HPA-1a2 CR
67.79	73.68	4.00	3.00	ggtgagcccagaggcaggg HPA-1a3 CR
66.92	72.22	5.00	5.00	agcccagaggcagggcct HPA-1a4 CR
65.86	68.42	7.00	3.00	cccagaggcagggcctgta HPA-1a5 CR
69.93	73.68	7.00	3.00	tacagccctgcctccggg HPA-1b1
70.94	77.78	5.00	3.00	aggccctgcctccgggct HPA-1b2
68.84	77.78	4.00	0.00	ccctgcctccgggctcac HPA-1b3
66.21	72.22	4.00	0.00	ctgcctccgggctcacct HPA-1b4
66.33	72.22	4.00	0.00	ctccgggctcacctcgct HPA-1b5
66.33	72.22	4.00	2.00	agcgaggtgagcccggag HPA-1b1 CR
66.21	72.22	4.00	1.00	aggtgagcccggaggcag HPA-1b2 CR
68.84	77.78	4.00	3.00	gtgagcccggaggcaggg HPA-1b3 CR
70.94	77.78	5.00	5.00	agcccggaggcagggcct HPA-1b4 CR
69.93	73.68	7.00	3.00	cccggaggcagggcctgta HPA-1b5 CR



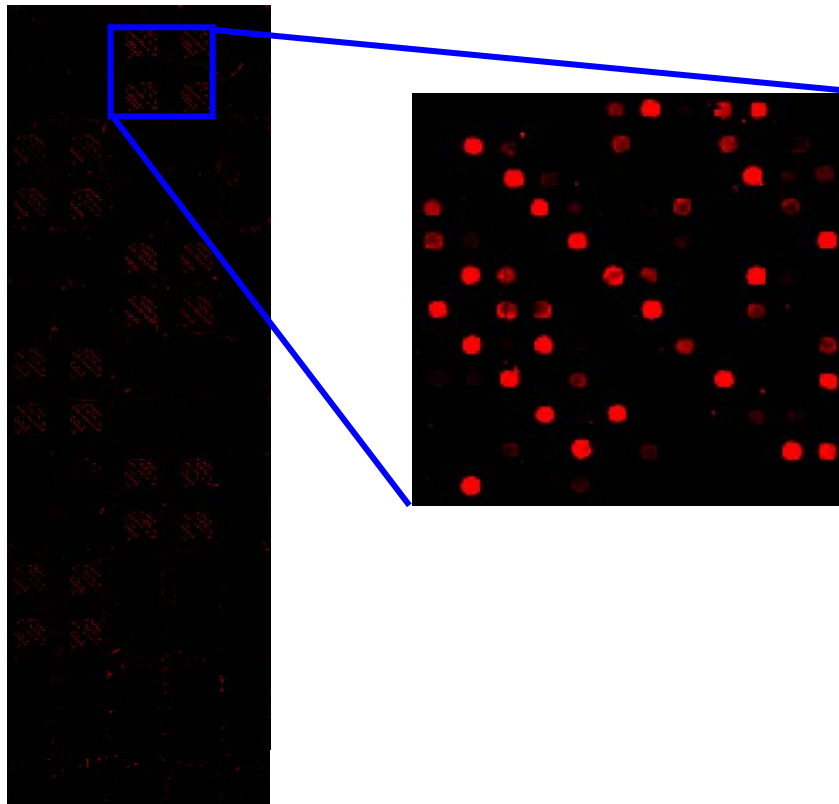
HPA glass array

xxxx	3ad2	1aa	2aa	3aa	4aa	5aa	6aa	1ba	2ba	3ba	xxxxx
4ba	5ba	6ba	1aacr	2aacr	3aacr	4aacr	5aacr	6aacr	1bacr	2bacr	3bacr
4bacr	5bacr	6bacr	3ad2cr	C	1ab	2ab	3ab	4ab	5ab	6ab	1bb
2bb	3bb	4bb	5bb	6bb	1abcr	2abcr	3abcr	4abcr	5abcr	6abcr	1bbcr
2bbcr	3bbcr	4bbcr	5bbcr	6bbcr	3bd2	C	1ac	2ac	3ac	4ac	5ac
6ac	1bc	2bc	3bc	4bc	5bc	6bc	1accr	2accr	3accr	4accr	5accr
6accr	1bccr	2bccr	3bccr	4bccr	5bccr	6bccr	3bd2cr	C	1ad	2ad	3ad
4ad	5ad	6ad	1bd	2bd	3bd	4bd	5bd	6bd	1adcr	2adcr	3adcr
4adcr	5adcr	6adcr	1bdcr	2bdcr	3bdcr	4bdcr	5bdcr	6bdcr	3ad1	C	1ae
2ae	3ae	4ae	5ae	6ae	1be	2be	3be	4be	5be	6be	1aecr
2aecr	3aecr	4aecr	5aecr	6aecr	1becr	2becr	3becr	4becr	5becr	6becr	3ad1cr
xxxx	CS05	C	3bd1	3bd1cr							

- Similar oligo's are equally distributed
- Poly-L-lysine coated glass slides (Erie scientific)
- C: irrelevant sequence, negative control for background subtraction
- CS05: positive control, hybmix contains Cy5 labelled C&R oligo



Hybridization on blood group array



- 16 blocks per slide
- 2 blocks per donor sample
- 4 donor samples per slide



Define matrix

- ◆ Collect 3 samples per genotype with known geno- or phenotype
- ◆ Rare bloodgroups
 - ◆ SCARF (Serum, Cells and Rare Fluids exchange)
 - ◆ Exchange DNA samples with other labs
- ◆ Define matrix for all probe sets
 - ◆ Threshold: $F_{th} = F_{tot} - av.F_{bg} - 3xSD_{av.F_{bg}}$
 - ◆ Ratio of signal intensities per probe set: $a / (a+b)$
 - ◆ Define matrix for all probe sets
- ◆ Exclude probe sets yielding no or incorrect genotypes



Define matrix

ID	F _{total}	F _{th}	ratio	genotype		
C a33	861					av. C 692
C a35	579					SD 105
C a38	688					3*SD 315
C a42	640					
C a43	692					
CS05	64433					
HPA-1a1	1311	304.2	0.0	bb		aa > 0.75
HPA-1b1	29825	28818				bb < 0.25
						0.35 < ab < 0.65
HPA-1a1	tacaggccctgcctctg ^t ggg					
HPA-1b1	tacaggccctgcctc ^c ggg					

Av.C + 3*SD= 1007

$1311 / (1311 + 29825) < 0.25$

↓

|

1311-1007 = pos → keep
29825-1007=pos → keep



Define matrix

Exclude probe sets yielding no or incorrect genotypes

HPA	number of selected probe sets		
	sense	antisense	total
1	5	2	7
2	3	5	8
3	0	6	6
4	0	5	5
5	0	5	5
15	4	5	9



Genotyping of two blinded panels

- Two batches of slides
- Two panels of 58 and 43 samples: overlap of 7 samples
- Panels were genotyped by different persons
- Second panel was genotyped 3 months after first panel

		Microarray- result																
		HPA1			HPA2			HPA3				HPA4	HPA5			HPA15		
Donors		AA	AB	BB	AA	AB	BB	AA	AB	BB	ND	AA	AA	AB	BB	AA	AB	BB
HPA1	AA	61																
	AB		19															
	BB			21														
HPA2	AA				64													
	AB					30												
	BB						7											
HPA3	AA							28										
	AB								45									
	BB									28	2							
HPA4	AA											101						
HPA5	AA												66					
	AB													26				
	BB														9			
HPA15	AA															28		
	AB																48	
	BB																	25



Genotyping of two blinded panels

Results:

- ◆ No discrepancies
- ◆ No decision could be made between HPA-3ab and HPA-3bb for 2 samples: no problem after retyping
- ◆ 94 different samples typed for HPA-1,-2,-3,-4,-5 and -15 with good reproducibility

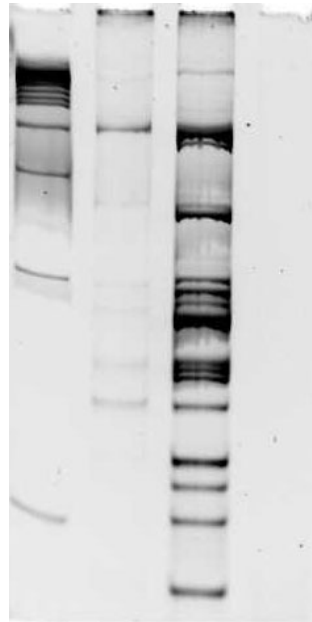


Genotyping blood group antigens

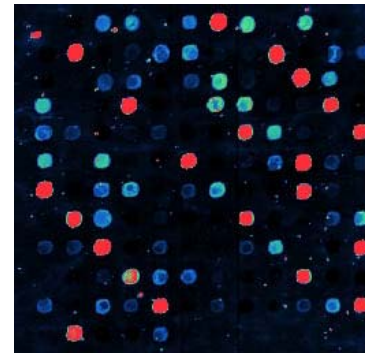
Automated DNA extraction



→ Multiplex PCR reaction



→ Hybridisation on DNA array



→ Scanning, Typing:

HPA-1, -2, -3, -4, -5, -15, RHD, RHD Ψ , RHDVIneg., r's, RHC/c, RHE/e, K/k, Kp^a/Kp^b, Js^a/Js^b, Jk^a/Jk^b, Fy^a/Fy^b, GATA-1 site, Fy^x, M/N, S/s, U, Yt^a/Yt^b, Do^a/Do^b, Jo^a, Di^a/Di^b, Wr^a/Wr^b, Co^a/Co^b, Lu^a/Lu^b,



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Amsterdam

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🔴 ServiceXS, Leiden

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🔴 Central Microarray Facility, NKI-AVL,
Amsterdam

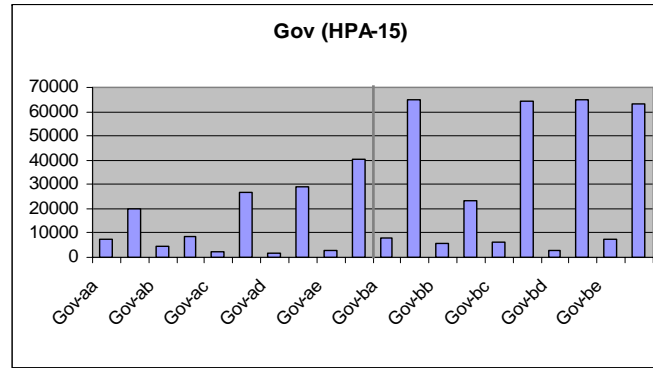
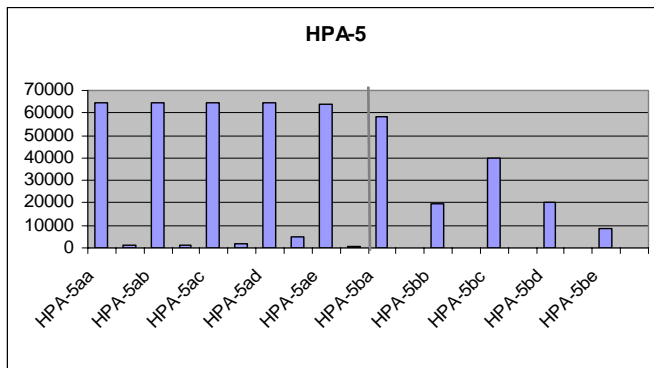
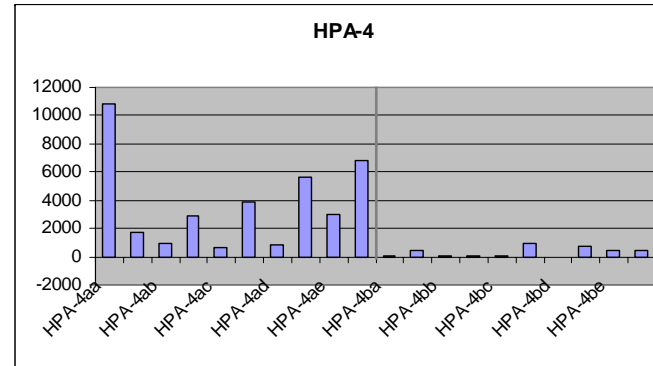
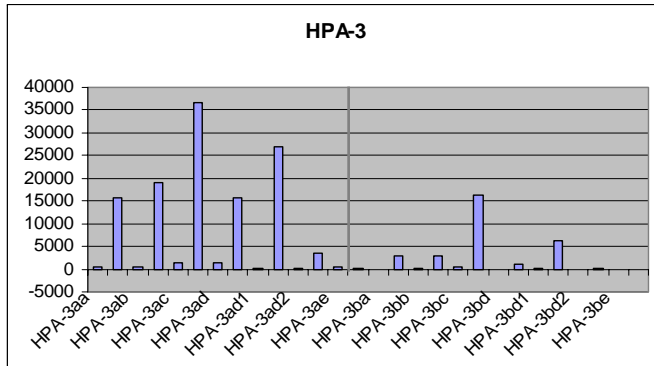
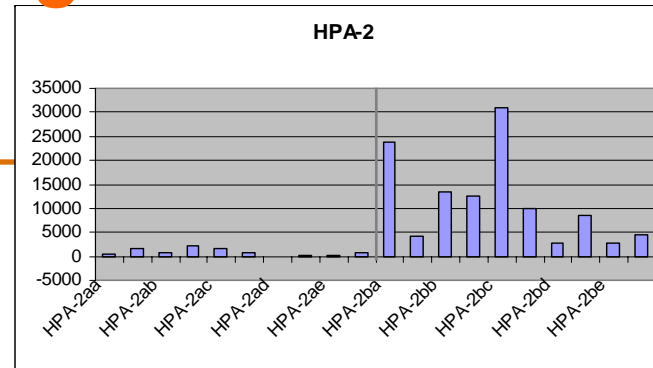
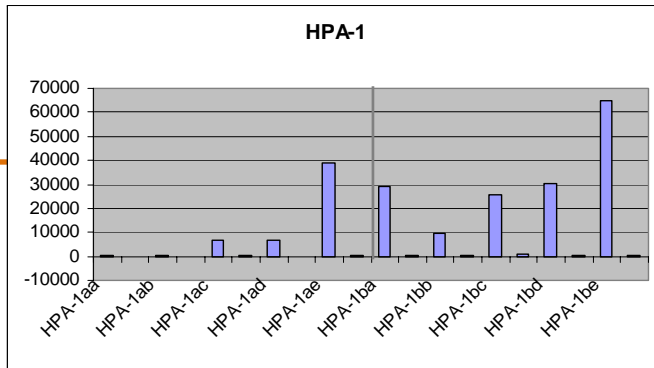
Ron Kerkhoven
Mike Heimerikx



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Probe signals





Development of a high-throughput blood group antigen genotyping assay; international interest. Transfusion, May 2005.

- **Beiboer et al.** *Rapid genotyping of blood group antigens by multiplex PCR and DNA microarray hybridisation.*
- **Bugert et al.** *Microarray based genotyping for blood groups: comparison of gene array and 5' nuclease assay techniques with HPA as a model.*
- **Denomme and van Oene.** *High-throughput MPX SNP analysis for RBC and PLT genotypes.*
- **Hashmi et al.** *A flexible array format for large-scale, rapid blood group DNA typing.*





prehybridization

	'ons' protocol	Protocol 1	Protocol 2	
			PREHYB 1	PREHYB 2
Herringsperm	X			
Yeast tRNA	X			
Denhardts	X			
20xSSC	X	X	X	X
10%SDS	X	X	X	X
Formamide ☠		X		X
BSA		X		X
Natrium boorhydride ☠			X	
incubatie	30' 65°C	45' 42 °C	30' 42 °C	45' 42 °C



hybridization

	'ons' protocol	Protocol 1	Protocol 2
Voor hybridisatie	75' 65 °C*		
Herringsperm	X	X	X
Yeast tRNA	X	X	X
Denhardts	X		
20xSSC	X	X	X
10%SDS	X	X	X
Formamide 		X	X
BSA			
Natrium boorhydride 			
incubatie	3.5 hrs 55°C	16-20 hrs 42 °C	16-20 hrs 42 °C

