New Mile Stone of ELISPOT Assay

CTL ImmunoSpot System

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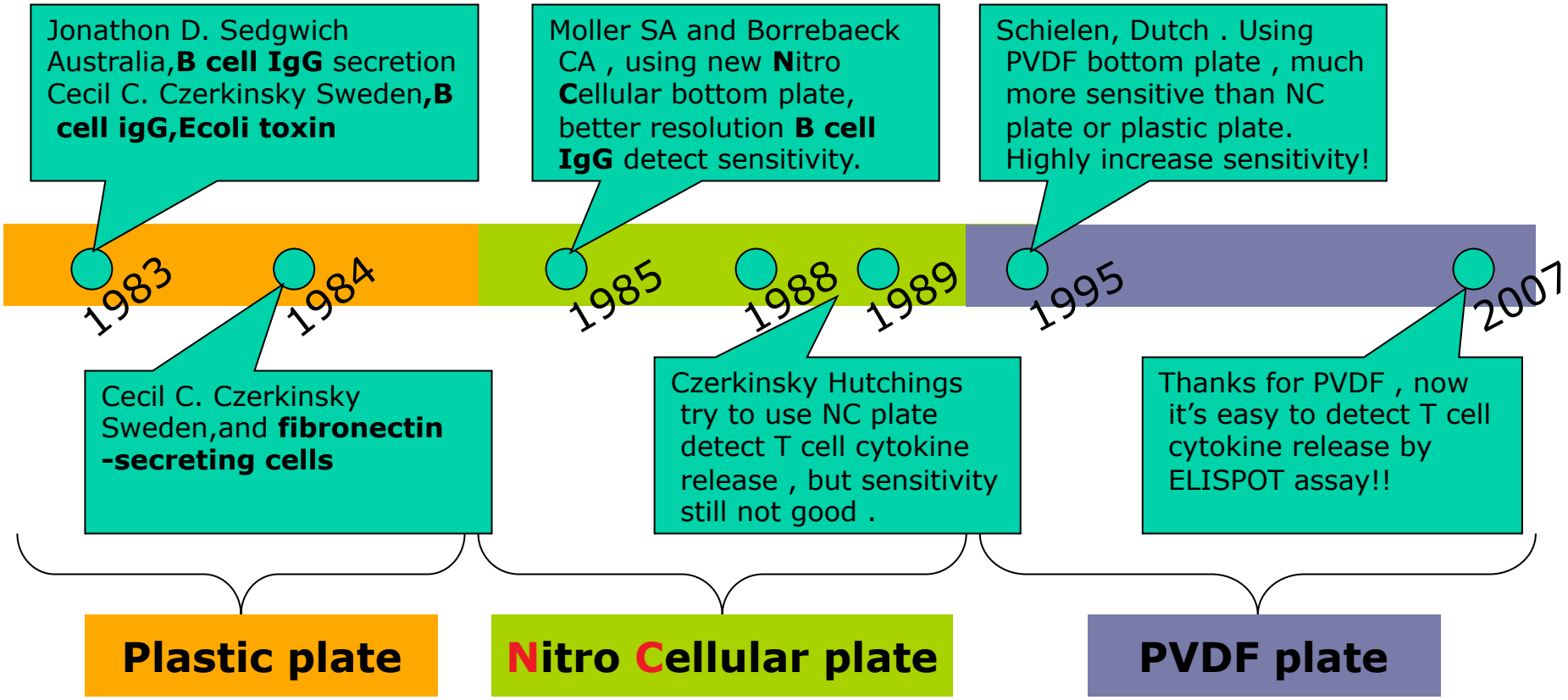
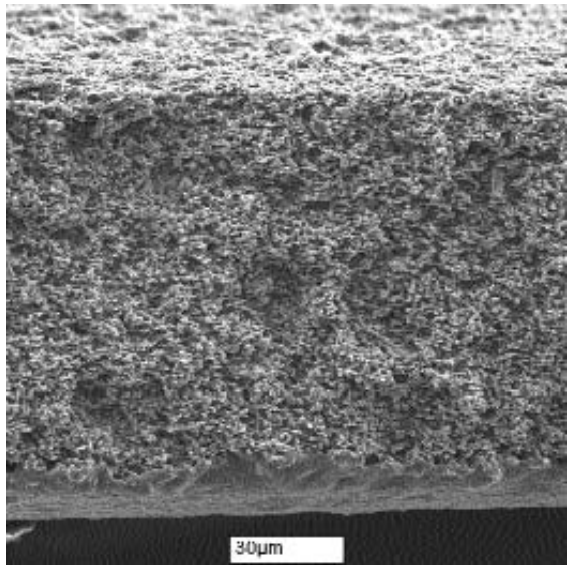
History of ELISPOT

Enzyme-Linked Immunospot = ELISPOT,an old technique base on sandwich ELISA,was used to detect antibody release frequencyof B cell.

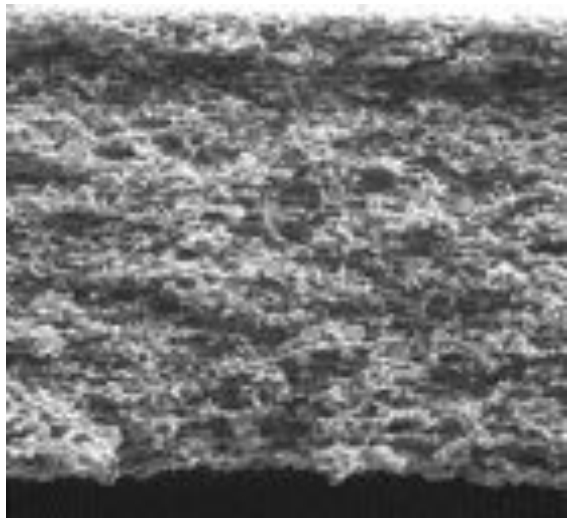


Membrane Attribute	NC (used in ELISPOT) [nominal or average values]	PVDF (used in ELISPOT) [nominal or average values]
Pore Size[a]	0.45 microns (µm)	0.45 microns (µm)
Porosity[b]	70 – 75%	65 – 70%
Thickness	150µm	135µm
B.E.T. Surface Area[c]	6.5m^2/gram	6m^2/gram
Surface Area Ratio[d]	250	350
Saturation Binding Capacity (IgG)	250µg/cm^2	350µg/cm^2
(IgG) Binding Capacity of Top 1µm	2µg	3µg
Wettability	Wettable due to the addition of surfactants or detergents to the membrane during membrane manufacture	Not directly wettable in water. Must be pre-wet with alcohol and then exchanged with water
Additives	Glycerin	None
Solvent Compatibility	Not compatible with methanol or ethanol	Broadly compatible with a wide range of aqueous and organic solvents. Avoid prolonged exposure to strong alkali (e.g., pH >12)
Mechanism of Binding	Electrostatic	Hydrophobic
Things which will interfere with or destabilize binding of anti-cytokine antibodies	Chaotropes (e.g., Tween-20, Triton-X 100, etc.). Water (if never dried), Proteins , especially larger molecular weight proteins	Detergents (e.g., SDS), low polarity solvents (e.g., dimethyl formamide, etc.)
Compatibility with different detection modes	✓ Colorimetric × Fluorescence ✓ Chemiluminescence	✓ Colorimetric ✓ Fluorescence (marginal) ✓ Chemiluminescence

PVDF membrane



NC membrane



Principles of ELISPOT :

<Day1>

Capture Antibody : Coat micro well with anti-cytokine capture antibody

<Day2>

(a)Blocking : Block unoccupied well sites with protein

(b)Add Cells : Incubate cells in well with Ag stimulus etc.

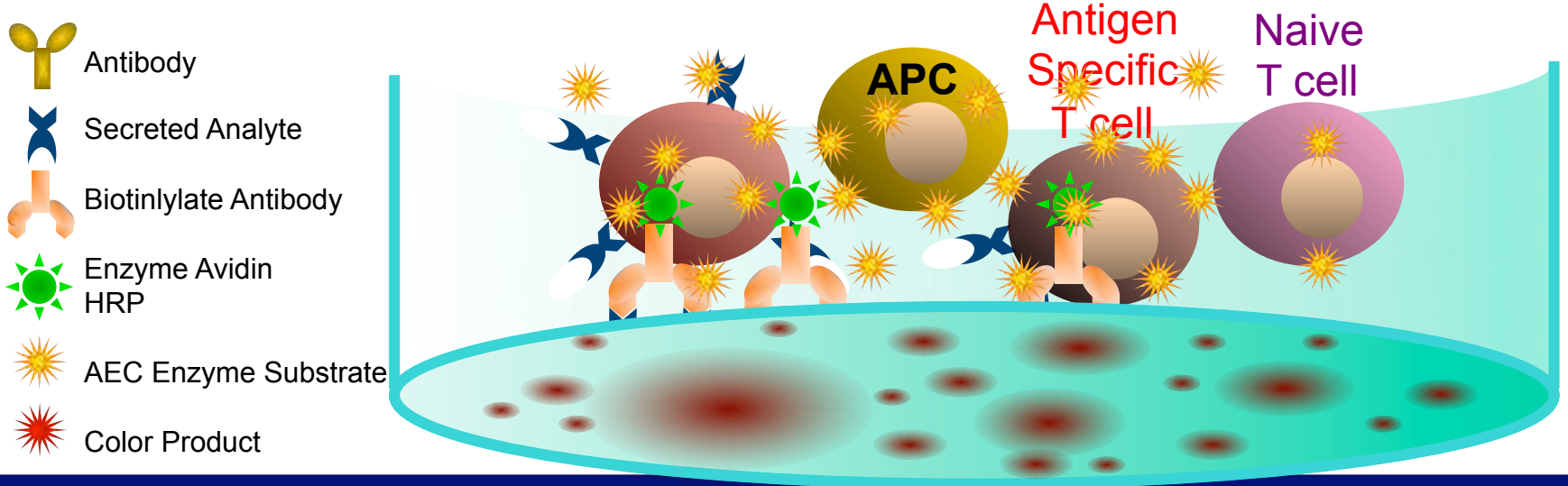
<Day3>

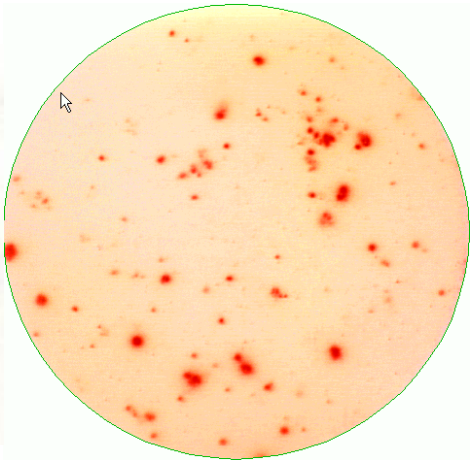
(a)Wash : Cells are washed off

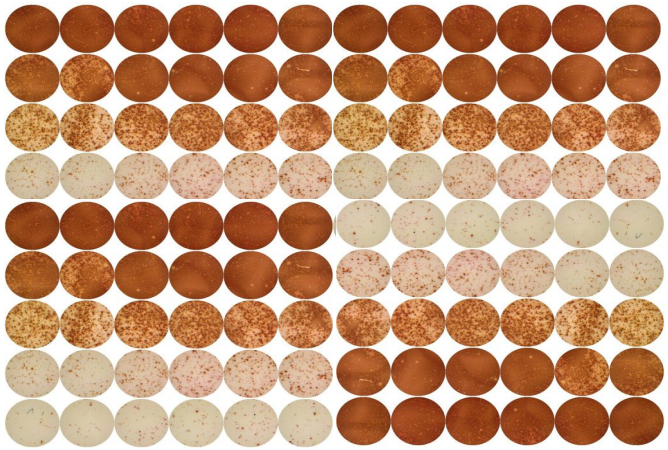
(b)Detection antibody : Add biotinylated anti-cytokine detection antibody

(c)Enzyme-Avidin : Add avidin-HRP

(d)Develop With Substrate : Add substrate and monitor formation of colored spots





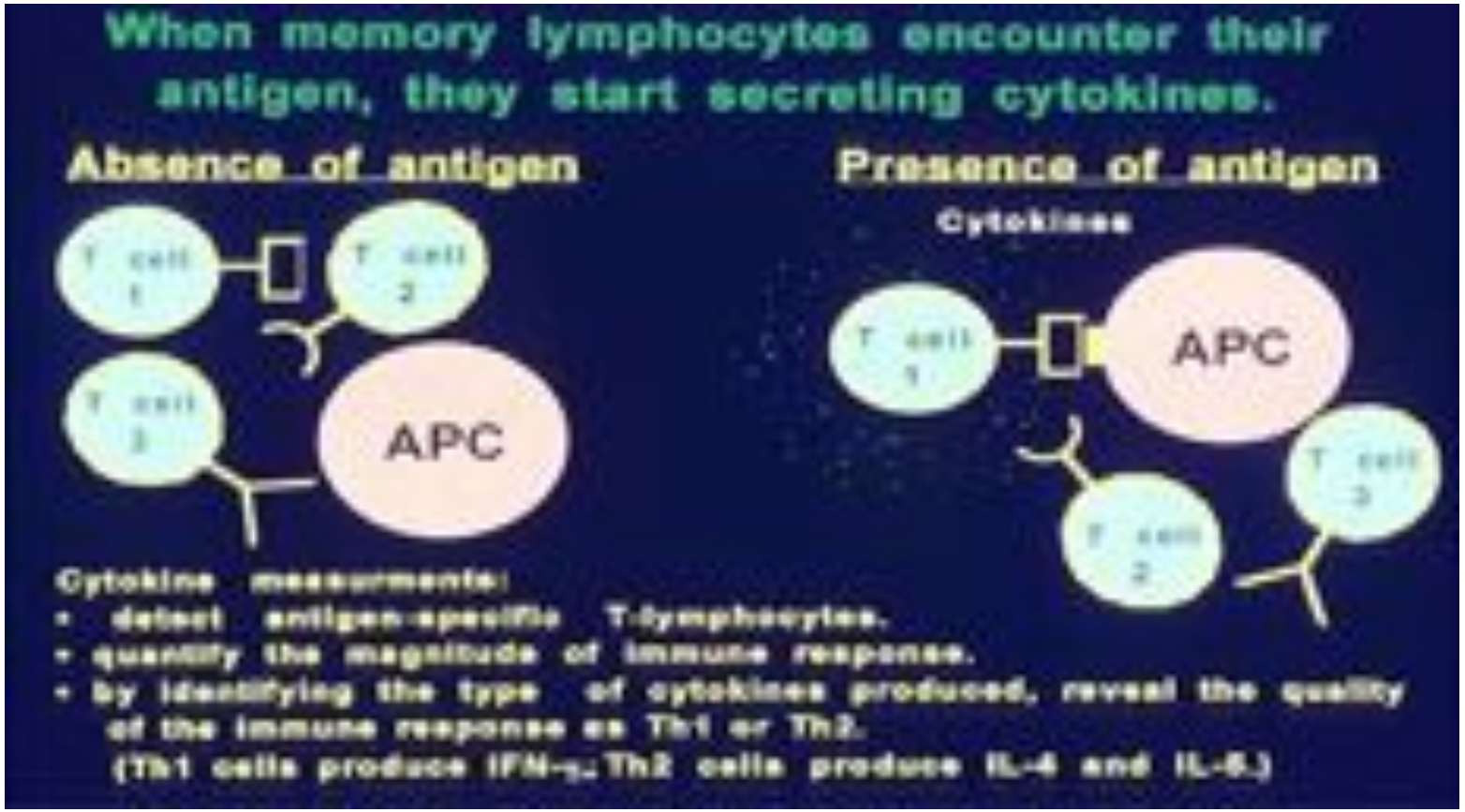


Why ELISPOT?

- The best solution of Ex-vivo T Cell Diagnostic…
- Ultra-High Sensitivity Suit to Detect Ultra-Low Frequency of Antigen Specific T Cell…
- ELISPOT Assays Visualize the Secretory Product of Individual Cells.
- Spot size and morphology directly reflect the secretion of cytokine…
- High throughput and Ease of use
- Low cost instrumentation, reagents and labor are very low cost relative to flow based techniques…

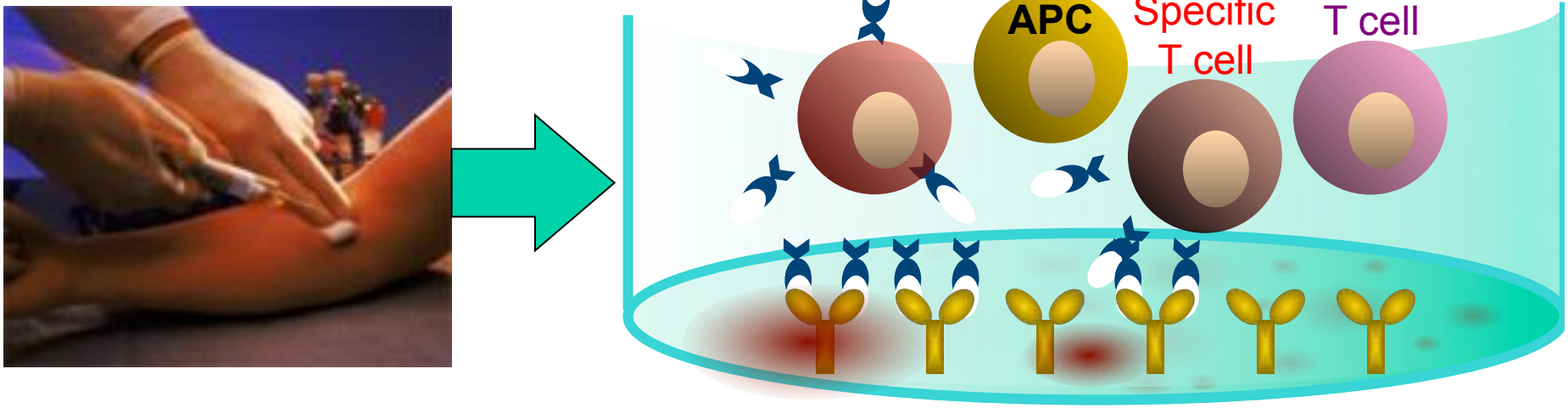
The best solution of Ex-vivo T Cell Diagnostic:

Old way :Using T Cell Cone or Immune-Transfect Mouse to "MIMIC" the T cell respond ,they're only module not close to reality because…..

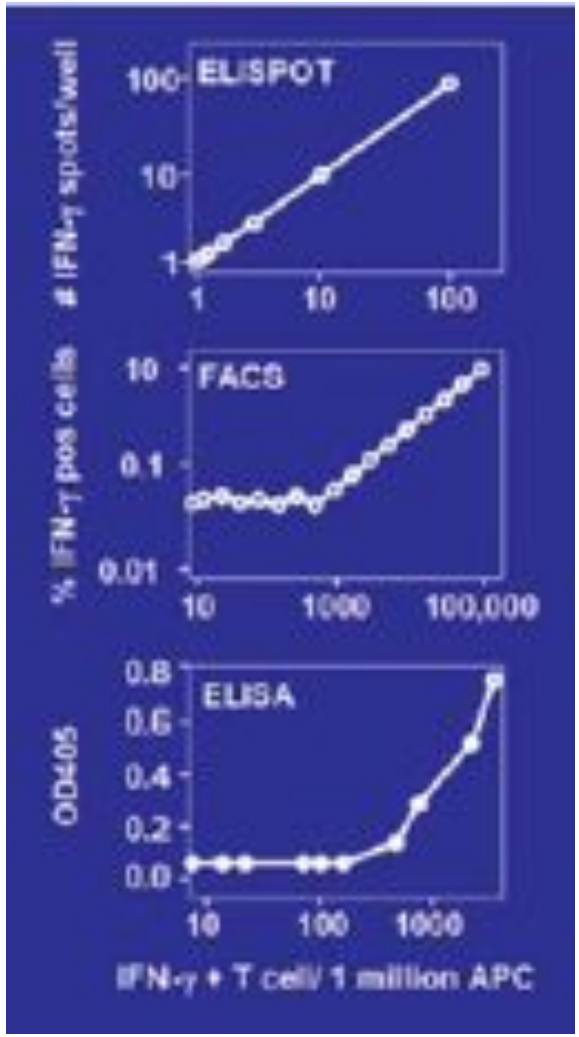


The best solution of Ex-vivo T Cell Diagnostic:

ELISPOT Assay is using fresh (or Cryopreserveed) PBMC as samples , which include whole ammonal T Cell .



Ultra-High Sensitivity Suit to Detect Ultra-Low Frequency of Antigen Specific T Cell

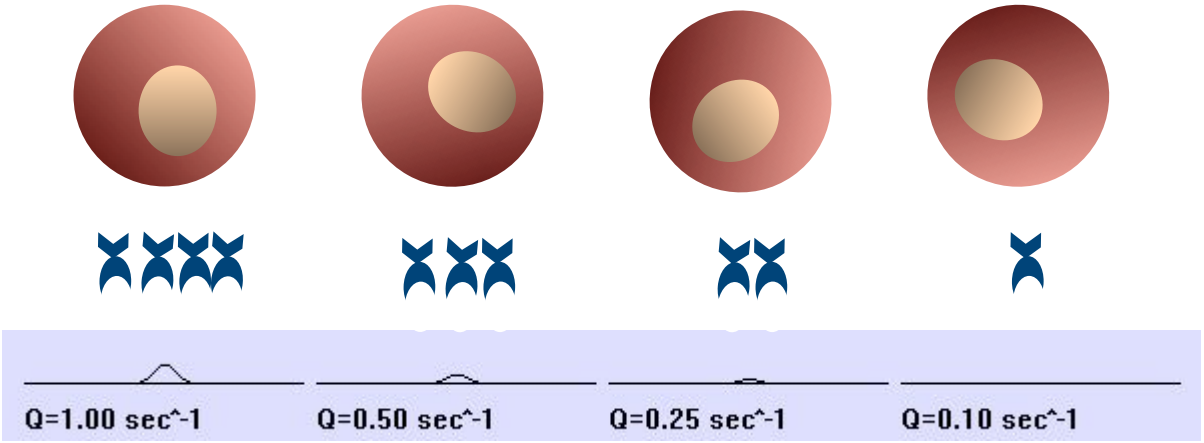


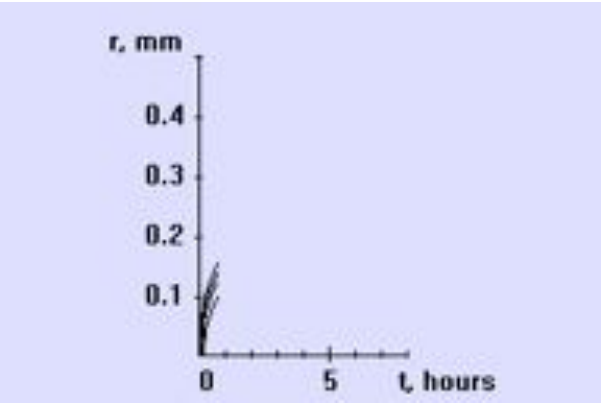
Sensitivity:

ELISPOT permits accurate frequency measurements down to the 1/1,000,000 cell range –

ICS and ELISA (also CBA) reach detection limit at 1:1,000

How does spot morphology reflect productivity?





Competing Techniques vs. ELISPOT

- **1) ELISA (Bulk Assay)**

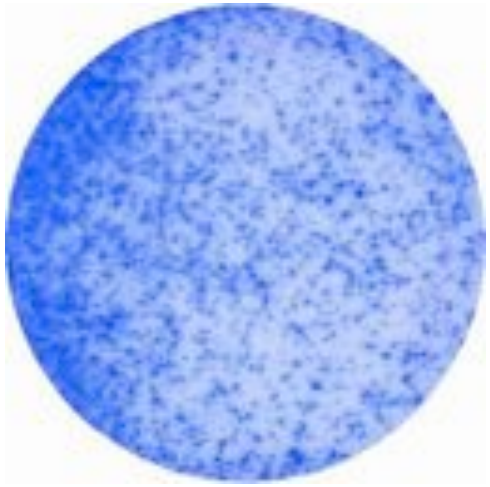
- much less sensitive (200-400 times) which makes it frequently fail for ex vivo T cell measurements

- no frequency information, semi quantitative

- low signal to noise resolution: does not permit to distinguish between many cells making little cytokine (frequently "background" spots in medium control) vs. few cells making lots of cytokine (the relevant T cells in the antigen-induced "foreground").

Strength: low cost, ease of use

Medium (NK cells)



Antigen (T cells)

Competing Technique vs. ELISPOT

- **2) Bead Arrays**

(same weaknesses as ELISA):

- relatively low sensitivity

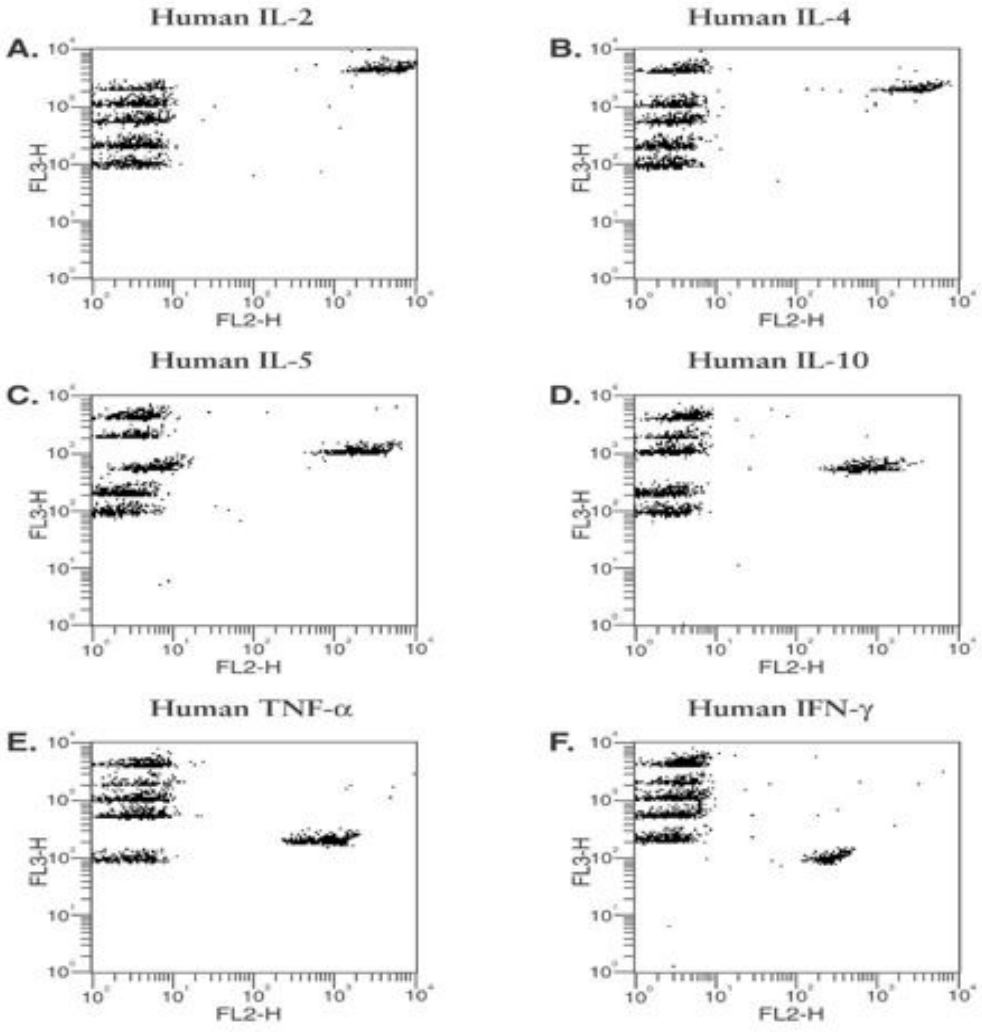
- no frequency information

-low signal to noise resolution

(plus weaknesses of flow cytometry)

- high cost of instrumentation, high maintenance effort and fee.

Strength: simultaneous analysis of multiple cytokines, relative fast analysis vs. other flow based measurements (but still much slower than ELISPOT analysis



Competing Technique vs. ELISPOT

- **3) Tetramers and Pentamer**

-Not suited to measure T cell function: tetramers activate and induce apopotosis in T cells; detect non-functional cells

-Frequent false positive results: temperature dependent unphysiologic multivalent binding

-Unsuited for studies of outbred populations (like humans): MHC polymorphism, polygenism, and variable determinant hierarchy.

-Cumbersome and expensive: specific construct needs to be made for each MHC allele of a donor, each each peptide

-Very slow analysis when low frequency cells are to be detected (many hours, vs. few minutes by ELISPOT for 100 samples)

Strength: Permits studies of cell surface phenotype ex vivo (without reactivating the cell, but only if fixed cells are stained.

Competing Technique vs. ELISPOT

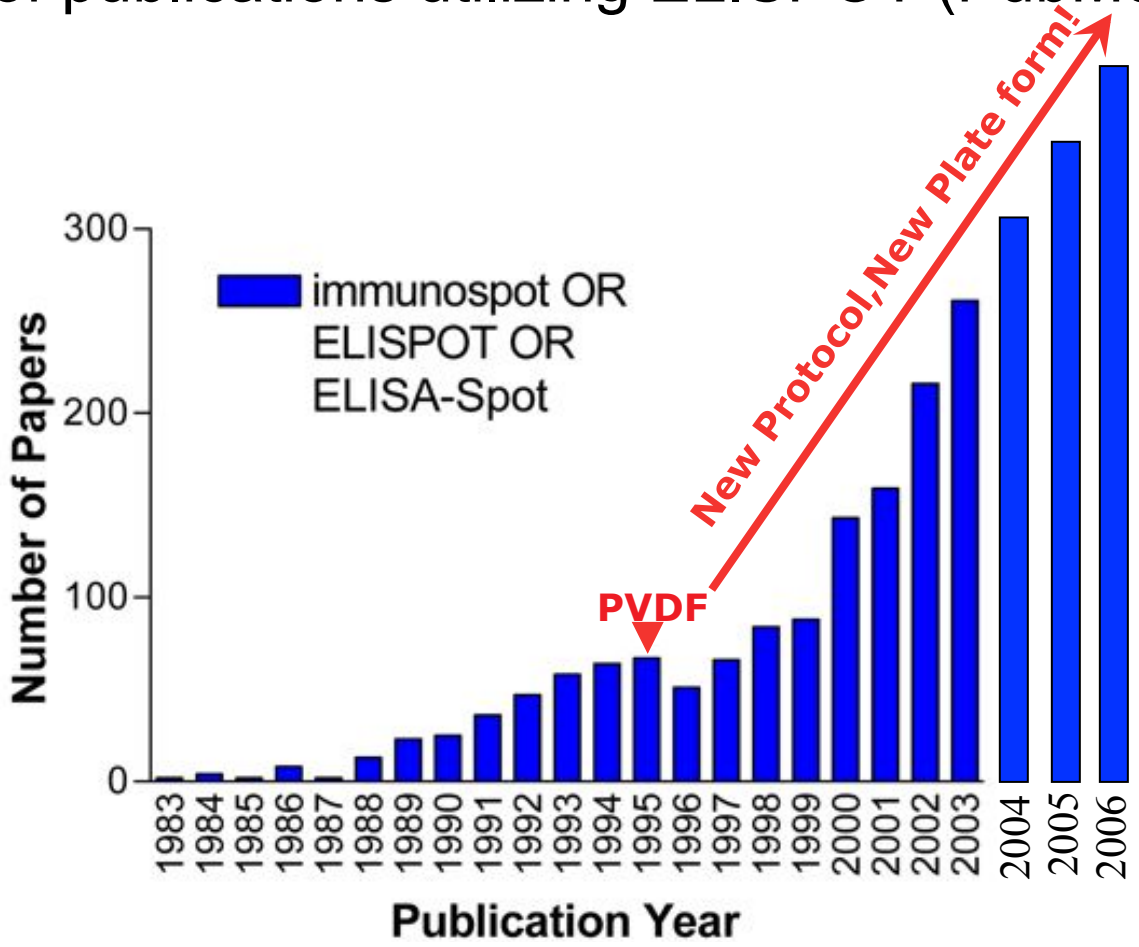
- **4) Intracytoplasmic Staining (ICS)**

- Does not account for post-transcriptional regulation: synthesis of does not mean biologically relevant secretion, e.g., IL-2.
- Does not permit to distinguish between storage and release of protein e.g., Granzyme B and Perforin.
- Does not permit endpoint measurement: e.g., actual killing (unlike Lysispot)
- Pharmacological treatment: altered cell functions vs. the untreated cells in ELISPOT. Cells do not survive analysis: can not be rescued and recultured like in ELISPOT.
- Very long analysis time when it comes to low frequency cells.
- Expensive analysis instrumentation, high maintenance fee.

Strength: permits simultaneous determination of CD4/CD8 lineage (other cell surface markers are uninformative if these are activation dependent since all cytokine expressing cells have been recently activated).

Rapidly growing ELISPOT market size:

Number of publications utilizing ELISPOT (PubMed)



Applications:

- Basic T cell immunology
  - Antigen-Specific Immune response / Exact Measurements of Antigen-Specific T cells Ex Vivo.
- Infectious diseases
- Autoimmunity
  - Measuring Th0/Th1/Th2 transition
- Tumors
  - Measurements of Functional T Cell Avidity
- Hypersensitive
  - Epitope mapping
- Allergies
- Vaccine development

Can we trust ELISPOT assay ?

Standardized Counting

Another important reason is that evaluation of even a pristine result is close to impossible when the spots are counted visually.

At the very heart of science, however,are exact, objective, and reproducible measurements.

Standardization Your ELISPOT Assay

CTL's Serum-Free Media

Serum is the largest variable in ELISPOT assay performance. Therefore CTL has a fine tuned serum-free media portfolio for standardized, high-performance T cell monitoring with PBMC:

- CTL Test™
- CTL Wash™
- CTL Cryo™
- CTL Anti-Aggregate™

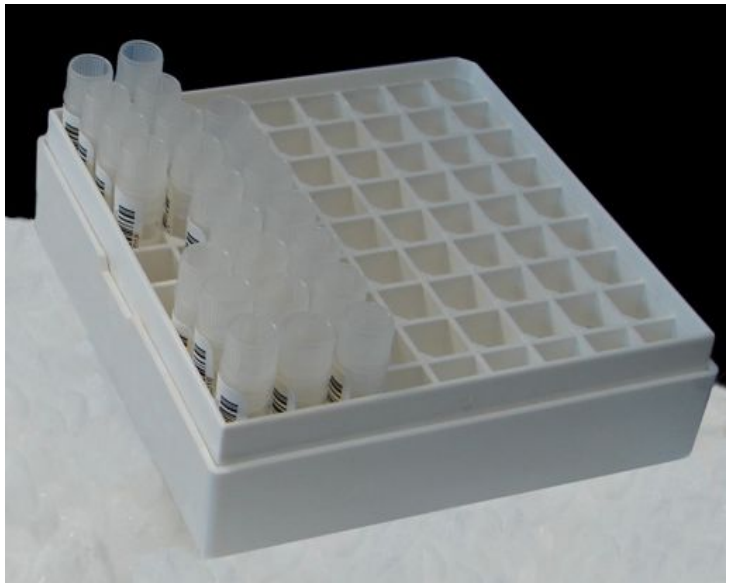
"Performs better than serum"

Standardization Your ELISPOT Assay

CTL's Cryopreserved PBMC

- PBMC frozen serum-free for >90% viability at thawing, with >95% functionality in ELISPOT and cytokine recall assays vs. the fresh PBMC.
  - Uncharacterized PBMC*
  - Characterized PBMC
  - HLA-typed: at low and high resolution*
    - Immunotyped: characterized for antigen reactivity*
  - Custom characterization available*

**Large donor libraries available for selection!*



"Why do it the cumbersome and irreproducible way?"

Standardization Your ELISPOT Assay

CTL's CEF Peptide Pools:

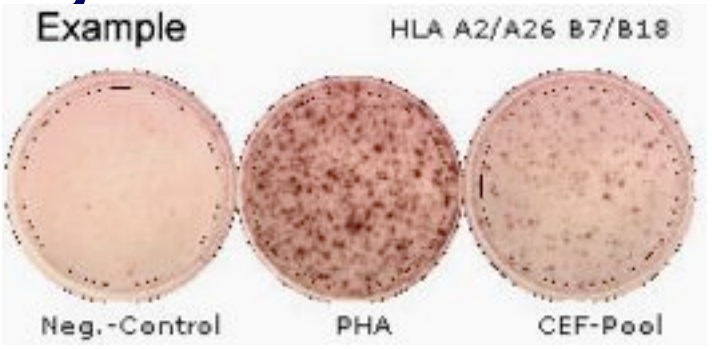
Peptides of CMV, EBV and Flu virus to stimulate cytokine release in peptide-specific CD8 memory cells.

- 23 CEF Class I Peptide Pool

The "classic" ***23 peptide*** pool that covers determinants restricted by ***11 HLA class I alleles.***

- 32 CEF Class I Peptide Pool

The extended peptide pool consisting of ***32 peptides*** that cover ***15 HLA class I alleles.***



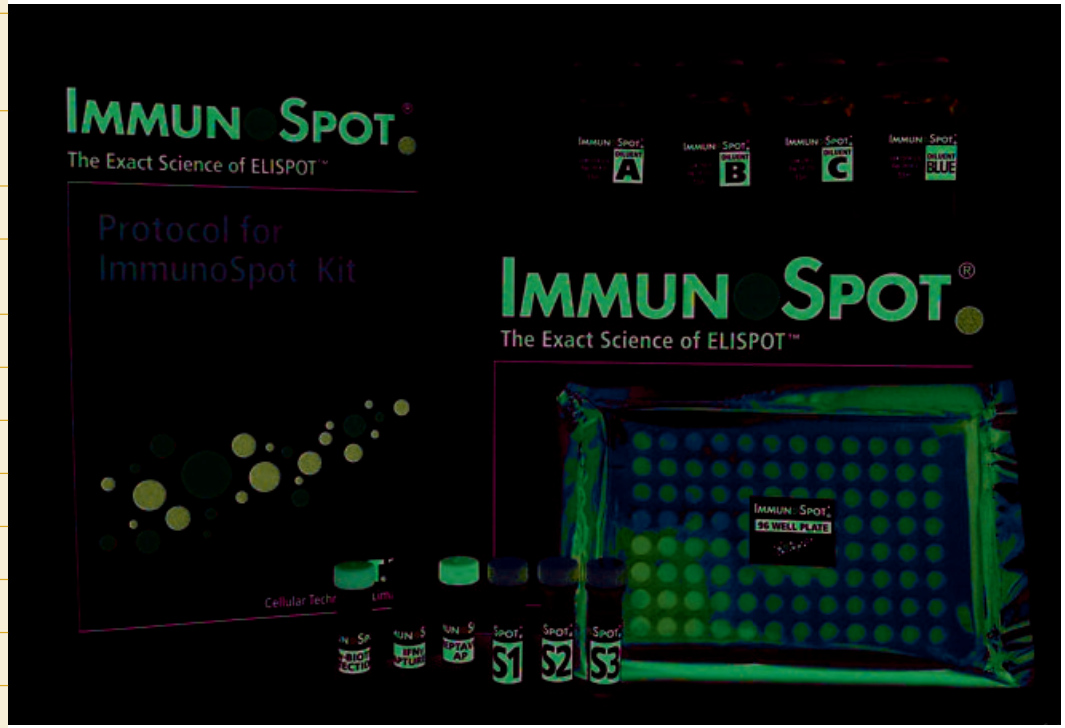
"The ultimate positive control for CD8 cell function in PBMC"

Standardization Your ELISPOT Assay

ELISPOT Kit

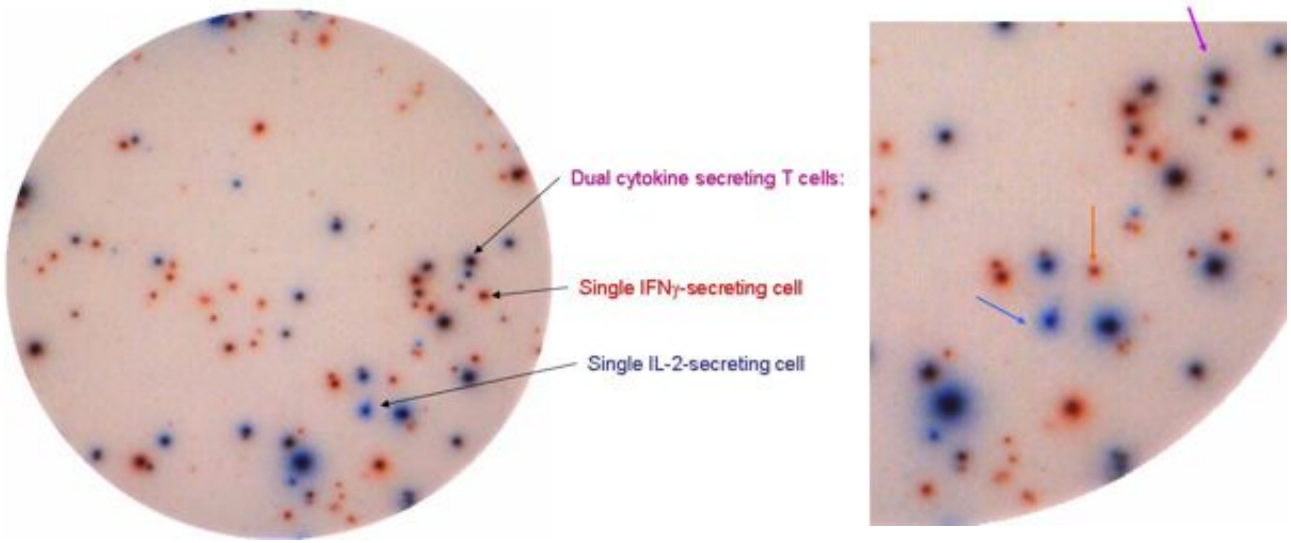
ImmunoSpot® Kits vs. the Competition*

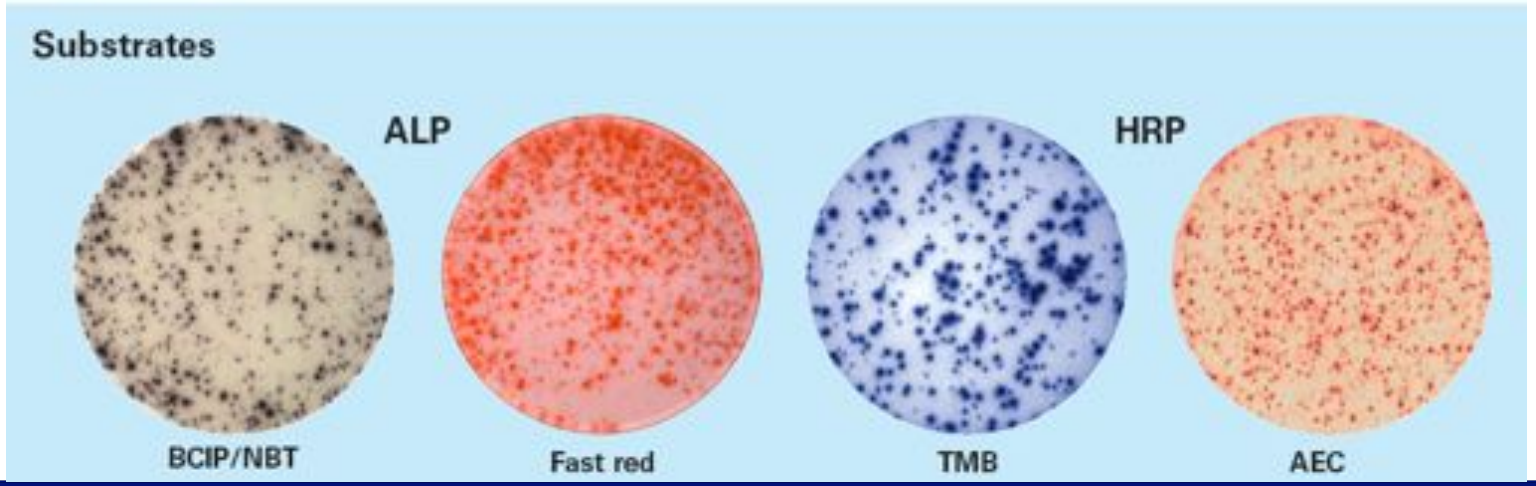
Features and Benefits	ImmunoSpot®	Competition
Ease of Standardization Assay will provide the same test results when performed by the same or different individuals.	✔	?
Sterile Plates Avoids false-negative or false-positive wells. Sterile plates come standard in all ImmunoSpot® Kits. Competition charges a substantial premium for this specialty item.	✔	For a Premium
Serum-free Medium Included Special medium that has been developed for low-background and high-signal performance in ELISPOT assays.	✔	No
All Buffers Included Adds convenience, saves time and expense.	✔	No
No Prewetting with Alcohol Avoids leaking wells, saves 5 washing steps, and false-negative wells if alcohol gets trapped in underdrain.	✔	?
No Blocking Saves time and effort.	✔	?
Fixed Substrate Development Time Standardizable spot numbers.	✔	No
No Potential for Substrate Overdevelopment Low background, clear spot separation, precise gating.	✔	No
Total Number of Washing Steps Saves time and effort.	11	Over 20
Plate Scanning Available Get started immediately, even without an analyzer.	✔	?
Reference PBMC Available Facilitates assay development, qualification, and validation.	✔	No
Wet Lab Training Available Obtain hands-on experience, network, consult with experts.	✔	No
Assay Consultation Available CTL's experts help you reach your goal faster and safer.	✔	?



New Application of ELISPOT:Dual Color ELISPOT

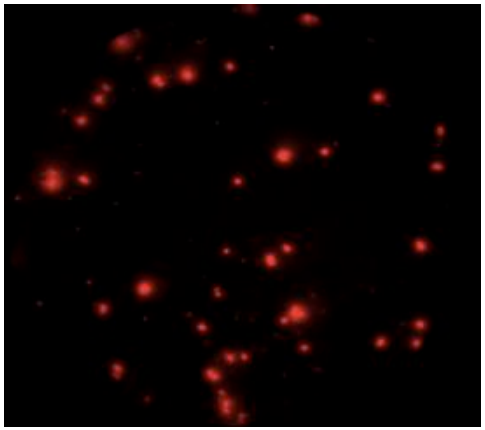
Figure 1A

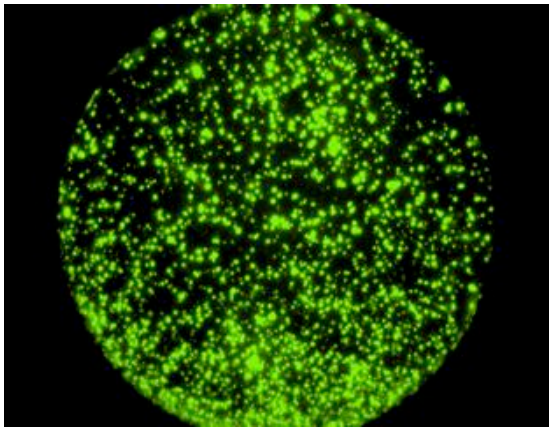




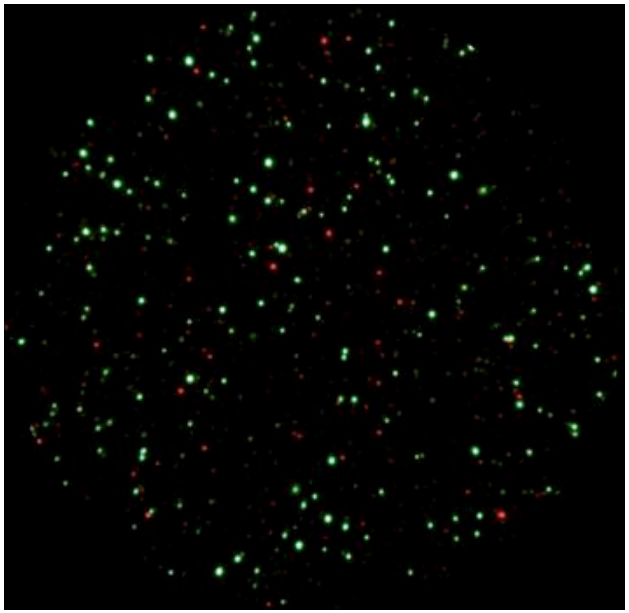
More Application… BioSpot®

Fluorescence-based ELISPOT and Cell Counting :





Examples of FluoroSpot Cytokine Assays



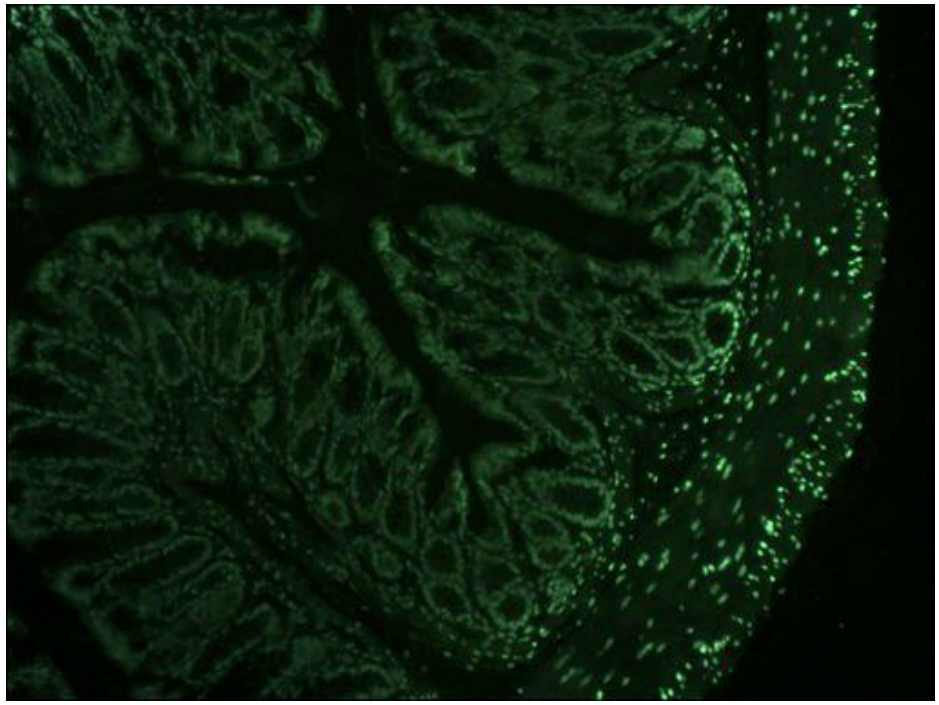
Example of In Vivo Killing Assay (CFSC /PKH-26)

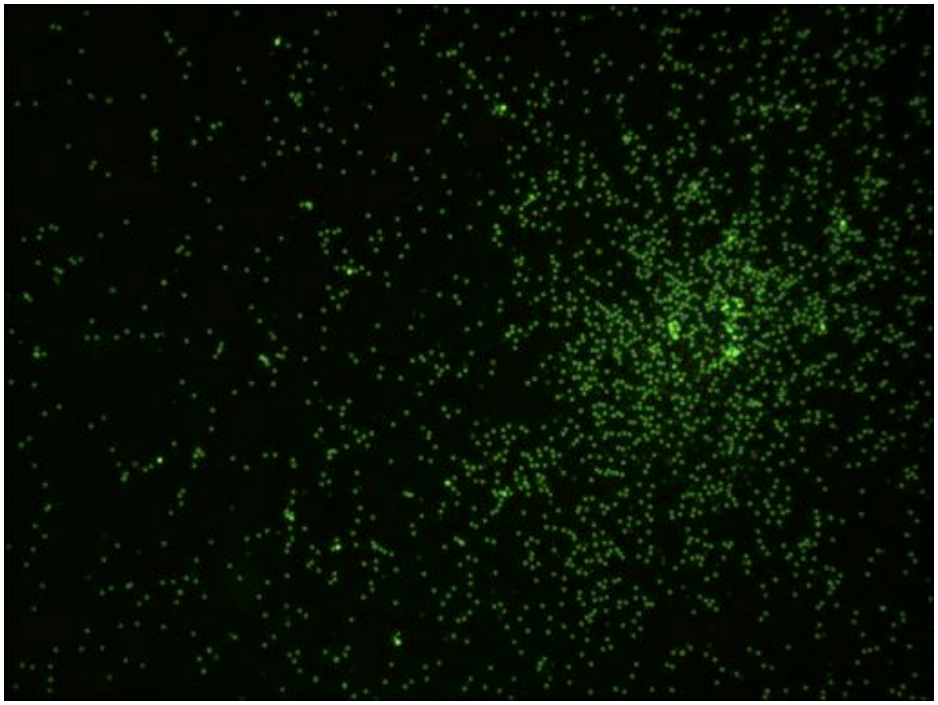
New function of ELISPOT Reader : BioSpot® Counting

- **Fluorescence-based Cell Counting :**
  - Fluorescence ELISPOT assay
  - Live/ dead/apoptotic cell counting
  - Nuclear counting
- **Colony Assay**
  - Microbial colony counting
  - Clonogenic Assay
  - Viral Plaque Assay
  - Genotoxic Assays
  - Stem Cell Assay

More Application… BioSpot®

FluoroSpot nuclear counting:



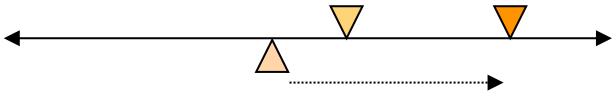


More Application… BioSpot®

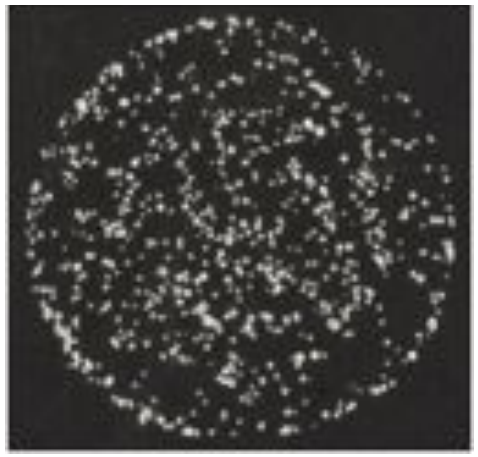
Microbial Colony Counting

Back light:
Detect ~100um spot

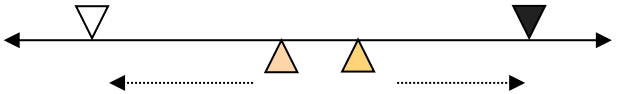
Backlighting



SmartLux Plus™:
Detect <25um spot



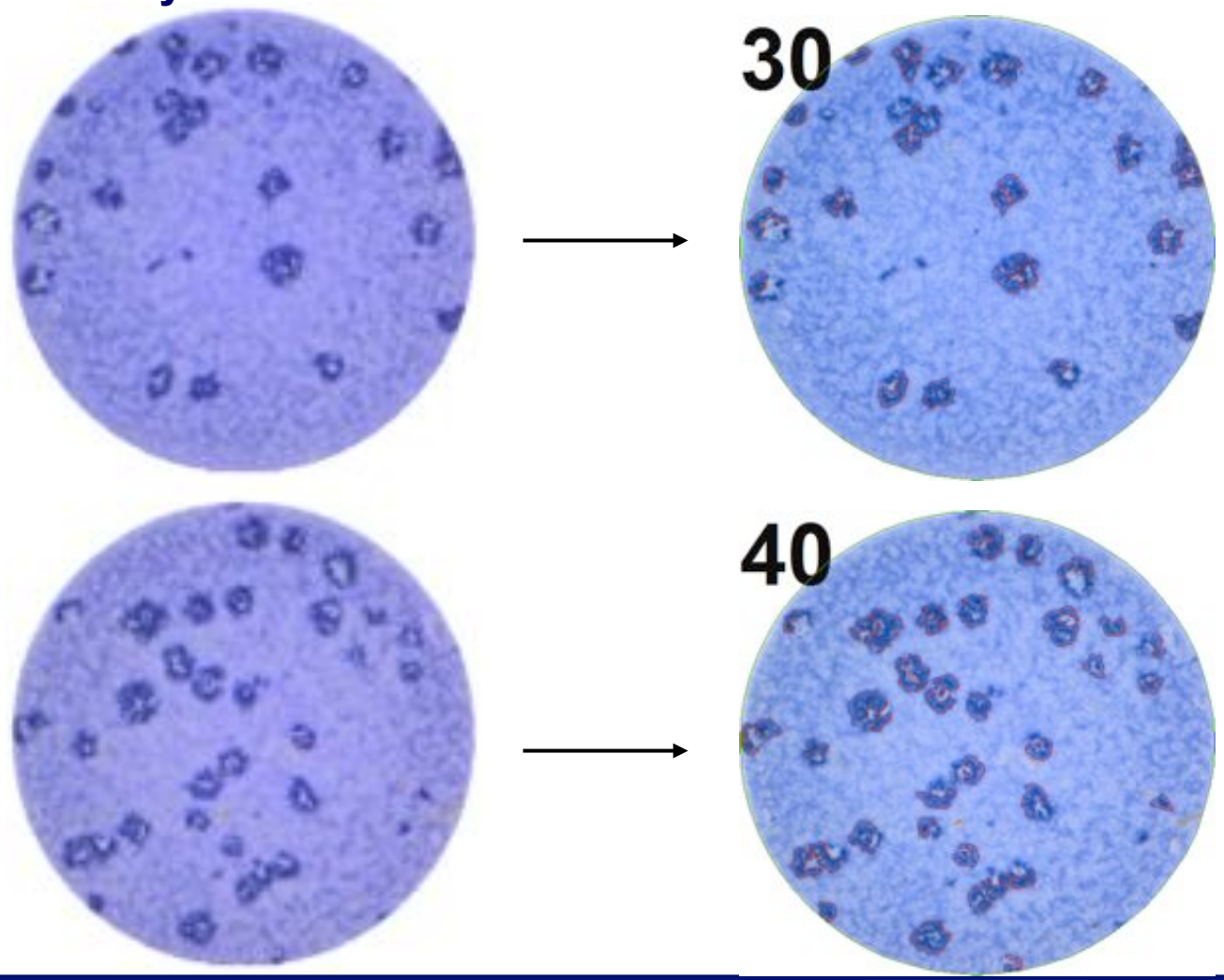
Darkfield



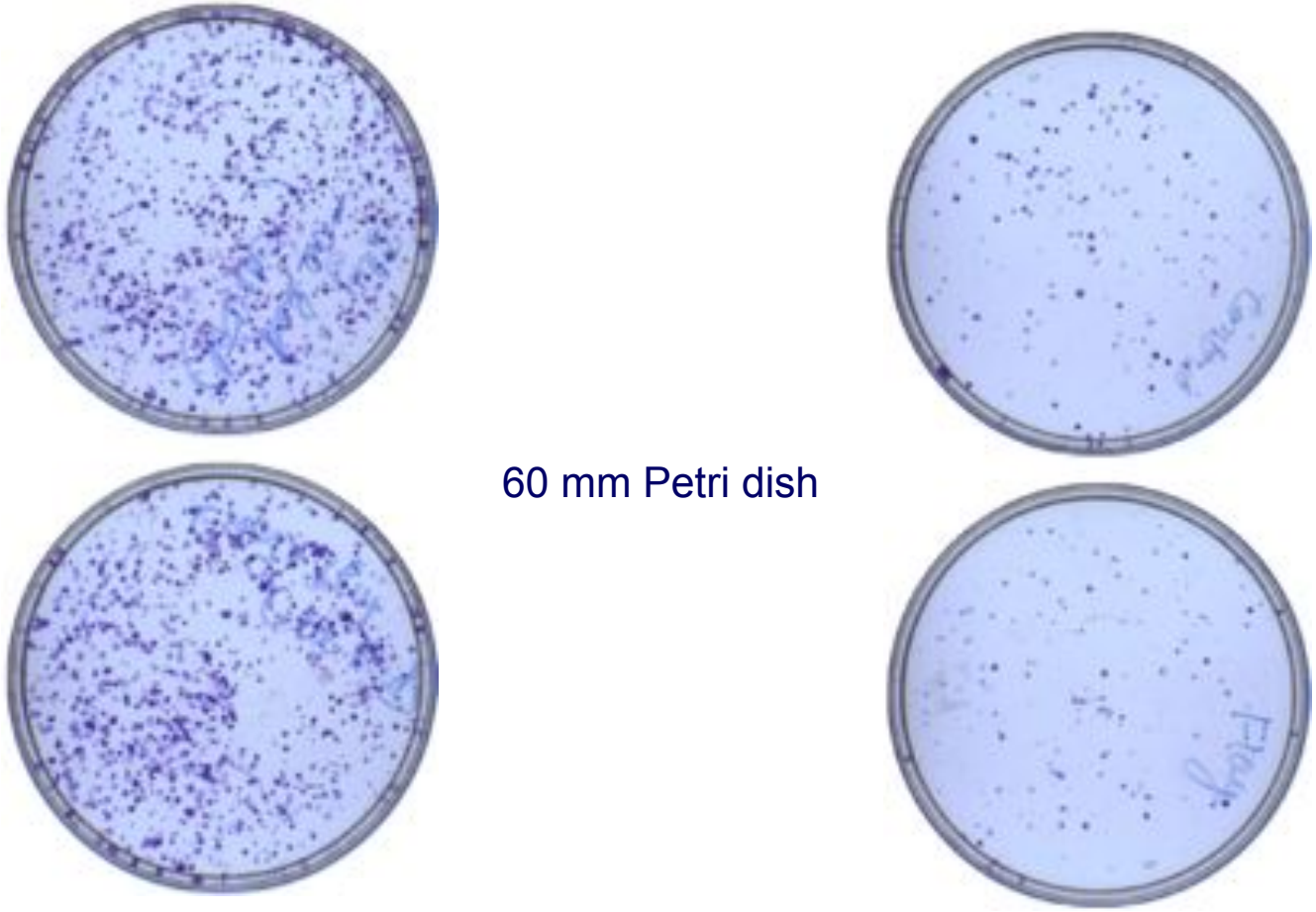
Maximum Contrast

More Application… BioSpot®

Viral Plaque Assay

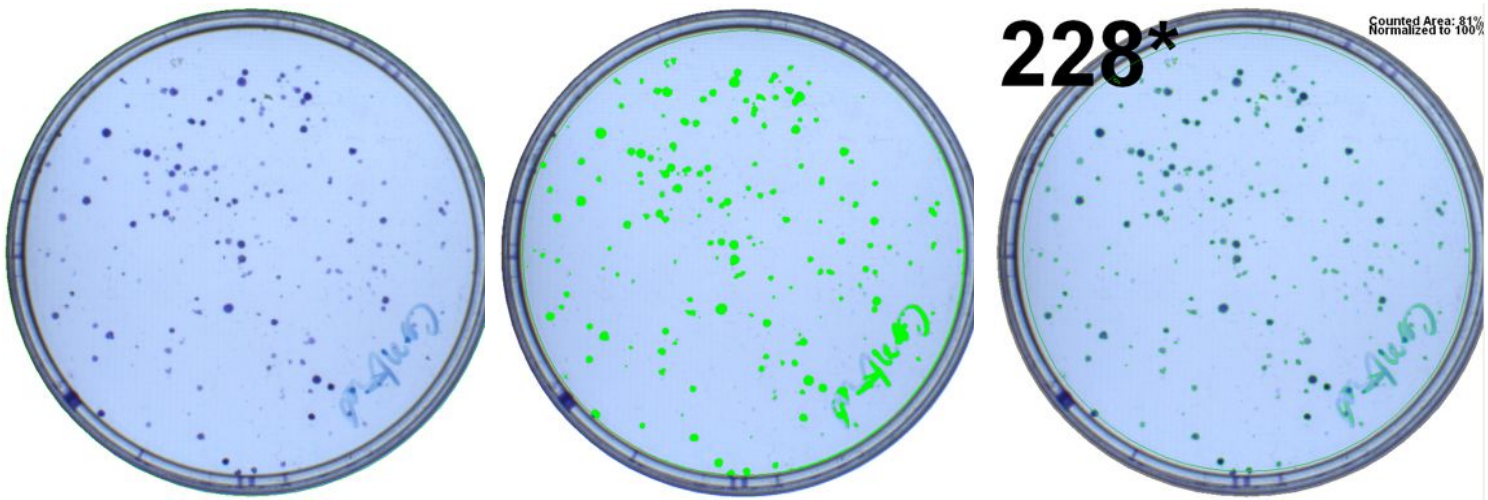


Clonogenic Assay



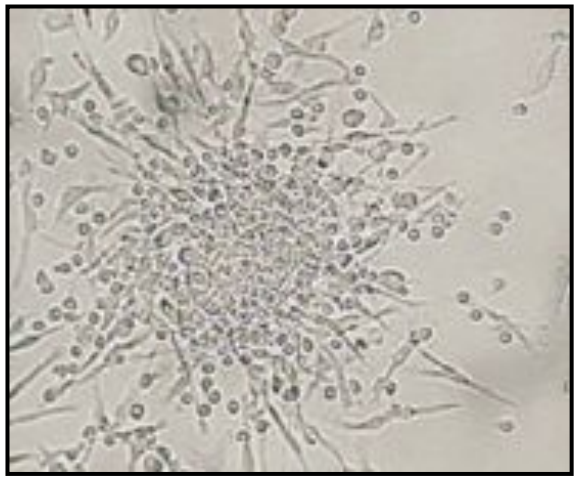
60 mm Petri dish

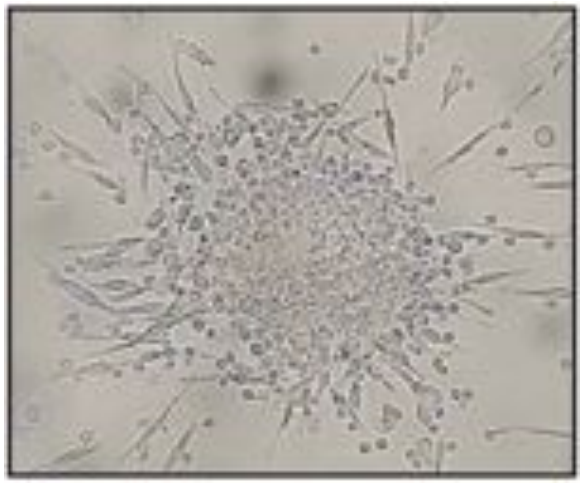
Size Gating Identifies Viable Colonies



Stem Cell Assay:

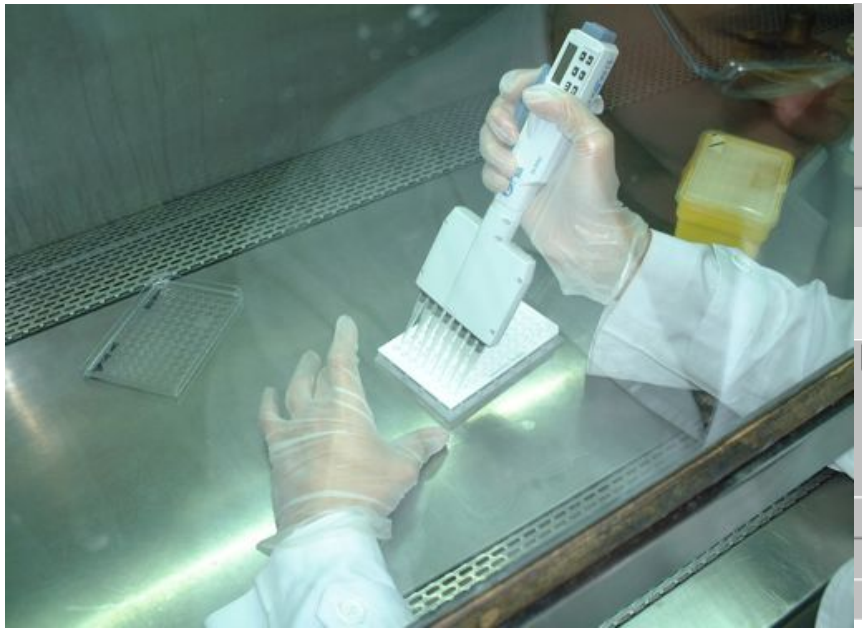
- Identifies multipotent progenitor cells from bone marrow, cord blood and peripheral blood.
  - CFU-EC endothelial cell progenitor
  - CFU-GM granulocyte and macrophage progenitor
  - BFU-E erythroid cell progenitor
- Progenitor cell colonies are identified by microscopic analysis.
  - Tedious and time consuming.
- Automated visualization and enumeration of stained colonies enables high throughput.





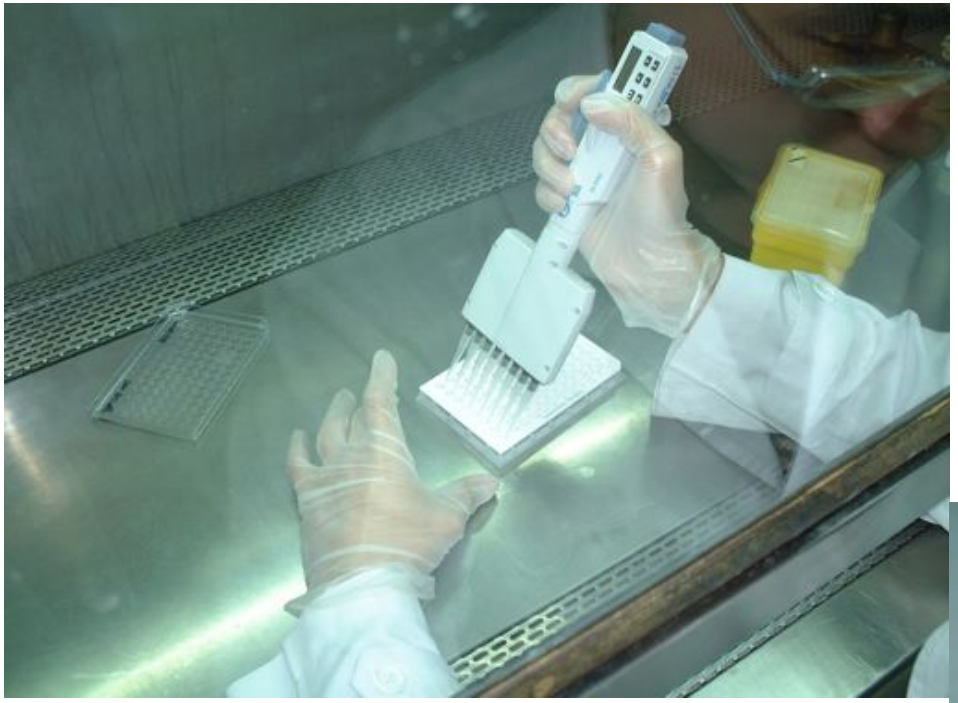
Detect INF-r Cytokine Secretion of Antigen Specific CD8+T Cell Immune Respond by ELISPOT Assay ♪

Pre-wet PVDF Membrane



Pre-Wetting Condition	Bound Antibody (µg/well) Average ± Std. Dev.
No Pre-Wetting	0.04 ± 0.001
15µL 35% MeOH	0.91 ± 0.011
15µL 70% MeOH	0.89 ± 0.016

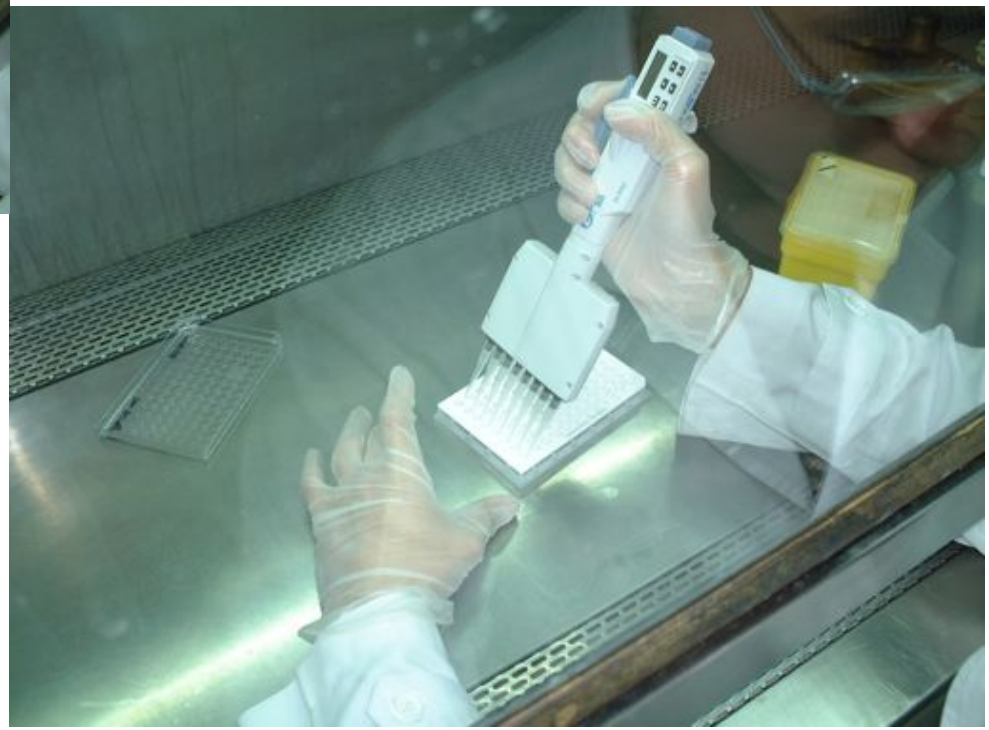
Experiment Number	Pre-Wet Spot Number Mean ± Std. Dev*.	Non Pre-Wet Spot Number Mean ± Std. Dev*.	Non Pre-Wet As A Percent of Pre-Wet
1	606 ± 46	413 ± 37	68%
2	577 ± 37	416 ± 34	72%
3	604 ± 35	440 ± 42	73%
4	609 ± 40	391 ± 28	64%



Coat micro well with
anti- INF-rcapture antibody

Reactivate at 4℃ overnight

Blocking with
Complete Medium



Reactivate 2hr at RT

Incubate ***2-5×10^5*** PBMC in Well with Ag stimulus (5 ng/ml PMA and 500 ng/ml Ionomycin)

Setup plate layout

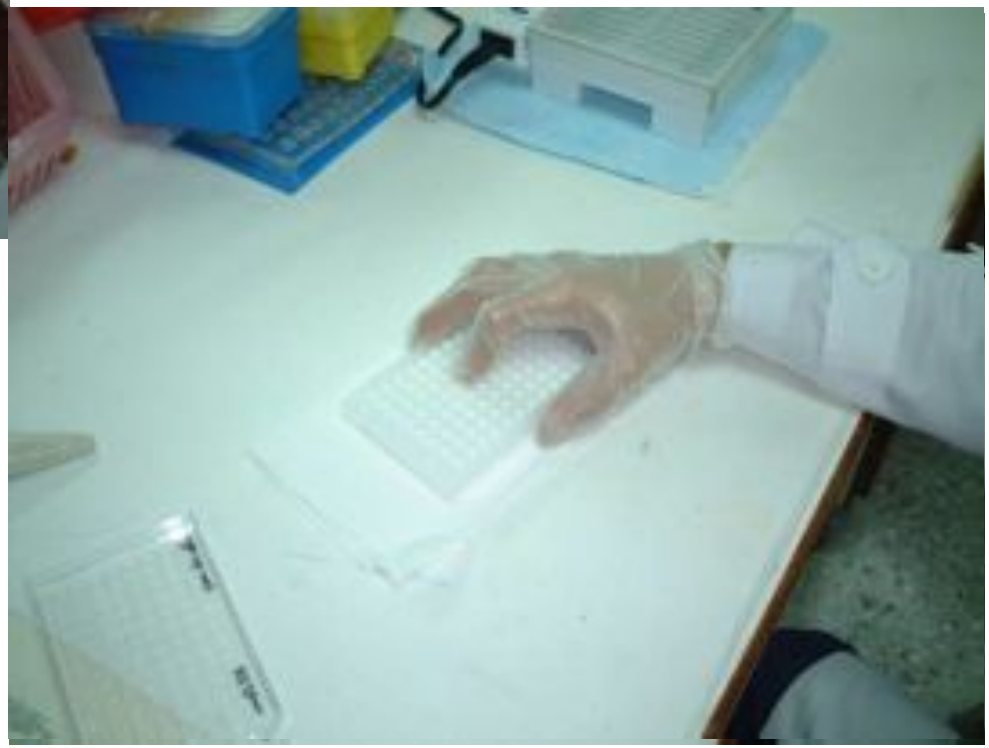
Plate Layout

	Stimulated, 1 X10E6 c/ml				Non-Stimulated							
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:1				1:1							
B	1:3				1:3							
C	1:9				1:9							
D	1:27				1:27							
E	1:81				1:81							
F	1:243				1:243							
G	1:729				1:729							
H	No Cells	No Cells	No Cells	No Cells	No Cells	No Cells	No Cells	No Cells				

Reactivate in incubator
for 12~16hr

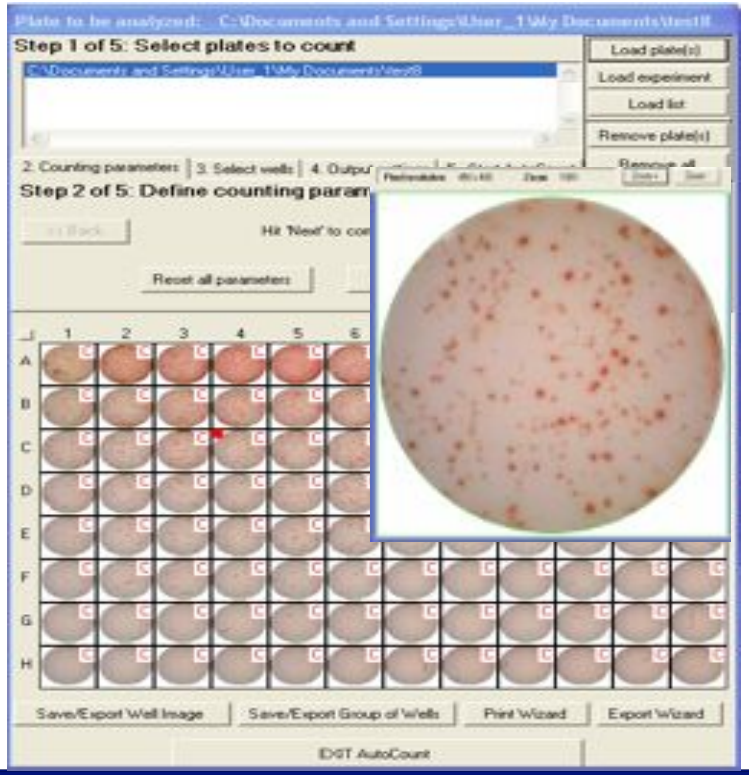
Note it! DO NOT MOVE
PLATE WHEN STIMULATION

Remove cell
(or collect for other experiment)



Rinse by ddWater 3 mins~Twice
Remove Water by shaking.

Analyze plate by Immunospot



- Add biotinylated anti-INF-r detection antibody
- Add SAv-HRP
- Add AECsubstrate and monitor formation of colored spots

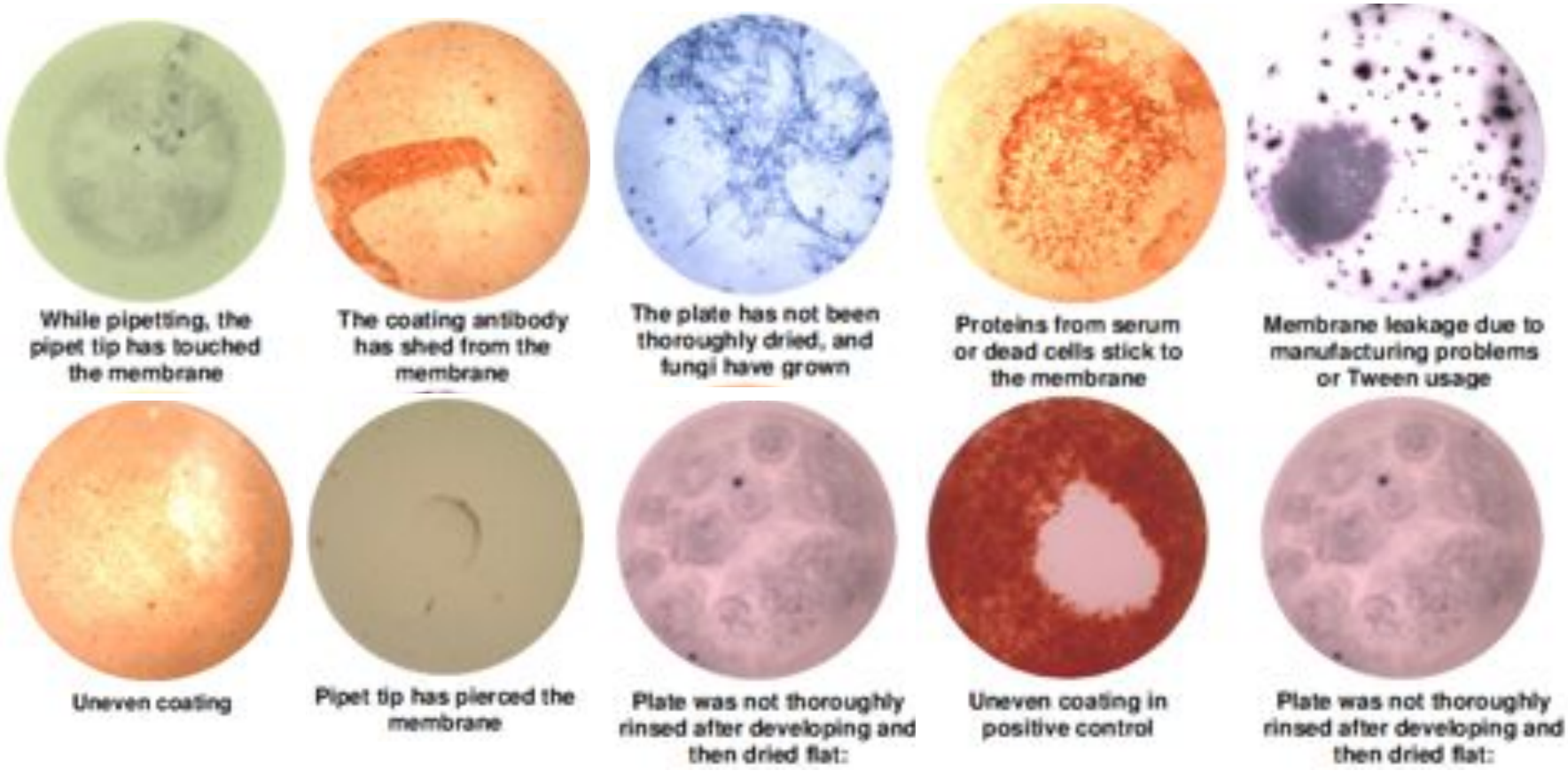
Comparison of Substrates for ELISpot

Substrate	Format	Enzyme	Color Development	Sensitivity
TrueBlue	1-C	HRP	Blue	√√√√
BCIP/NBT	1-C	AP	Dark Purple	√√
AEC	2-C	HRP	Red	√√

C= Component √=Least sensitive √√√√=Most Sensitive

IMMUNOSPOT®

Artifact Image Library



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