

**COST CLINIMARK TRAINING SCHOOL**  
**Approaches for Biomarker Discovery and Validation**  
**September 23<sup>rd</sup> to 27<sup>th</sup> 2019**  
**Spetses, Greece**

# Biomarker clinical implementation

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CA16113 - CliniMARK

**eatris**

European infrastructure  
for translational medicine

**TRANS  
COLON  
CAN**



**cost**  
EUROPEAN COOPERATION  
IN SCIENCE AND TECHNOLOGY

# HUGTiP-IGTP Barcelona



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# Clinical diagnostic kits



## REFERENCE VALUES

**Range** of values present in 95% of the “healthy” population

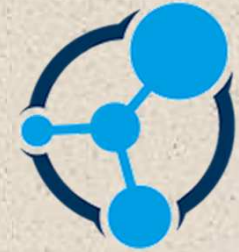
**EFFECTIVENESS:** capability of a given biomarker to identify/support the presence or absence of a given disease

### ➤ **Four categories of results:**

- ✓ True positive / True negative
- ✓ False positive / False negative

### ➤ **Four indexes:**

- Sensitivity
- Especificity
- Positive predictive Value (PPV)
- Negative Predictive Value (NPV)



	Presence of Disease	Absence of disease	
Positive Test	True positive (TP)	False Positive (FP)	total positive
Negative Test	False negative (FN)	True negative (TN)	total negative
	total with disease	Total without disease	

- **Sensitivity (S):** Proportion of patients with the disease that give a positive result. **Ability to detect patients**  
$$S = TP / (TP + FN)$$
- **Specificity (E):** Proportion of individuals without the disease that give a negative result. **Ability to detect healthy**  
$$E = VN / (VN + FP)$$
- **Positive predictive value (PPV):** probability that a subject with a positive result has the disease  
$$PPV = TP / (TP + FP)$$
- **Negative predictive value (NPV):** probability that a subject with a negative result doesn't have the disease  
$$NPV = TN / (TN + FN)$$

# Characteristics of the “ideal” diagnostic test

- High sensitivity and specificity
- High PPV and NPV
- Reproducible
- Objective - quantitative
- Automatable
- Low cost
- ...



But... how is real life?????



**Clinical laboratory North of Barcelona (Spain)**

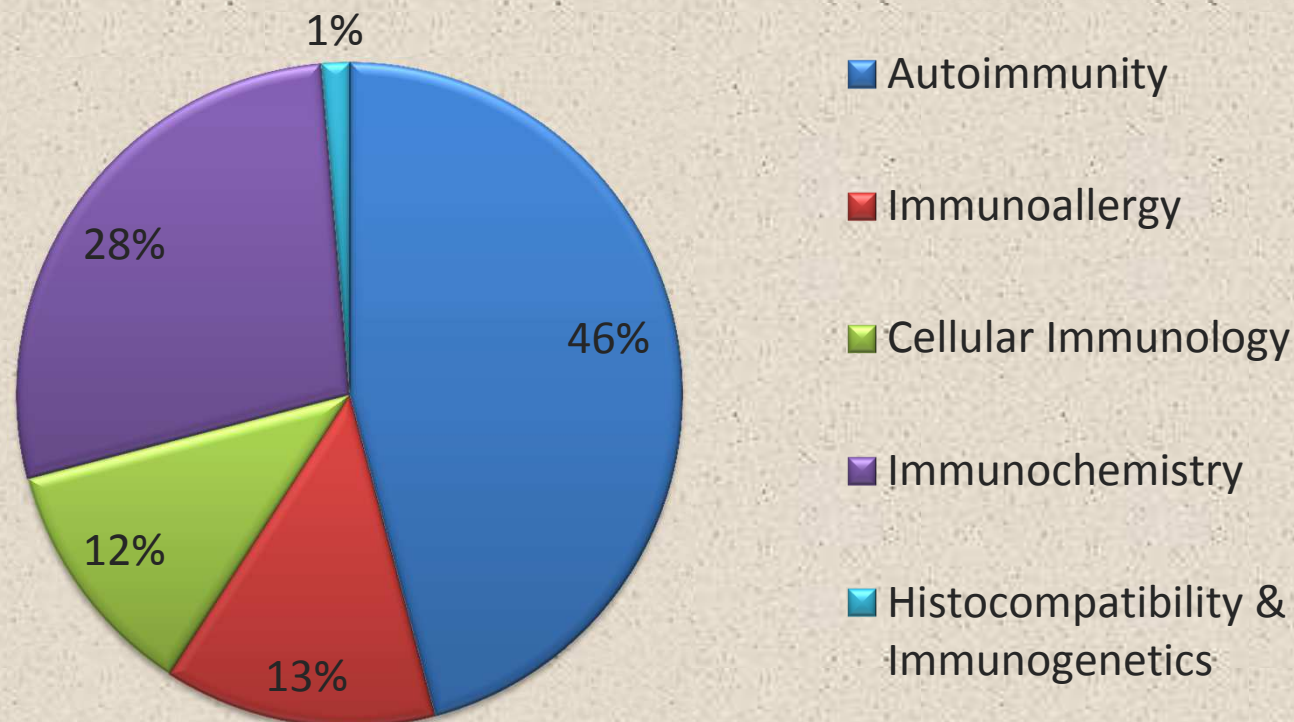
**1,5 million people**

**>10.000.000 test in 2018**

**<https://www.youtube.com/watch?v=7bYU6Gd> DBs**

## Immunology Division

**203.161 test in 2018**



Atenas sept.mov

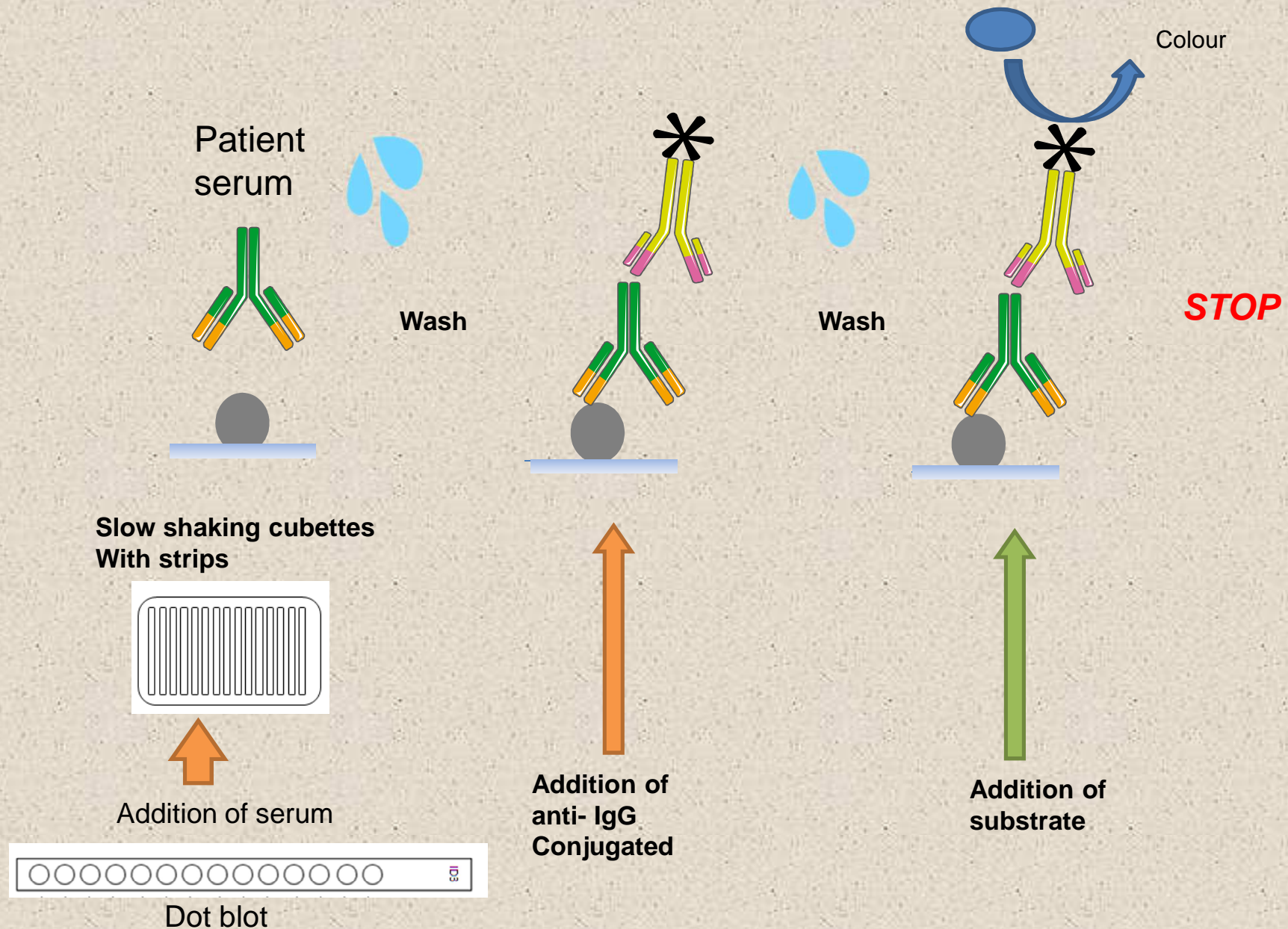
# Commercial kits



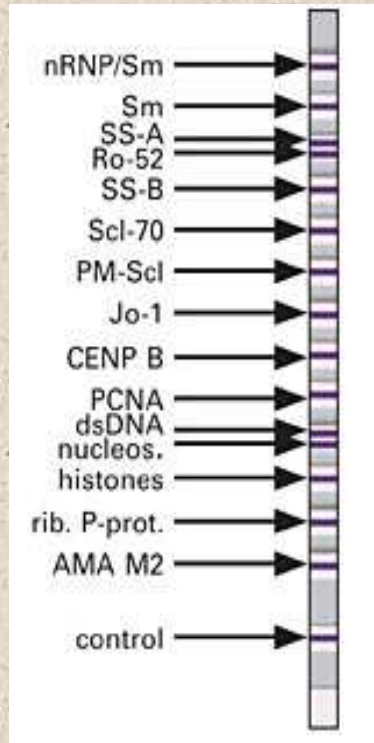
- ✓ Commercial kits with biomarkers very well standardized  
<https://www.youtube.com/watch?v=7bYU6Gd> DBs
- ✓ Commercial kits with biomarkers not enough validated (minoritary diseases)
- ✓ Prognostic biomarkers ( i.e. biomarkers of cancer associated to a disease)



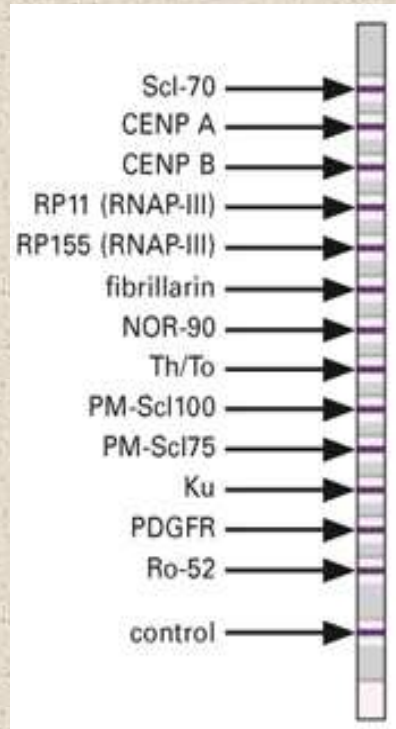
# *Immunoblot. Enzyme immunoassay*



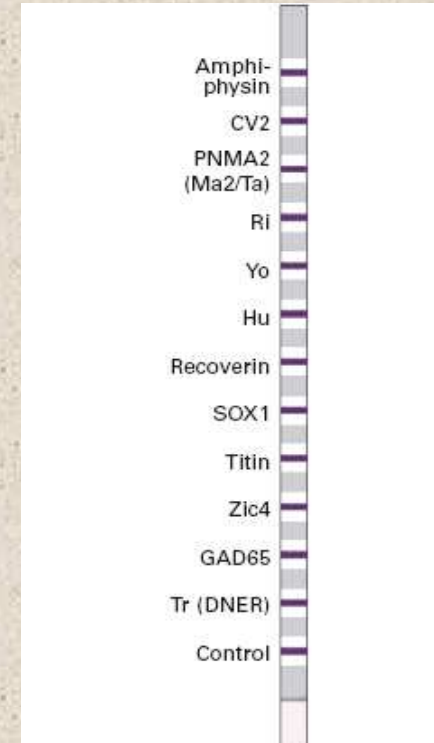
# *Immunoblot. Examples of different commercially available profiles*



Antigen related to  
systemic autoimmune  
diseases  
(Extractable nuclear  
antigens-ENA-)

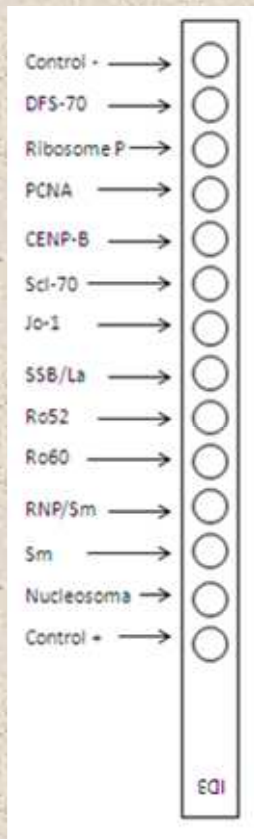


Systemic  
sclerosis

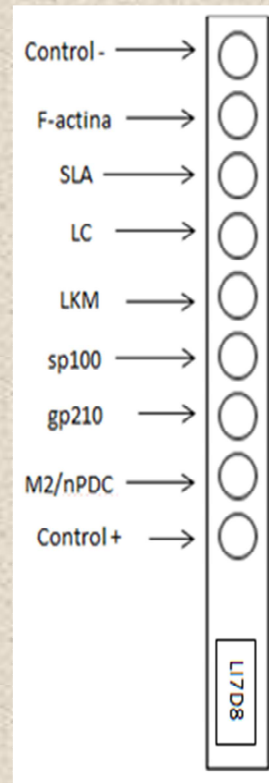


Paraneoplastic  
neurological  
diseases

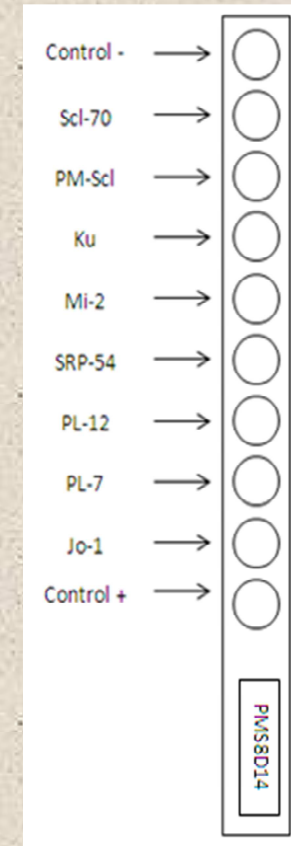
# ***Immunoblot. Different profiles (II)***



Extractable  
nuclear  
antigens



Autoimmune  
hepatic  
diseases

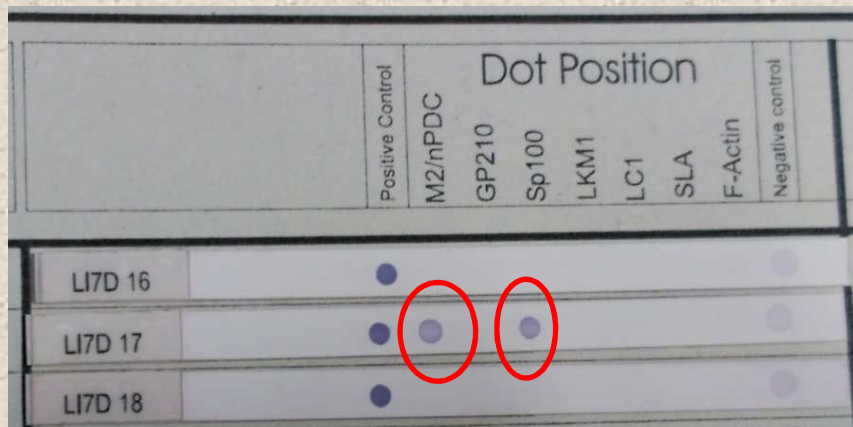


Myositis



# Interpretation of the results

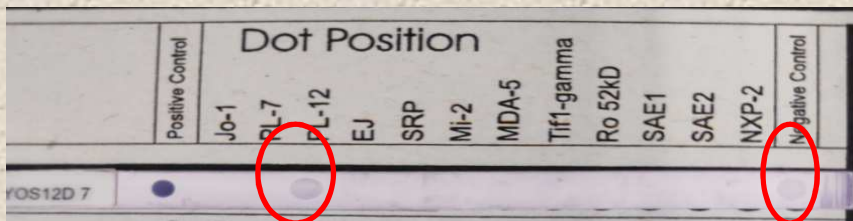
- How to read the results? Visual or Scanner (semiquantification)
- How to give a “positive” result?



Good example



Bad example



Difficult example

# Insert information commercial kits

## Interpretation of Results

Compare the specific antigen dots to the Negative Control Dot (which always is the last bottom dot). The color intensity of the Negative Control Dot may vary depending on the sample characteristics. If the sample is free of interfering substances the Negative Control Dot may be even close to uncolored. In contrast, a highly colored Negative Control Dot indicates a high rate of unspecific binding in the sample.

### **POSITIVE RESULT:**

A sample is positive for a specific antibody if the color intensity of the corresponding antigen dot is higher than the intensity of the Negative Control Dot.

### **NEGATIVE RESULT:**

A sample is negative for a specific antibody if the color intensity of corresponding antigen dot is lower than or equal to the intensity of the Negative Control Dot.

A weak coloration of an antigen dot, when close to the color intensity of the Negative Control dot may be difficult to differentiate by visual inspection only. In such cases, it is recommended to use

scanning system

### **POSITIVE RESULT:**

A sample is positive for a specific antibody if the colour intensity of the corresponding Antigen dot is higher than the intensity of the Negative Control Dot.

### **NEGATIVE RESULT:**

A sample is negative for a specific antibody if the colour intensity of corresponding Antigen dot is lower than or equal to the intensity of the Negative Control Dot.

NB: A weak coloration of an antigen dot, when close to the colour intensity of the Negative Control Dot may be difficult to differentiate by visual inspection only. In such cases, it is recommended to use software and scanning system

# Clinical case 1. When everything fits

- Woman, 52 yr.
- Several weeks with symmetrical and progressive muscular weakness
- Problem doing routinary things (combing, sitting down...)
- Suspected diagnosis: autoimmune myositis

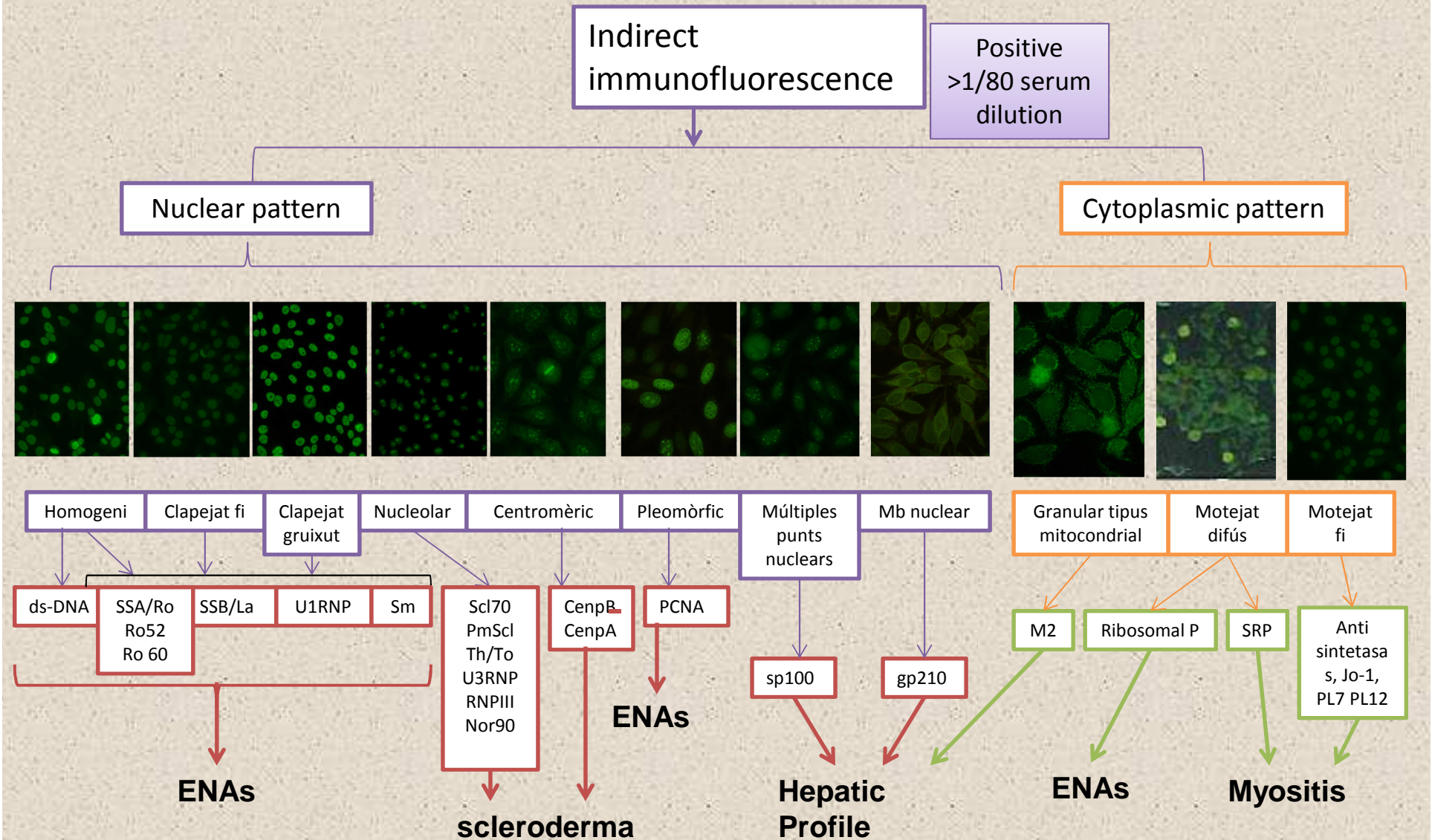
Immunology lab:

- 1) Indirect immunofluorescence (gold standard)
- 2) Confirmation by immunoblot

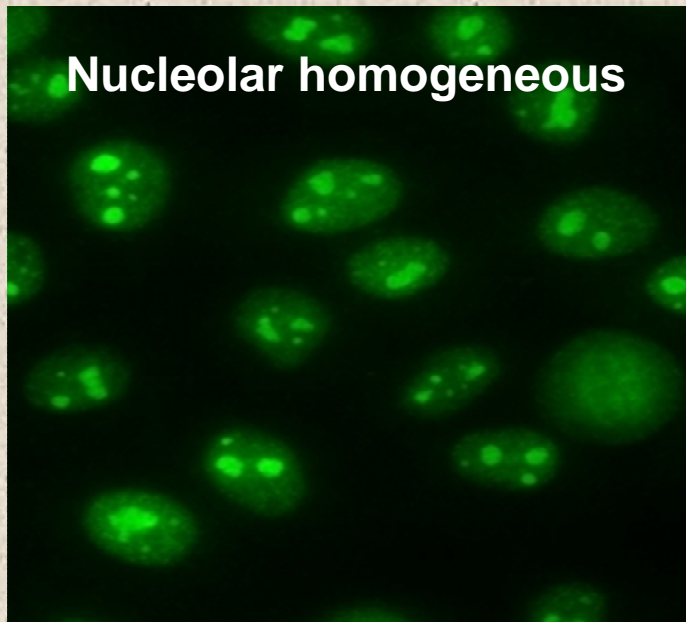


## GOOD APPLICATION

### Algorithm with IFI+ immunoblot + clinical context



# Clinical class 1: Nucleolar Pattern



	Positive Control	Dot Position							Negative Control
		Jo-1	PL-7	PL-12	SRP-54	Mi-2	Ku	PM-Scl	
PMS8D 16	•								•
PMS8D 17	•							•	•

Very specific biomarker  
of polymyositis

Which profile?

# Clinical clase 1:

- Commercial kit information:

## 10.2 Sensitivity and Specificity

Characterized samples (confirmed positive or negative for specific antibodies by reference laboratories and/or methodologies) were assayed following the test instructions. Sensitivity and Specificity were calculated from the results generated by the DOT software.

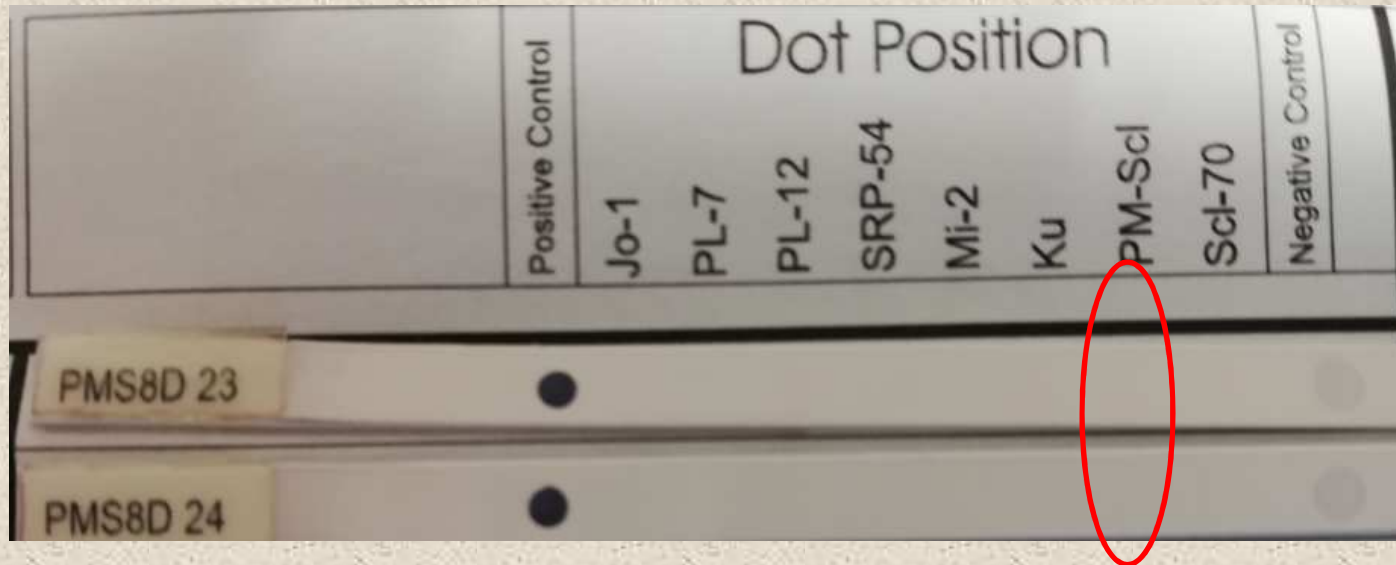
<div><div><div>+</div><div>-</div></div><table><tr><td>true positive</td><td>false positive</td></tr><tr><td>22</td><td>0</td></tr><tr><td>false negative</td><td>true negative</td></tr><tr><td>0</td><td>119</td></tr></table><div><div>Sensitivity</div><div>100%</div></div><div><div>Specificity</div><div>100%</div></div></div>	true positive	false positive	22	0	false negative	true negative	0	119	<div><div><div>+</div><div>-</div></div><table><tr><td>true positive</td><td>false positive</td></tr><tr><td>3</td><td>0</td></tr><tr><td>false negative</td><td>true negative</td></tr><tr><td>0</td><td>50</td></tr></table><div><div>Sensitivity</div><div>100%</div></div><div><div>Specificity</div><div>100%</div></div></div>	true positive	false positive	3	0	false negative	true negative	0	50	<div><div><div>+</div><div>-</div></div><table><tr><td>true positive</td><td>false positive</td></tr><tr><td>3</td><td>0</td></tr><tr><td>false negative</td><td>true negative</td></tr><tr><td>0</td><td>50</td></tr></table><div><div>Sensitivity</div><div>100%</div></div><div><div>Specificity</div><div>100%</div></div></div>	true positive	false positive	3	0	false negative	true negative	0	50	<div><div><div>+</div><div>-</div></div><table><tr><td>true positive</td><td>false positive</td></tr><tr><td>18</td><td>0</td></tr><tr><td>false negative</td><td>true negative</td></tr><tr><td>0</td><td>20</td></tr></table><div><div>Sensitivity</div><div>100%</div></div><div><div>Specificity</div><div>100%</div></div></div>	true positive	false positive	18	0	false negative	true negative	0	20
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22	0																																		
false negative	true negative																																		
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false negative	true negative																																		
0	24																																		
true positive	false positive																																		
13	0																																		
false negative	true negative																																		
0	91																																		

What do you think?

What would you ask the company?



# Clinical case 1. Alternative result



Can we decide -based on sensitivity and specificity- that the patient DOES NOT have polymyositis?

Is the information/robustness of the kit strong enough?

What would you do?

<b>TEST LIMITATIONS</b>	
1.	A clinical diagnosis should not be made on the basis of a single in vitro diagnostic method only.
2.	A complete clinical investigation, as well as other laboratory test results, should be considered to state a diagnosis, since no technique used alone can rule out the possibility of false-positive or false-negative results. In this respect, more particularly an indirect Immunofluorescence test, when applicable, should be performed in parallel with the determination of autoantibodies

# Clinical case 2. Borderline result

- Woman , 44 yrs.
- Cutaneous lesions in face, neckline and hands, suggestive of dermatomyositis
- No muscle weakness
- 4 years follow–up with exacerbations. Worsening
- Rest of analysis: no significant



## Autoanticossos cribatge

Srm-Ac. Anti-nuclear Result: Positive (1:100)  
(IFI sobre cel. lúles Hep2) Patró ( )  
Homogeni (AC-1) 1/80  
Comentari estudi ANA

### Malalties autoimm.

### Srm-Factor reumatoide

Srm-Ac.Anti-péptids c

**Negativu Negativo <20 L**

Positiu feble 20 - 39 UA

### Positiu moderat 40 - 59

Positiu alt &gt;59 UA

Srm-Ac. Anti-DNA do

**Negativu** Negativu  $<30$  UI

Indeterminat 30 - 75 UIM

Positiv  $>75$  UI/mL

Srm-Ac Anti-Sc170 (td

**Negative**

Negativ

### Perfil miositis (Immu

Immunoblot

**Srm-Ac. Anti-Ro52 Ne**  
**Immunoblot**

Srm-Ac. Anti-isoleucil-

Immunoblot

100

### **Perfil miositis (Immunodot múltiple 16 Aqs)**

Immunoblot

**Srm-Ac. Anti-Ro52 Negativ**  
Immunoblot

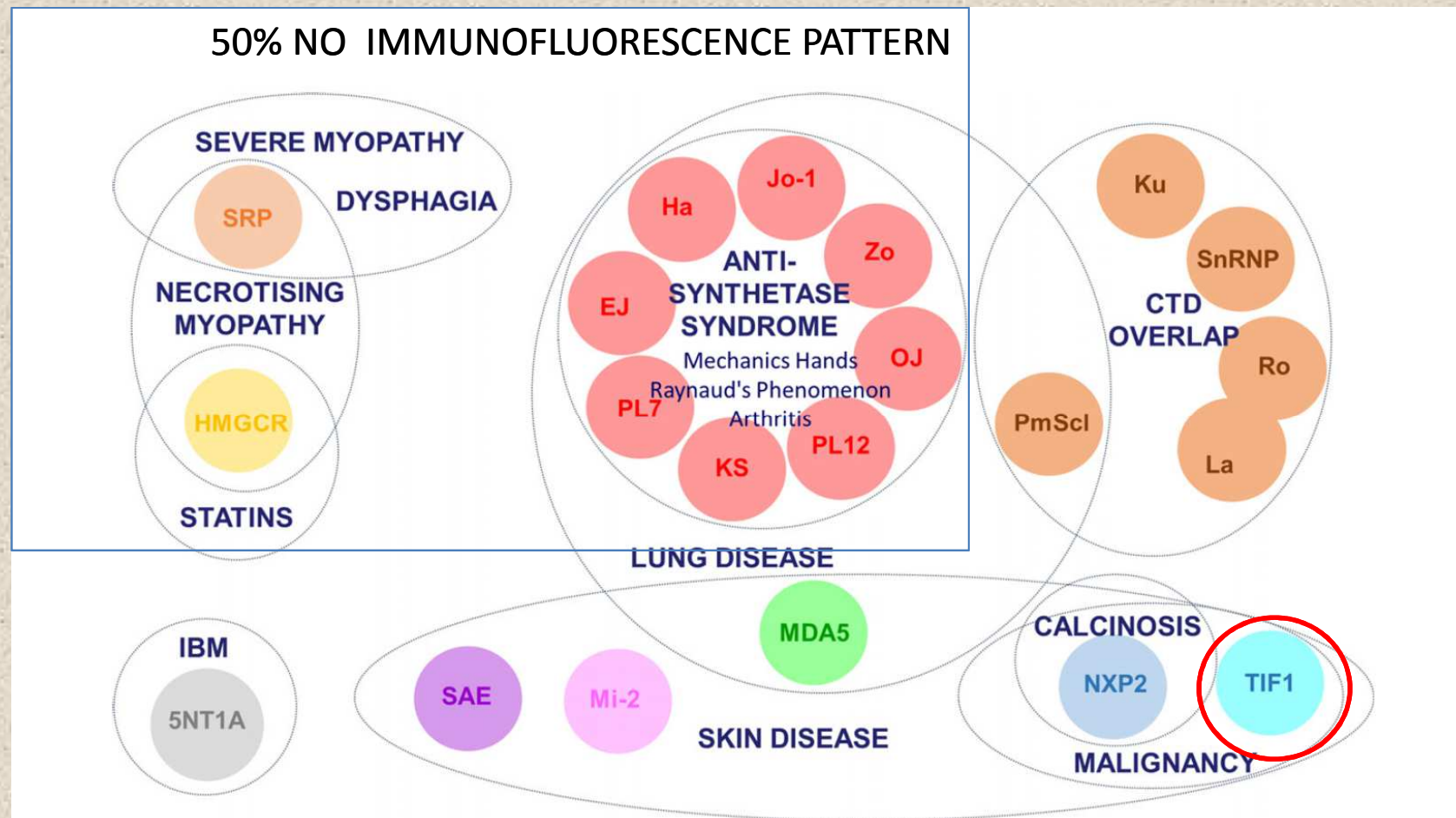
Srm-Ac

Immunoblot

—

Western blot analysis of MYO12D protein levels. The blot shows bands for MYO12D 6, 7, 8, 9, 10, and 11 across various lanes. A red arrow points to the MYO12D 10 band in the Tif1-gamma lane. A blue box highlights the Tif1-gamma lane. Two red circles highlight the MYO12D 10 bands in the Tif1-gamma and NXP-2 lanes.

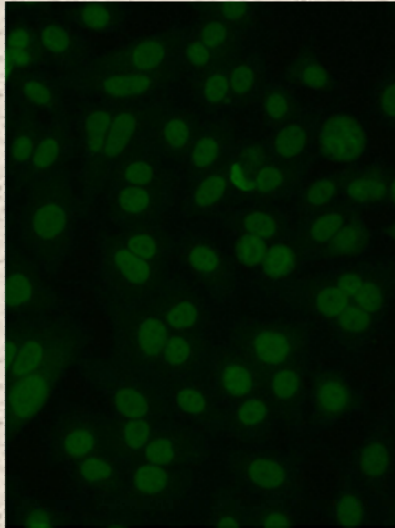
# Polymyositis/Dermatomyositis. Antibody related



**No specific pattern by IFI. Immunoblot very important**



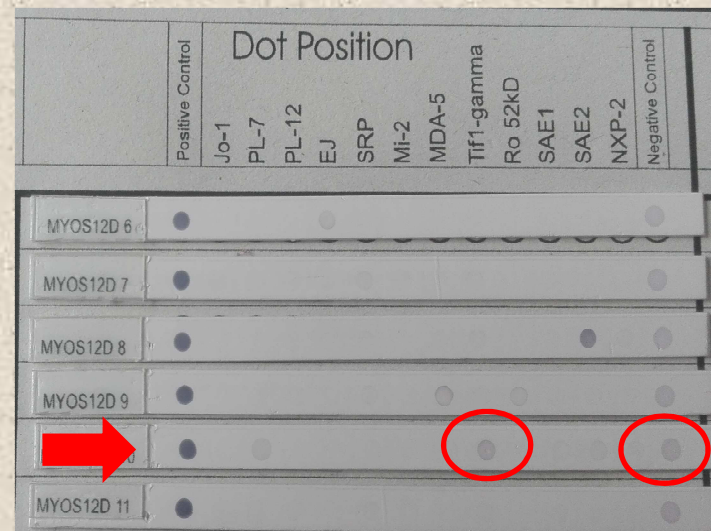
# Clinical case 2



TIF1-  $\gamma$  is a biomarker related to cancer

INSERT:

TIF1- $\gamma$			
	+	-	
+	true positive 3	false positive 0	
-	false negative 0	true negative 261	
Sensitivity		100 %	
Specificity		100 %	



Result: Positive? Negative? Borderline?

What would you do?

# Clinical case 2. Follow-up

- If “POSITIVE”
  - ✓ High resolution radiography and chest CT scan
  - ✓ Mamography, gynecological examination
  - ✓ Positron Emission Tomography (PET)
  - ✓ Tumoral markers (CEA, CA-10.9, CA-125)
  - ✓ If cancer no detected, follow –up 5-10 years
  - Impact of giving a FP result
- If “NEGATIVE”
  - ✓ Nothing
  - Impact of giving a FN result

In our case, a lung cancer was detected in the patient and treatment started

# Clinical case 3. Very rare disease

- Woman of 31 yr. old
- Seizures
- Refers memory disorders (words, faces...)
- Suffers disconnection from the environment and automatisms
- Since 15 days ago, the family refers she has delusions
- Psychiatry: treatment, but not responding
- Apathia, adinamia
- Eating disorders
- Suspected diagnosis: Autoimmune encephalitis



# **Autoantibodies against surface or intracellular synaptic antigens**

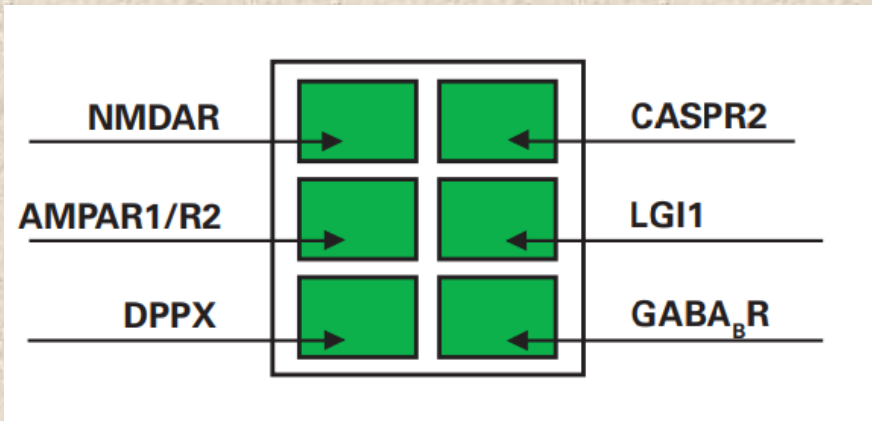
- Recognize conformational epitopes
- Present in serum and cerebrospinal fluid (CSF)
- Are pathogenic: interfere in the synapsis
- Clinical manifestations: seizures and psychiatric symptoms

# Clinical signs and antibodies

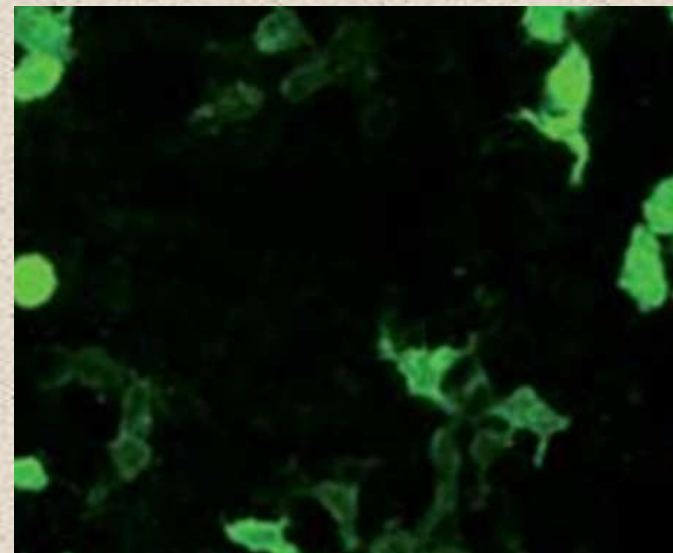
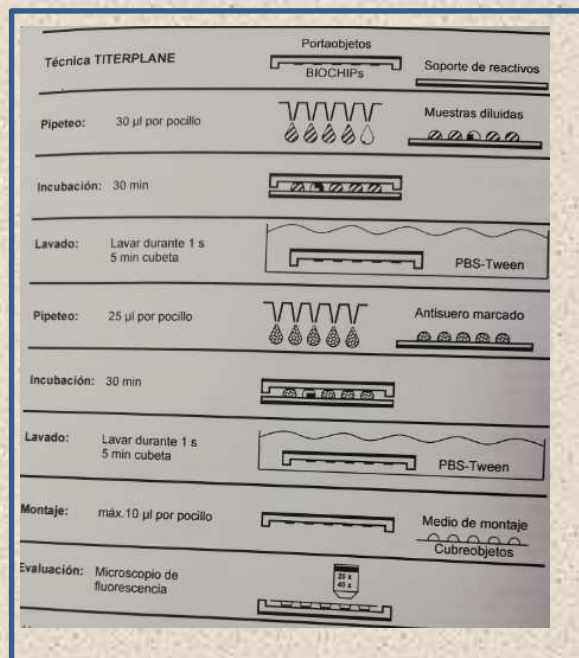
Autoantibodies	Clinical syndrome	Frequent symptoms	Paraneoplastic cases	Associated tumours
Anti-glutamate receptor (type NMDA)	Anti-glutamate receptor (type NMDA) encephalitis	Psychosis, memory-/language impairment, seizures, impaired consciousness, dyskinesia, movement disorders, dysautonomia, hypoventilation	35%–40% (depending on sex, age, ethnicity)	Ovarian teratoma; in rarer cases: teratoma of the testis, breast carcinoma, neuroendocrine ovarian tumour, ovarian germ band stroma tumour, pseudopapillary pancreatic neoplasia, SCLC, neuroblastoma, Hodgkin lymphoma
Anti-glutamate receptor (type AMPA)	Limbic encephalitis, atypical psychosis	Memory deficits, confusion, disorientation, seizures, agitation, aggressive behaviour	70%–75%	SCLC and non-SCLC, thymoma, breast carcinoma
Anti-GABA <sub>A</sub> receptor	Limbic encephalitis	Seizures, confusion, memory deficits, behavioural disorders, paranoia, hallucinations	50%–80%	SCLC, thymic carcinoid
Anti-LGI1	Limbic encephalitis	Epileptic seizures, memory deficits, confusion, disorientation, hyponatraemia, myoclonus, dysautonomia	0%–10%	Thyroid carcinoma, thymoma, SCLC, renal cell carcinoma, ovarian teratoma
Anti-CASPR2	Neuromyotonia, Morvan's syndrome, Limbic encephalitis	Peripheral neuronal hyperexcitability, muscle spasms/fasciculations/myokymia, seizures, memory deficits, confusion, disorientation, neuropathic pains, sleeping disorders, dysautonomia, weight loss	0%–35%	Thymoma, endometrial adenocarcinoma
Anti-DPPX	Autoimmune encephalitis	Anxiety, forgetfulness, confusion, hallucinations, muscle spasms, tremor and pleocytosis (in CSF)	unknown	–

Abbreviations: **NMDA**: N-methyl-D-aspartate, **AMPA**: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, **GABA**: γ-amino butyric acid, **LGI1**: leucine-rich glioma-inactivated protein 1, **CASPR2**: contactin-associated protein 2, **SCLC**: small-cell lung cancer, **DPPX**: dipeptidyl aminopeptidase-like protein 6

# Indirect immunofluorescence in transfected cells



Initial dilution 1:10 serum; 1:1 CSF  
anti-human IgG, FITC-conjugated



CASPR2



# Interpretation: Insert of the kit

## Clinical specificity and sensitivity

	Antibody Substrate	Ig class	Sample characterisation clinical patient panels	n	Prevalence	
					Antibody-positive	%
Serum	Anti-NMDA-R Transfected cells	IgG	Anti-NMDAR encephalitis	235	201	85.5
			Other encephalitides	130	0	0
			Apparently healthy blood donors	200	1	0.5
CSF	Anti-NMDA-R Transfected cells	IgG	Anti-NMDAR encephalitis	216	215	99.5
			Other encephalitides	13	0	0
			CSF samples without a positive neurological finding	60	1	1.7
	Anti-GABA <sub>B1/B2</sub> -R Transfected cells	IgG	Limbic encephalitis	17	14	82
			Apparently healthy blood donors	200	0	0
			Limbic encephalitis	14	14	100
	Anti-LGI1 Transfected cells	IgG	Apparently healthy blood donors	150	0	0
			Limbic encephalitis	9	9	100
			Apparently healthy blood donors	150	1	0,7
Serum	Anti-AMPA1/R2 Transfected cells	IgG	AMPA2 antibody-positive patients	5	5	100
			AMPA2 antibody-negative patients, control panel	33	0	0
			Patients with mental disorders, control samples, patients with Hashimoto's thyroiditis	771	0	0
			Apparently healthy blood donors	206	0	0
			DPPX antibody-positive patients	6	6	100
	Anti-DPPX Transfected cells	IgG	DPPX antibody-negative patients	9	0	0
			Patients with mental disorders, control samples, patients with Hashimoto's thyroiditis	771	0	0
			Apparently healthy blood donors	200	0	0

What do you think? How would you increase the specificity? Increase serum dilution?

# Interpretation: Insert of the kit

What do you think?

How would you increase the specificity? Increase serum dilution?

Relevance of the sample: cerebrospinal fluid versus serum? (Not specified in the insert)

Recommended both. Necessary?

Important to know if the patient already started treatment (False Negative!)

Relevance of the most adequate patient sample (Serum, CSF ...)

Relevance of the dilution of the sample

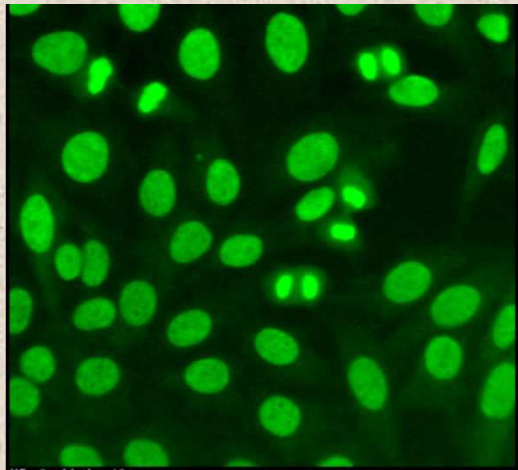
# Clinical case 4: When we have “too many” data

- Woman, 23 years old
  - Inflammatory bowel disease
  - Starts treatment with anti-TNF $\alpha$  antibodies
  - After 3 months starts with cutaneous lesions and polyarthralgias
  - Biopsy of the lesions: lupic band (deposits of immunoglobulins and complement in the skin)
- ¿Systemic Lupus erythematosus?



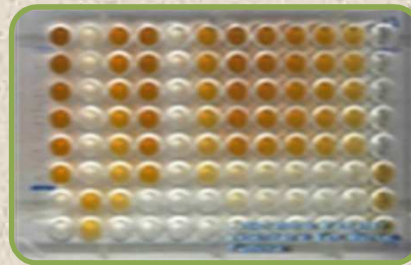


# Clinical case 4: Immunological study



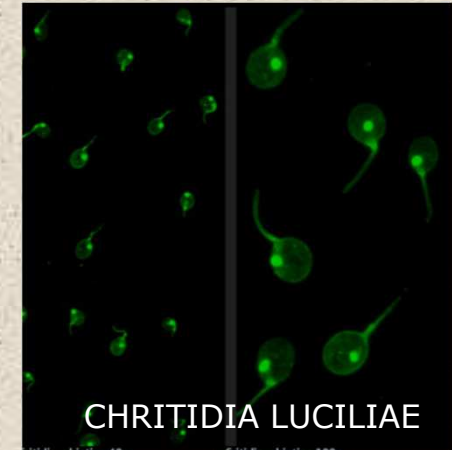
**HOMOGENEOUS pattern**  
**HEP-2 cells**

Suggestive of antibodies anti- ds-  
DNA Histones ([H2A-H2B]-DNA  
complex



**ELISA anti-dsDNA**

**Positive**



**Negative**

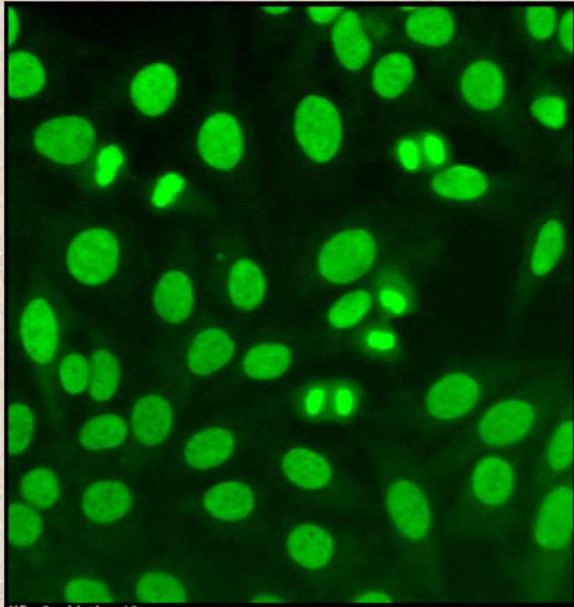
?

# Clinical case 4: insert kit ELISA

Panel (Source: Charité Universitätsmedizin Berlin)	n	Anti-dsDNS NxX- ELISA positive	Anti-dsDNS RIA positive	Anti-dsDNS ELISA positive	IIFT (Crithidia luciliae) positive
SLE	209*	125	108	88	57
Sensitivity	209	59.8%	51.7%	42.1%	27.4%
Sjögren's syndrome	88	1	0	1	1
Progressive systemic sclerosis	81	2	2	4	6
Specificity	169	98.2%	98.8%	97.0%	95.9%
Sensitivity at 98 % specificity (according to ROC analysis)	378	60.8%	53.1%	35.4%	–

\* Only 208 SLE sera were incubated on Crithidia luciliae

# Clinical class 4: relevance of the antigen



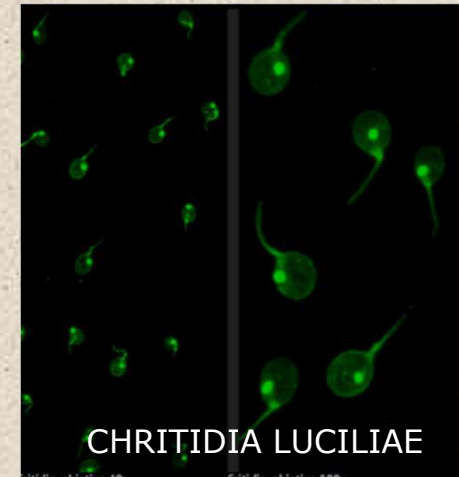
**HOMOGENEOUS pattern**  
**HEP-2 cells**

Suggestive of antibodies anti-complex ds-DNA Histones ([H2A-H2B]-DNA



**ELISA anti-dsDNA**

**Positive**



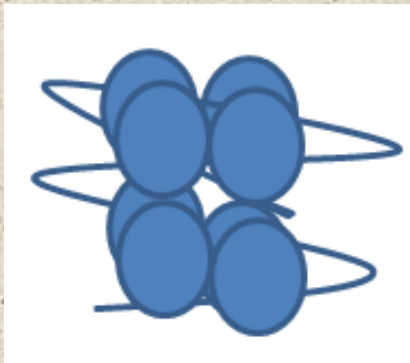
**CHRITIDIA LUCILIAE**

**Negative**



# What are we looking for ?

*“Who does not know what he is looking for,  
Does not understand what he finds”*



Nucleosome complex

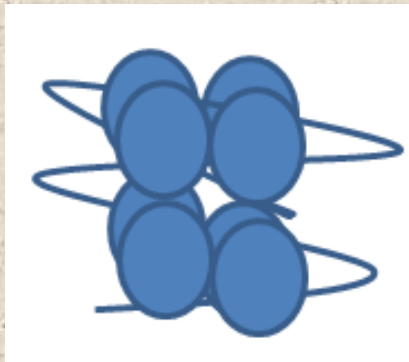
Relevance of the antigen!!!

Do all reagents/kits have the same antigen?

*Chritidia luciliae*: dsDNA

ELISA: dsDNA –nucleosome complex

# What does it mean?



## Clinical association:

Anti-dsDNA: Systemic lupus erythematosus (LES)

Anti-nucleosomes: very specific of systemic LES

Anti-Histones → Drug-induced Lupus



Different treatment and different prognosis

ANA-3/ 966-60																	
Et	Co	M2	RIB	HI	NUC	DNA	PCNA	CB	Jo	PM100	Sci	SSB	52	SSA	Sm	RNP/Sm	
-1	79	1	0	23	0	0	0	0	0	2	2	1	1	0	0	0	
0	+++	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	



Drug-induced Lupus: STOP treatment

# Key points for a “real life” diagnostic test

- **Sample:** (plasma, serum, CSF, urine....)  
Extraction and preservation  
Dilution
- **Antigen**
- **Algorithms: sensitivity *versus* specificity**
- **Interpretation.** More than one technique, if possible.  
Clinical context of the patient





# Take home messages

- ✓ **Antibodies are good biomarkers in the clinic**
- ✓ **Better validation and especification of kits is needed**
- ✓ **More research is needed**
- ✓ **International colaborations**
- ✓ **Multicenter studies**
- ✓ **Interpretation in the clinical context**



Most of the graphs and images are from our clinical practice using anonymous results from our patients and explanations of the commercial inserts from different commercial diagnostic pharmaceutical companies

NOTE: The tables, images or other graphic material included in this presentation have the exclusive intention to illustrate the explanations of this class, the author following article 32 of vigent Law of Intellectual Property (Spain)

Thank you!



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