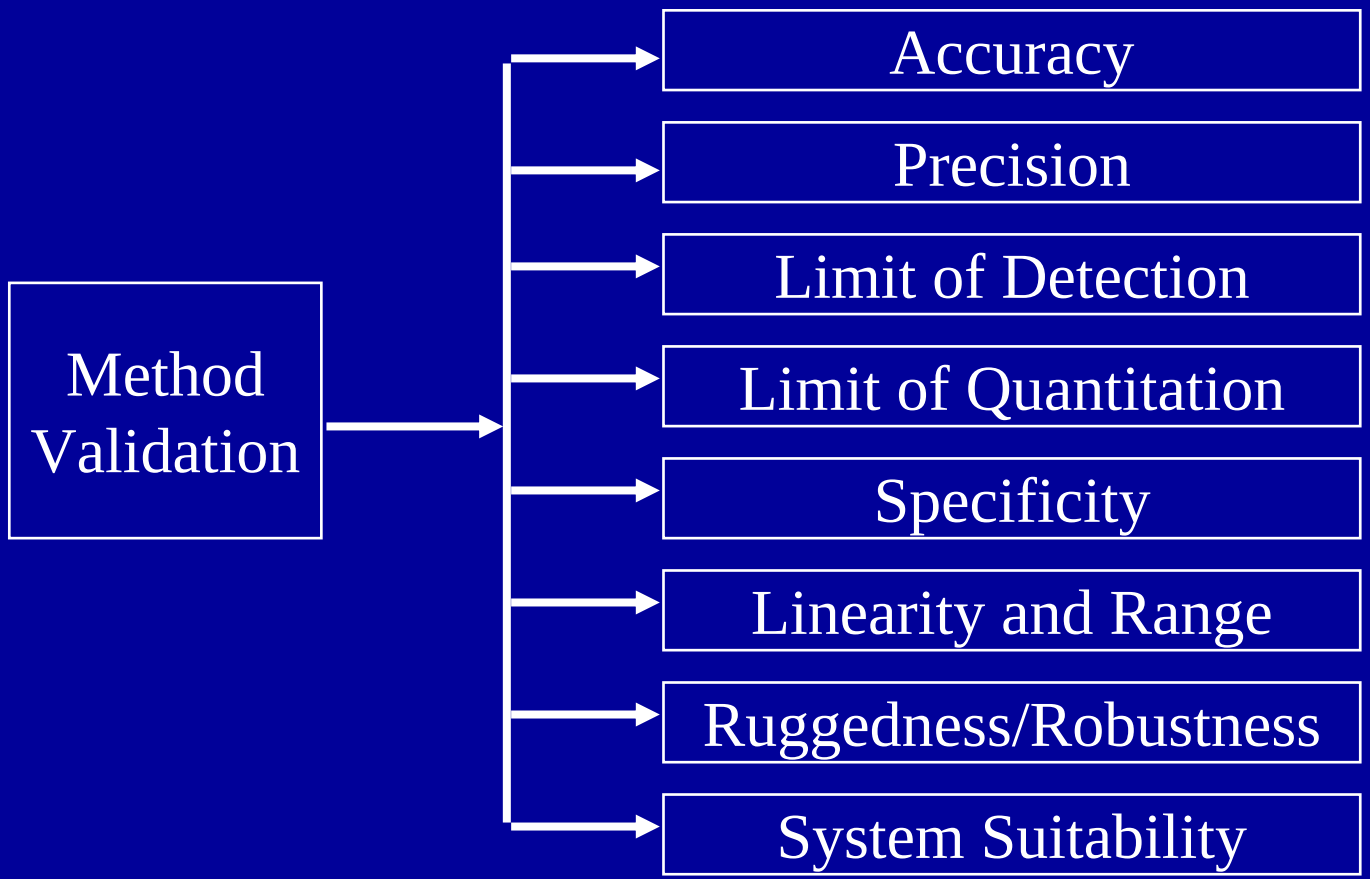
Status of Immune Monitoring: Quantitation and Reproducibility

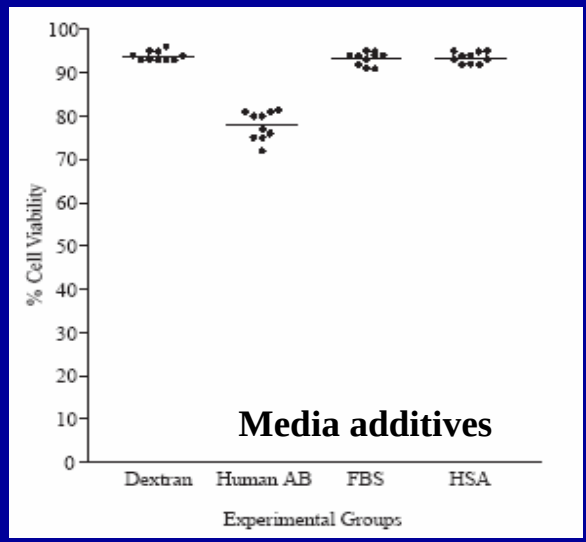


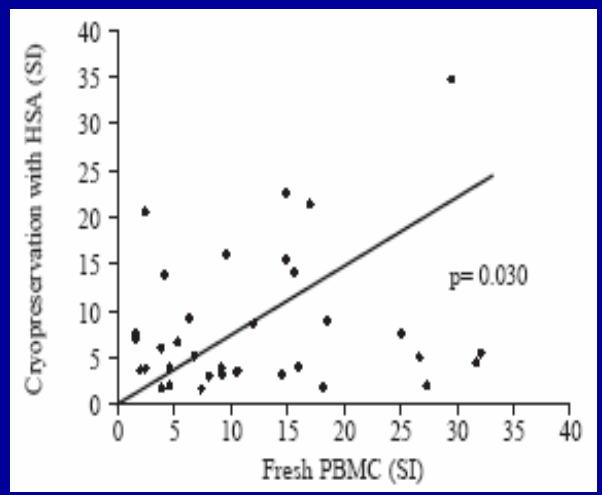
Dominique Pifat, FDA Website, 2003

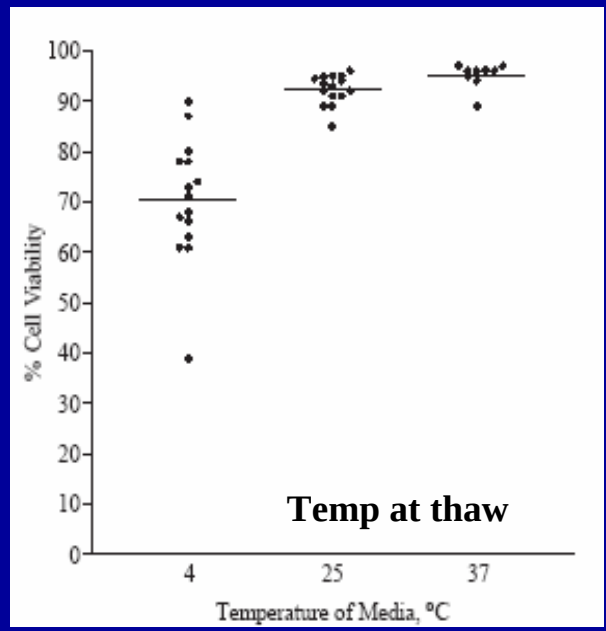
Need for Effective Cryopreservation

<u>Factors That Did Not Matter</u>

- Dry ice, 24, 48, 72 hours (p>0.05)
- Large vs. small volume thaw (p>0.05)
- Speed of spin at wash (p>0.05)
- # cells/vial (p>0.05)



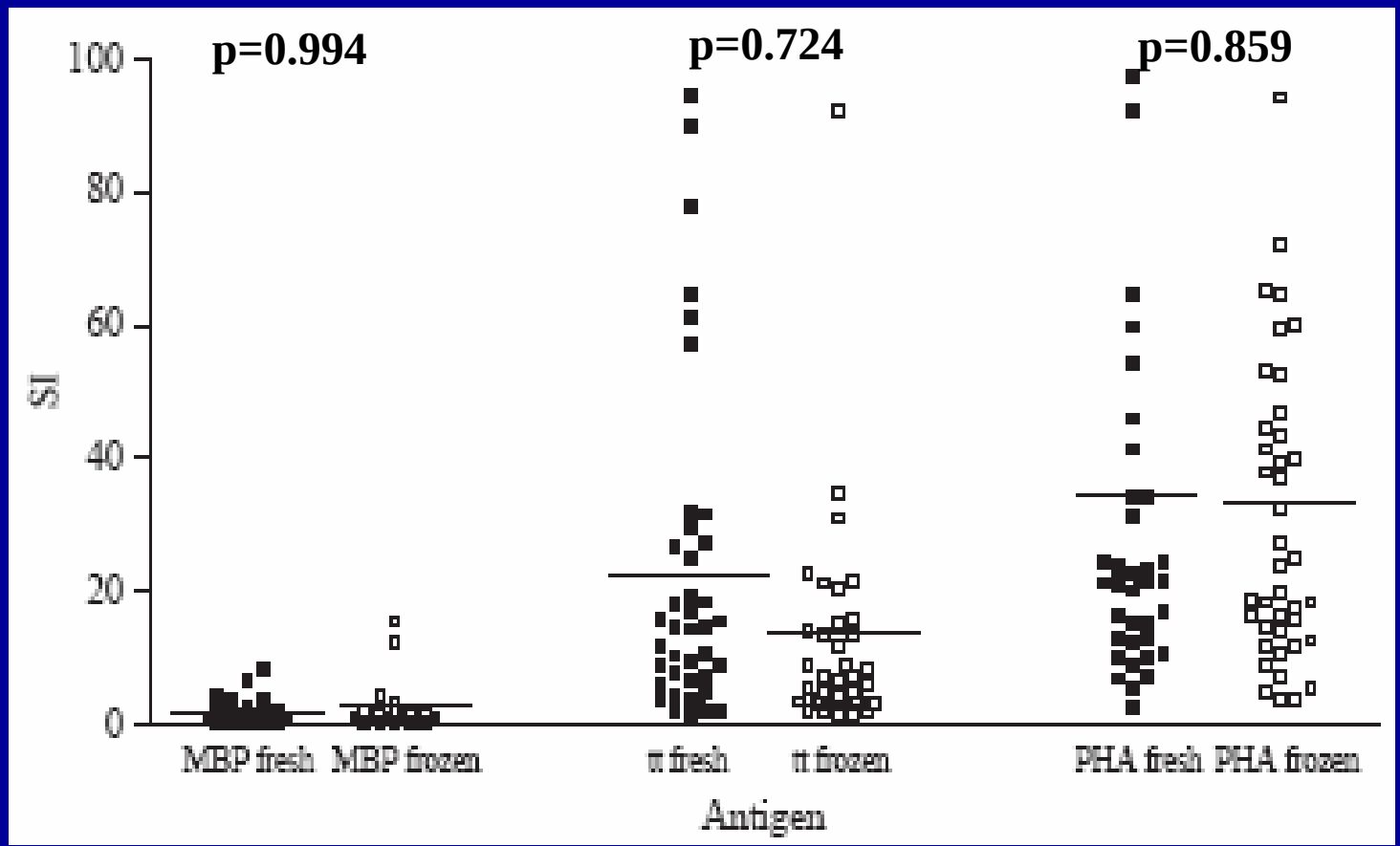




Optimized parameters based on viability

Disis et al, JIM, 2005

Fresh vs. Frozen T Cell Responses using Optimized Cryopreservation Method



Lymphoproliferation Assay

Disis et al, JIM, 2005

Public Access Validated Cryopreservation Protocol

Protocol for Isolation, Cryopreservation, and Thawing of PBMCs

Description

Cryopreserved PBMCs are a common specimen source for studies of immunological responses to vaccines, immunotherapies, etc. The health and viability of cells recovered post-cryopreservation are of course critical to the success and accuracy of immunological assays performed on them. We have developed this protocol to help standardize PBMC isolation and cryopreservation techniques, specifically for the assessment of thawed cells by cytokine flow cytometry.

Cryopreservation of PBMCs

The following protocol for freezing PBMCs uses a final concentration of 10% dimethylsulfoxide (DMSO) and 11.25% protein (human serum albumin) in cRPMI. Cryoprotectants, such as DMSO, reduce the amount of ice present during freezing and reduce solute concentration, thus reducing ionic stress. However, these compounds can themselves cause osmotic injury since they are hypertonic and can cause damage during their addition or removal.

1. Resuspend PBMCs (from **Isolation** section of **Processing of Fresh PBMCs**, above) at 1 x 10^7 viable lymphocytes/ml in 4°C 12.5% HSA in RPMI medium, in a 50 ml conical polypropylene tube.
2. While *gently* swirling the tube, add dropwise enough 4 C 2X freezing medium to double the volume of the cell suspension.
3. Immediately place the tube on ice. Avoid any further mixing or agitation of the cells. Slowly remove the cell suspension into a pipet and dispense 1 ml per cryovial on ice.
4. Place the cryovials in a pre-cooled Mr. Frosty-style freezing container that has been filled with 70% isopropanol according to the manufacturer's instructions. Place the freezing container at –80°C.

Thawing of PBMCs

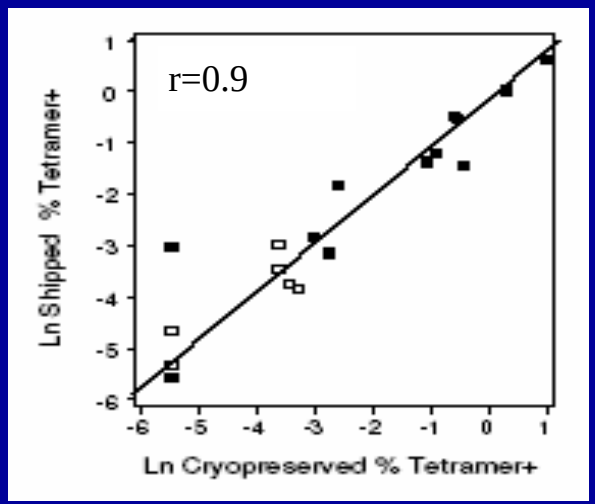
If PBMCs are not thawed properly, viability and cell recovery can be compromised; and cells may not perform optimally in functional assays. In general, cells should be thawed quickly but diluted slowly to remove DMSO. Cells with DMSO intercalated into their membranes are very fragile, and must be pelleted and handled gently.

1. Warm cRPMI to 22°-37°C in a 37°C water bath before beginning thawing procedure.
2. Transfer the cryovial from liquid nitrogen to a 37°C water bath. If liquid nitrogen has seeped into the cryovial, loosen the cap slightly to allow the nitrogen to escape during thawing.

Maecker et al, BMC Immunol, 2005

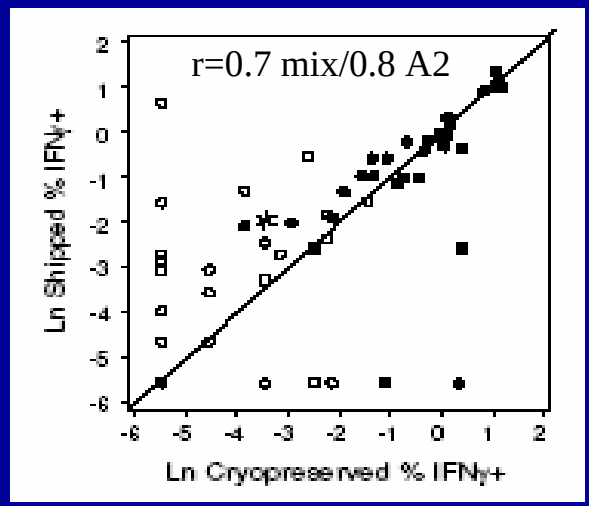
Correlation of Fresh Shipped vs. Frozen Samples

HLA-A2 Tetramer



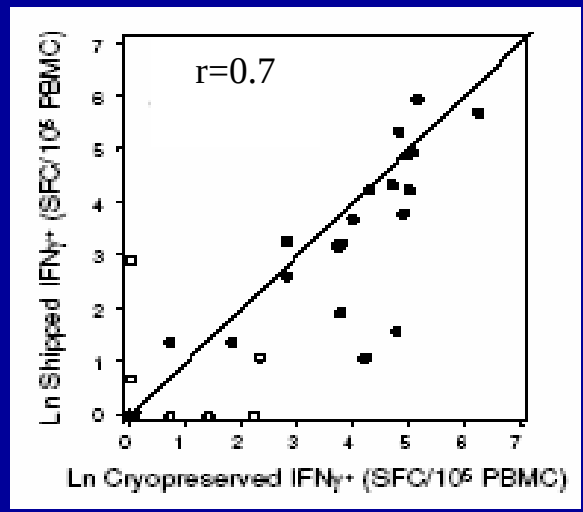
HLA-A2 Peptide

Intracellular Cytokine Staining



HLA-A2 Peptide
Peptide Pool-pp65

ELISPOT



Peptide Pool-pp65

Maecker et al, BMC Immunol, 2005

Intraassay Precision of Optimized Common Assays

CMV response: A2 Peptide
Low, Intermediate, High
Mean of 10-12 runs
6 replicates each

	CFC (A2 peptide)		Tetramer (A2 peptide)		ELISPOT (A2 peptide)	
	Mean	CV	Mean	CV	Mean	CV
L	0.06	0.33	0.06	0.25	8	1.08
I	0.28	0.12	0.25	0.12	31	0.41
H	0.93	0.07	1.27	0.05	28	0.40

Definition of negative set by analysis of 20 CMV- samples

- Reproducibility is compromised at the lower limit of detection
- ELISPOT has highest variability, but sensitivity and specificity were >90%

Maecker et al, 2006

Interassay Precision of Optimized Common Assays

Same donors
Triplicates on 8 different days
1 operator

CFC (A2 peptide)		Tetramer (A2 peptide)		ELISPOT (A2 peptide)		
Mean	CV	Mean	CV	Mean	CV	
0.06	0.57	0.05	0.27	11	1.33	L
0.27	0.17	0.25	0.17	38	0.44	I
0.96	0.12	1.29	0.07	41	0.45	H

- Reproducibility is compromised at the limit of detection
- ELISPOT has highest variability

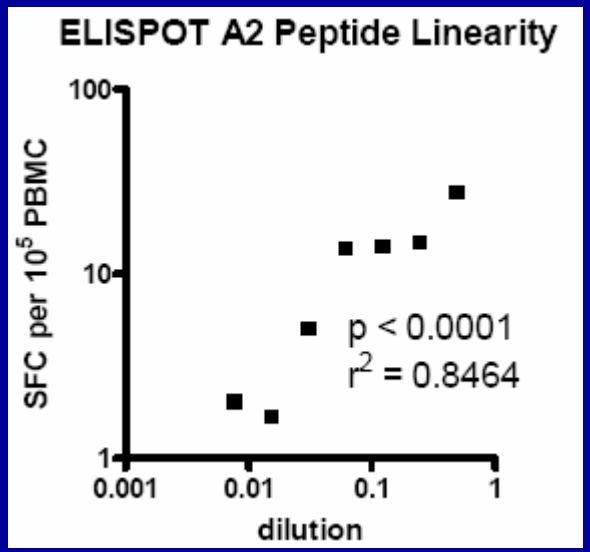
Same donors
Triplicates on same day
3 trained operators

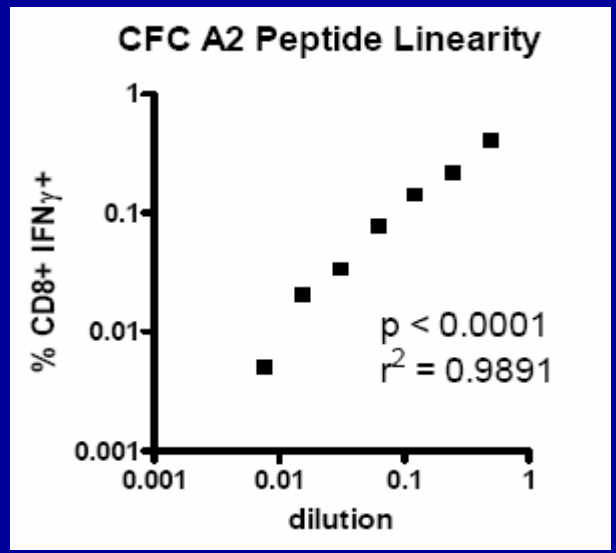
CFC (A2 peptide)		Tetramer (A2 peptide)		ELISPOT (A2 peptide)	
Mean	CV	Mean	CV	Mean	CV
0.05	0.35	0.06	0.30	14	0.77
0.28	0.19	0.28	0.11	51	0.47
0.86	0.13	1.46	0.04	35	1.08

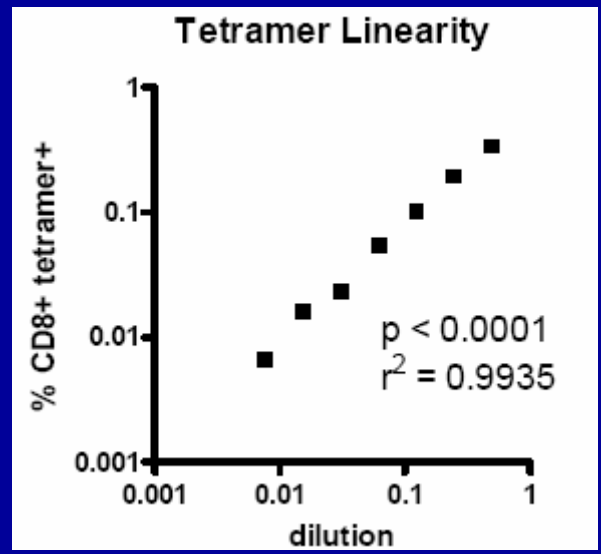
- Well developed training system can limit changes in reproducibility

Maecker et al, 2006

Linearity and Range of CMV Responses



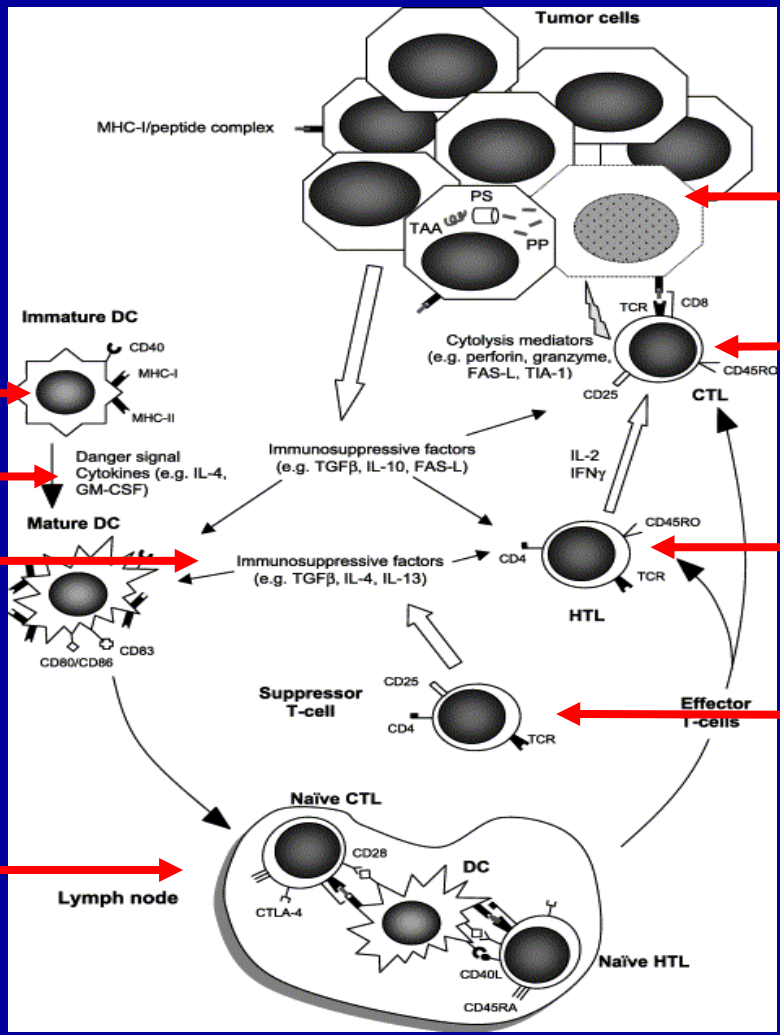




Triplicates of serial dilutions
Donor PBMC high CMV response
(>1%) into CMV-PBMC (no response)

Maecker et al, 2006

Complexity of the Tumor Specific Immune Response



Tumor and stroma

CD8+ T cells

APC

Cytokines

Suppressive factors

CD4+ T cells

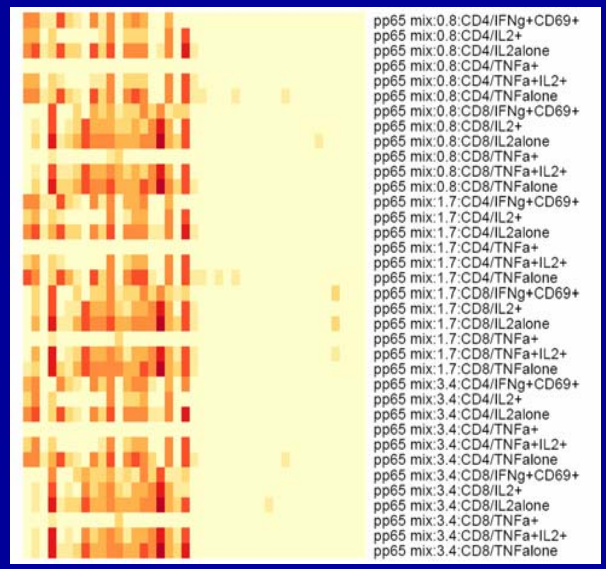
"Other" T cells

Co-stimulation

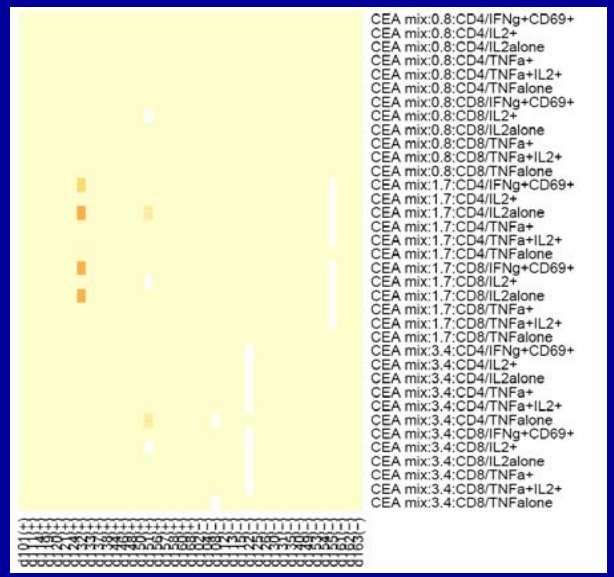
Mocellin, S., et al, Biochim Biophys Acta, 2003

Multiparametric Flow Based Assays

CMV

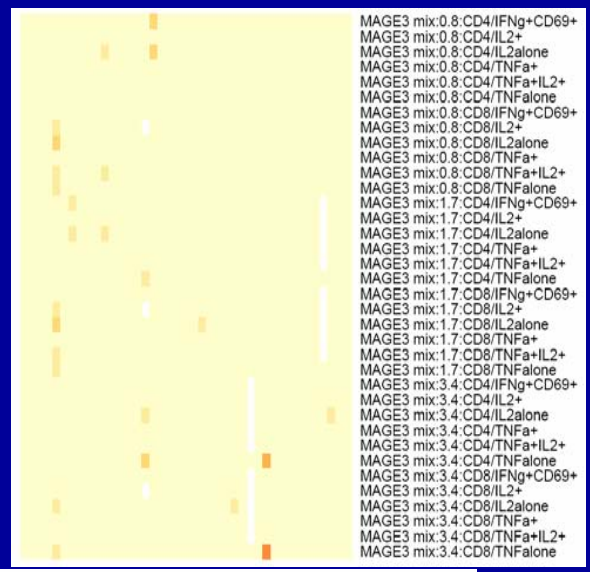


CEA



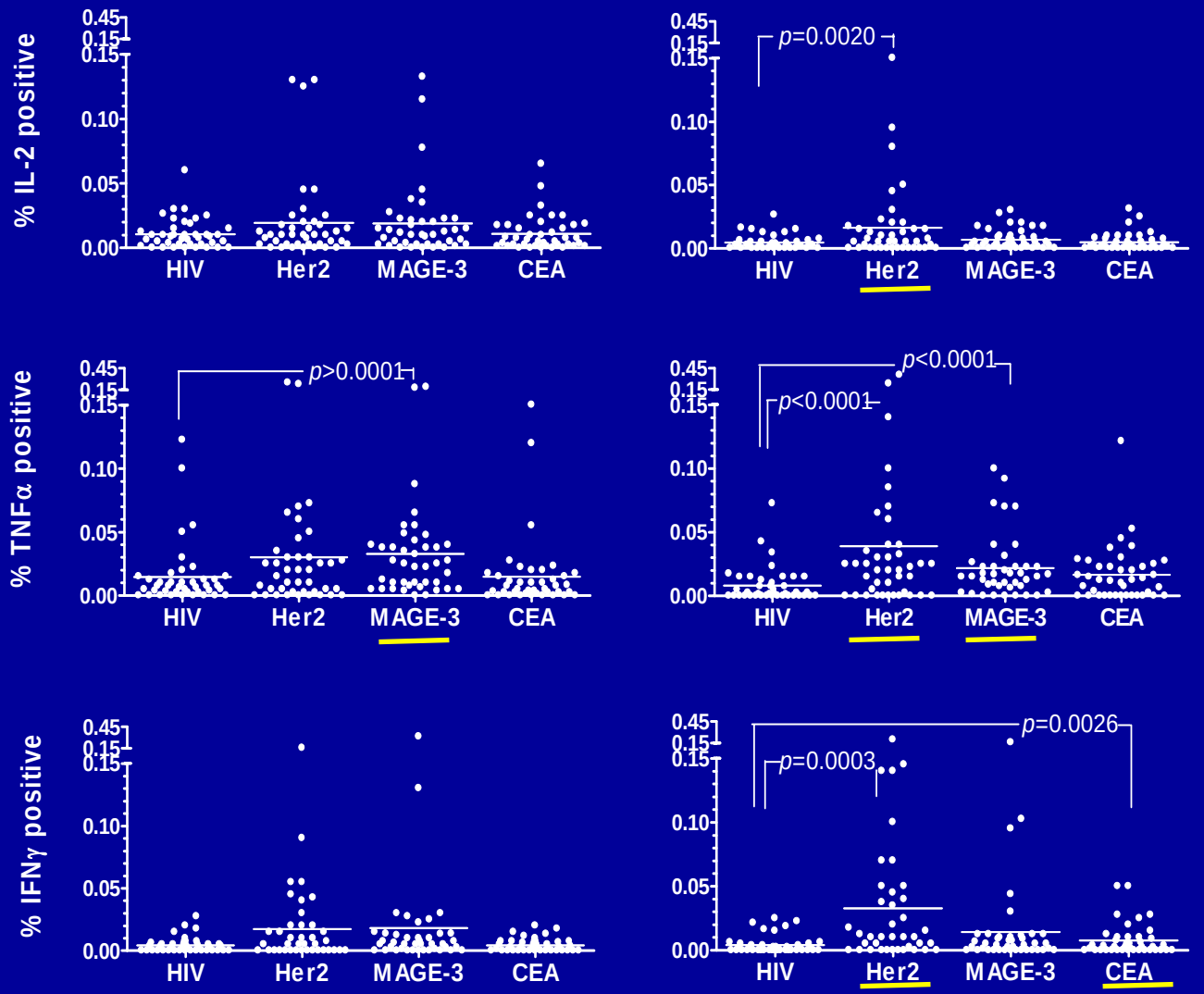
HER2

MAGE3



40 Volunteer Donors

T Cell Immunity to Tumor Antigens in Volunteer Donors



Inokuma M., et al, 2006

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