Detection of antinuclear Antibodies: Recommendations from EFLM, EASI and ICAP: QA, Lot Evaluation & Verification, a practical Approach

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Sciensano non infectious serology committee

EFLM Working group Autoimmunity testing.

Analytical verification/validation

Lot acceptance and monitoring

Internal quality control
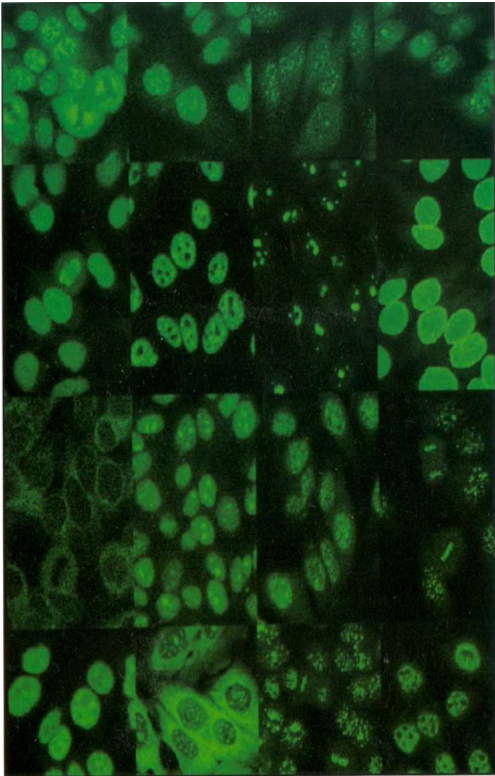
Recommendations EFLM EASI ICAP

References

Survey EFLM EASI ICAP

Topic	%

How to do it: literature – practical examples

HEp-2(000) IFA variability

Antigens: culture conditions
                      fixation
                      permeabilization

Secondary antibody: isotype, species, immunogen, purification
                      fluorescein/protein ratio, anti-fading

Media and buffers
Equipment   light source
                      CAD

Procedures
Lab collaborators

Antigen-specific
SSA, RNP/Sm, Cenp-F
Jo-1, PCNA
NUMA, Rods and rings

≠Brands can ≠
        positivity/negativity
        pattern

Bonroy et al CCLM 2023, 61(7), 1167–1198; Dellavance et al J Bras Patol Med Lab 2013,43:182-90; Wener et al Arch Pathol Lab Med 2021;145:937-42;

Analytical validation/verification

Validation: objective evidence (by documenting performance characteristics) that a method/application is adequate for the intended use

Verification: abbreviated process that confirms via objective evidence that an already validated examination procedure is appropriate for a specific intended use in one's own laboratory

WHO BS/95, 1973. 1995 ; Sarewitz SJ. 2013; webapps.cap.org; ISO 9000:2005; Directive 98/79/EC Regulation (EU) 2017/746 Commission Decision 2010/227/EU; National guidelines

Performing verification of commercial method (n=187)	%
Yes	80
No verification, rely on kit insert	14
No verification, rely on publications	4
No verification, rely on kit insert and publications	2

nroy et al CCLM, *61*(7), 1167–1198. https://doi.org/10.1515/cclm-2023-0209 and *Adapted from Vercammen et al. CCLM*, *61*(7), 1199–1208. https://doi.org/10.1515/cclm-2023-0210

Analytical verification

HEp-2(000) cells: density, distribution morphology, mitotic cells

Trueness: method comparison versus characterized samples:
              % positivity/negativity nucleus and cytoplasm
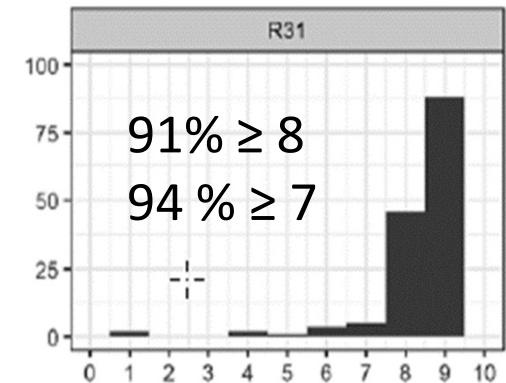   patterns
              titer

Precision

Pipetting device

R31: Analytical verification: Trueness

R 31.

Each laboratory should demonstrate that its Hep-2 IFA method detects the major clinically relevant patterns as well as the major clinically relevant antigen reactivities, both in the nuclear and cytoplasmic compartment.

Grade A/B

91% ≥ 8
94 % ≥ 7

Bonroy et al CCLM, 61(7), 1167–1198. https://doi.org/10.1515/cclm-2023-0209

R31: Analytical Verification of Pattern. Recommendations

Characterized samples

EQC target
solid phase assays
clinical information

Clinically relevant patterns/reactivities

AC-1	nuclear homogeneous	dsDNA
AC-4, 5	nuclear speckled	SSA/Ro60, Sm/RNP
AC-8,9,10	nucleolar	Scl70, RNA polymerase III
AC-3	centromere	CenpB
AC-6	multiple nuclear dots	sp100
AC-11,12	nuclear envelope	gp210
AC-19,20	cytoplasmic speckled	Jo-1
AC-21	reticular/antimitochondrial	AMA-M2

Number of samples

5/pattern
10 negatives

Analytical verification. Survey

Variability in approach towards verification > origin, level and characterization samples

Origin of samples (n=149)	%
EQC samples exclusively	9
Patient and EQC samples	45
Patient samples exclusively	46

Level of sample positivity (n=133)	%
Only stronly positive samples	10
Only weakly positive samples	4
Combination	86

Characterization of patient samples (n=136)	%
Clinically characterized	30
Laboratory characterized Method comparison and/or follow-up tests	44
Both clinically and laboratory characterized	26

R32: Analytical verification: Precision

Precision is an essential verification requirement

R 32.

Each laboratory should verify the precision of the method used.

The approach will depend on how the data are handled: binomial (positive/negative), ordinal (titers) or continuous (fluorescence intensity measure results)

Grade A/B

89% ≥ 8
95 % ≥ 7
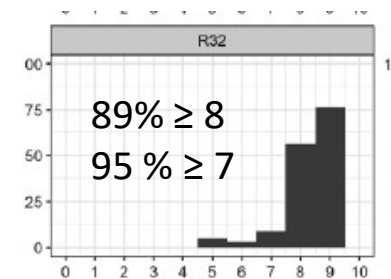
Verification of precision (n=225)	%
yes	72
- Between-run only	17
- Within-run only	5
- Between- and within-run	78

Analytical verification: Precision

Qualitative tests

EASI

10 replicates of a negative sample

10 replicates of positive sample (low, medium, high) +/- 1 titer ⎫ within and between run

Sack Auto Immun Highlights 2020;11:12

CAD FI

Results qualitative and semi-quantitative ⟵ quantitative results

CLSI EP05A3; CLSI EP15-A3

State-of-the-art publications   6-20 replicates

Bonroy CCLM 2013;51:1771-9; Bizzaro Autoimm Rev 2014;13:292-8; Bossuyt; Van Hoovels CCLM 2018;56:258-61; Bogaert CCLM 2019;57:990-8

Analytical verification: Pipetting device

Pipetting Verification Test

Date / Time	16-1-2024

Robot Serial No	

Test Results [mOD]
Difference 450 nm - 620 nm

	Disp. 1	Disp. 2	Disp. 3	Disp. 4	Disp. 5	Disp. 6	Disp. 7	Disp. 8	Disp. 9	Disp. 10	Disp. 11	Disp. 12	Mean	CV
Ch.1	480	485	484	498	507	507	509	509	492	494	494	459		
Ch.2	529	475	479	467	524	489	491	522	514	462	491	485		
Ch.3	483	517	509	517	499	497	511	498	504	479	483	459		
Ch.4	508	506	489	521	536	501	486	525	518	469	497	518		
Ch.1	481	484	506	495	493	525	510	480	493	514	484	450	493,0	3,4%
Ch.2	505	489	490	483	523	483	501	538	496	469	485	504	495,6	4,2%
Ch.3	480	524	503	480	502	507	490	490	512	534	477	457	496,3	3,9%
Ch.4	522	502	500	513	529	498	503	531	484	473	508	502	505,8	3,5%
													497,7	3,8%

	Criteria	Result	Pass/Fail
CV per Channel	5%	4,2%	Pass
CV over all	5%	3,8%	Pass

Date	16-1-2024		Operator	
			Operator Signature	

Analytical verification: Carry-over

A. HCG High 13522,96 IU/L
B1. HCG low
B2. HCG low
B3. HCG low

A. HCG High
B1. HCG low
B2. HCG low
B3. HCG low

A. HCG High
B1. HCG low
B2. HCG low
B3. HCG low

Data UZ Gent

mean B1   10,782 IU/L
mean B3   6,90 IU/L

(paired t-test 0,018 > 0,05)

% carry-over

$$\frac{\text{mean B3 - mean B1}}{\text{mean A}} *100$$

$$\frac{10,82 - 6,90}{13522,96} *100 = 0,03\% \ (< 0,1\%)$$

R34 R35 Clinical validation

R 34

According to ISO 1589, CAP directives and the new 2017 IVD
regulation, the manufacturer is responsible for the
clinical validation of a CE/FDA labelled test. National
regulation can formulate additional requirements.

Grade A

R34
93% ≥ 8
98 % ≥ 7
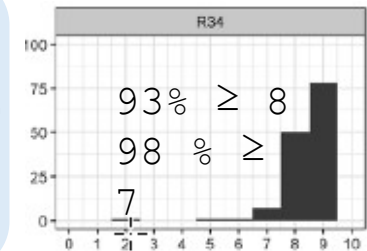
R35

Validation of a HEp-2 IFA method is preferentially done in
large multi-center studies including a sufficient number
of diagnostic samples of clinically characterized patients
and controls. Such studies should allow to estimate test
result specific LR

Grade B

R35
85% ≥ 8
92 % ≥ 7
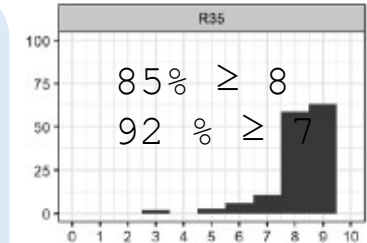
(Diagnostic sensitivity and specificity)

Test-result specific likelihood ratio's

collaboration clinical immunologists, clinicians, manufacturers

R26: Lot acceptance and monitoring

Literature review

Variation has been shown between brands

Variation linked to lot changes of the same brand have also been suggested

ISO 15189:2012> each new reagent lot and shipment should be verified before use

CLSI EP26-A

Francescantonio PL et al. Rev Bras Reumatol, 2014;54:44-50.

Cruvinel WM et al. Adv Rheumatol. 2019;59:28.

Dellavance A et al.J Bras Patol Med Lab. 2013;49:182-190.

Van Hoovels L et al. Clin Chem Lab Med. 2018;56:258-261.

Maenhout TM et al. Clin Am J Clin Pathol. 2012;137:825-830.

Copple SS et al. Am J Clin Pathol. 2012;137:825-830.

Silva MJ et al. Front Immunol. 2022;12:798322.

Bonroy et al CCLM, 61(7), 1167–1198. https://doi.org/10.1515/cclm-2023-0209

R 26.

Lot-to-lot variability of conjugate and/or substrate should be evaluated before implementing a new lot.

This can be done by patient-derived IQC samples supplemented with samples selected for purposes minimally covering different cell compartments (nucleus and cytoplasm) an different titer levels.

Grade A/B

79% ≥ 8
91 % ≥ 7
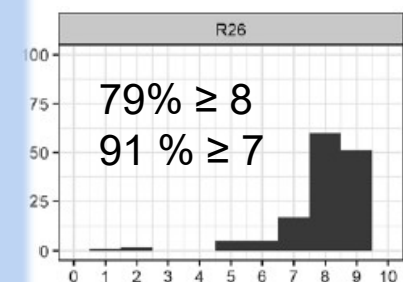
Lot acceptance and monitoring

CLSI LA02-A2

Low titer SSA
Panel negative and positive characterized sera

Brazilian guidelines

Panel broad array of patterns antigens emphasis susceptible to damage:
Jo-1, SSA, RNA polymerase, PCNA
Immunologically well characterized
morphologically well characterized

Francescantonio PL et al. Rev Bras Reumatol, 2014;54:44-50.;Cruvinel WM et al. Adv Rheumatol. 2019;59:28.; Dellavance A et al.J Bras Patol Med Lab. 2013;49:182-190

Lot acceptance criteria

IQC patient

Pattern: no deviation
Titer: +/- 1 dilution
Probability index/FI: target +/- 2 SD

Routine patients

n samples with pattern deviation
n samples with FI deviation
n samples with titer deviation
Statistical comparison of FI
Statistical comparison of median FI

Lot acceptance and monitoring: survey

Limited lot-to-lot evaluation was performed by 68% of the laboratories.

Lot change procedure > samples used (n=173)

Using exclusively patient samples	18 %
Using exclusively commercial samples	42 %
Combination of patient and commercial samples	40 %

For patient samples (n=89)

1 pattern	37 %
2 or 3 patterns	24 %
> 3 patterns	30 %
other	9 %

Topic	Number of respondents	%
Limitation of lot changes	259	47

Lot acceptance - Pattern and titer: example 1

IQC patient samples

	Auto titer Lot 1	Auto Titer Lot 2	FI Lot 1 1/80	FI Lot 2 1/80
Positive patient Ro-60 AC-4	640	640	634	771
Negative patient			38	33

Data AZ Sint-Jan

Routine samples

Pattern	Lot 1	Lot 2
	AC-3	AC-3

Titer	FI Lot 1	FI Lot 2
80	3489	4129
320	2071	1465
1280	261	235
2560	84	111
5120	40	46
autotiter	> 5120	> 5120

Lot acceptance - Routine patients example 1

dsDNA/ ENA	Lot 1 Pattern	Lot 1 Titer	Lot 1 Pattern	Lot 2 Titer
NEG	AC-1	640	AC-1	640
NEG	AC-1	160	AC-1	160
	AC-1 AC-8,9,10	160 160	AC-1 AC-8,9,10	160 80
NEG	AC-4,5 AC-19, 20	80 80	NEG	
NEG	AC-4,5	160	AC-4,5	80
NEG	AC-4,5	80 (n= 5)	AC-4,5 (n=4) NEG (n=1)	80
Ro-52	AC-4,5 AC-19, 20	320 320		320 160
	AC-21	1280	AC-21	640

Example 1: lot accepted

Data AZ Sint-Jan

dsDNA/ ENA	Lot 1 Pattern	Lot 1 Titer	Lot 1 Pattern	Lot 2 Titer
Cenp-B Ro-52 Ro-60 SSB	AC-3	5120	AC-3	5120
dsDNA cenpB	AC-1 AC-3	5120 5120	AC-1 AC-3	5120 5120
RNP Ro52 Ro60	AC-4,5 AC-19,20	2560 80	AC-4,5 AC-19,20	2560 80
Ro52 Ro60 SSB	AC-4,5 AC-19,20	1280 80	AC-4,5 AC-19,20	1280 80
NEG	AC-4,5 AC-19, 20	160 80	AC-4,5 AC-19, 20	80 80
	NEG (n=4)		NEG (n=3) AC-4,5(n=1)	80

Lot acceptance example 2

Samples with selected pattern and specificity

dsDNA/ENA	Lot 1 Pattern	Lot 1 FI	Lot 1 Autotiter/Titration	Lot 2 Pattern	Lot 2 FI	Lot 1 Autotiter/Titration
Scl70	AC-1	1807	640	AC-1	1141	640
RNP/Sm	AC-4,5	2692	1280	AC-4,5	2922	>1280
cenpB	AC-3	174	320	AC-3	163	320
Scl70	AC-8,9,10	1637	1280	AC-8,9,10	1040	640
M2	AC-21			AC-21		

Data GZA

Lot acceptance example 2

IQC positive patient SSA RO60
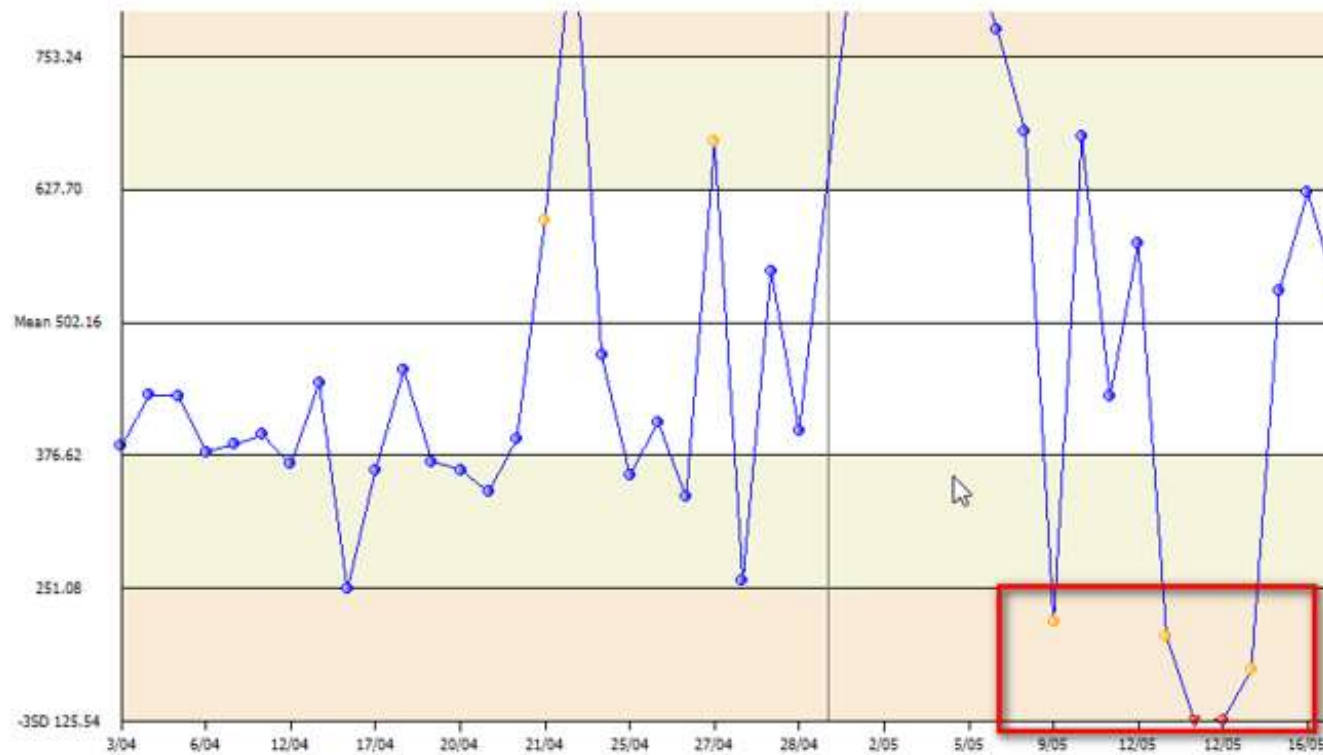
Data GZA

Lot acceptance example 2

IQC positive patient SSA RO60
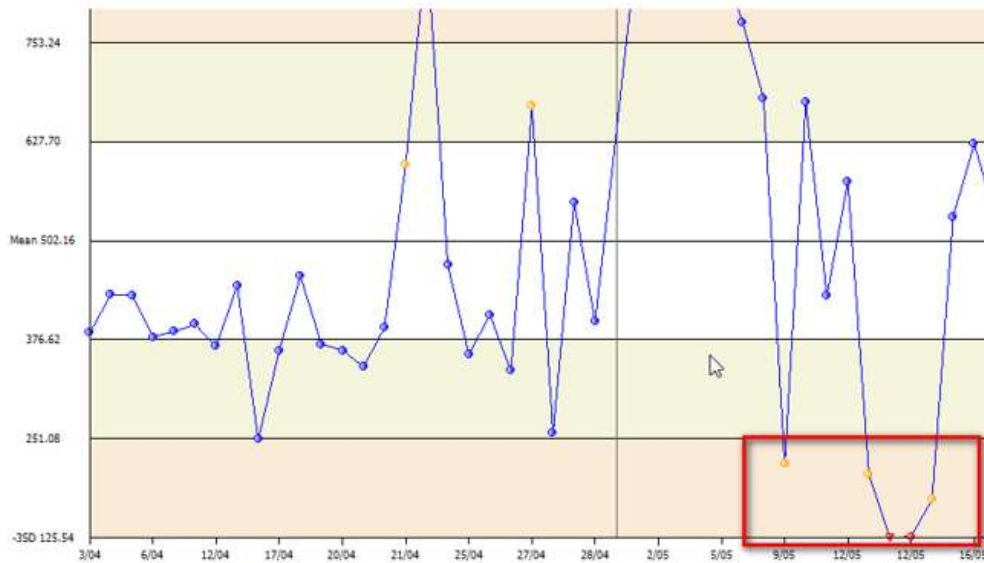
Lot not accepted

Data GZA

More SSA positive samples

Lot acceptance example 2

Data GZA

Lot monitoring

Fluorescence intensity

% Positive ANA
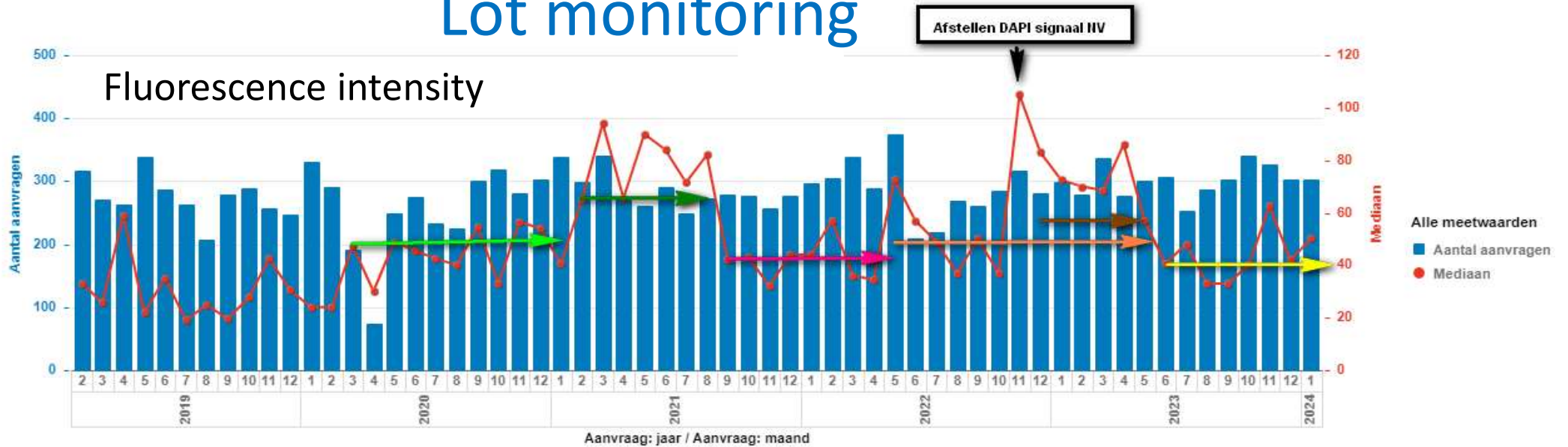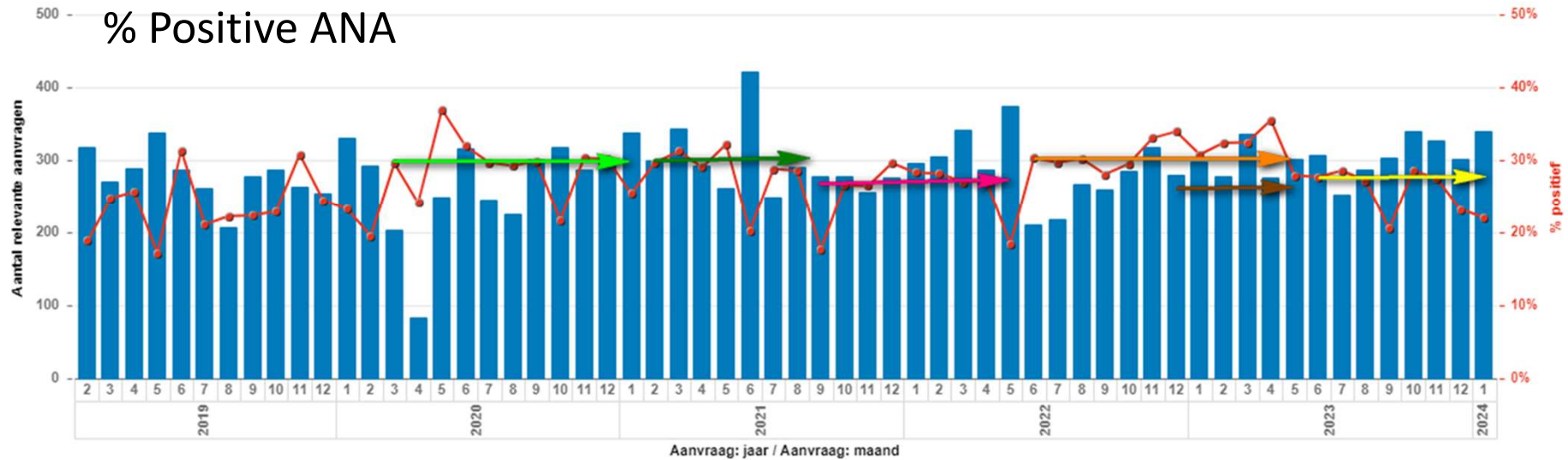
Data GZA

Lot monitoring

ENA
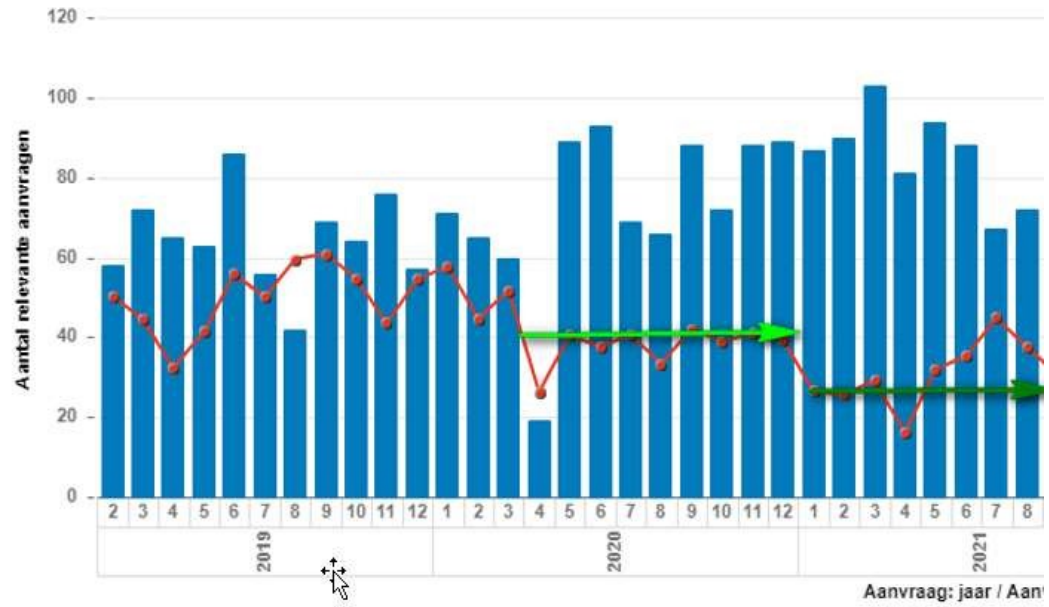
CTD screen
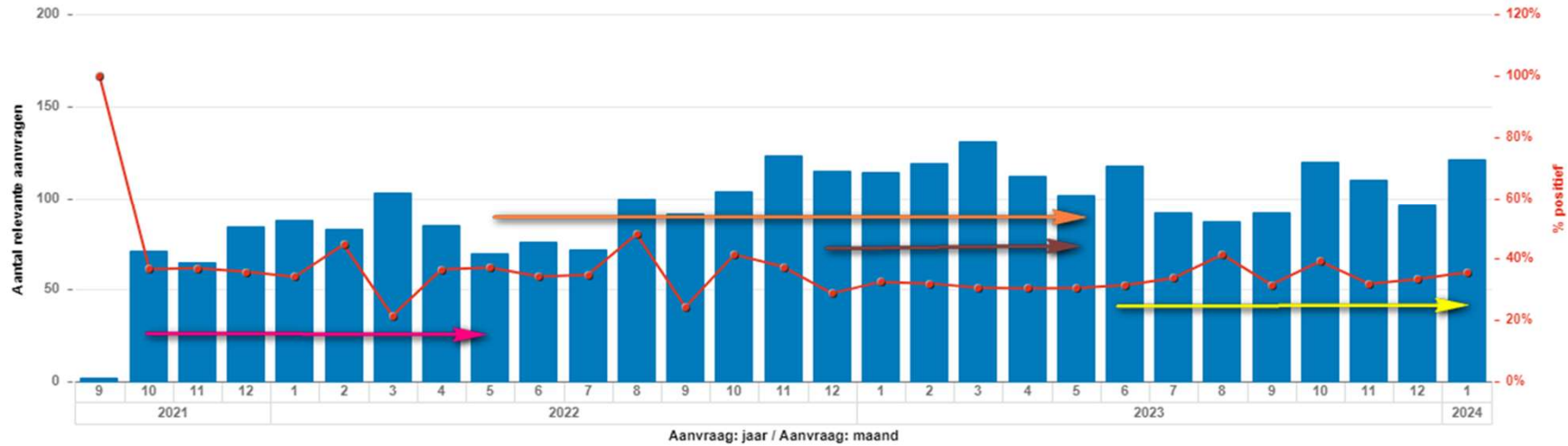
Data GZA

R19-R25: Quality approaches in HEp-2 IFA

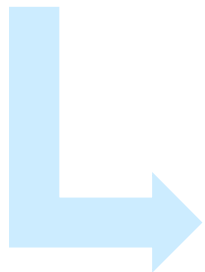International guidelines and EN/ISO 15189:2012 accreditation: challenge for ANA analysis

> **R 19.**
> Performance of HEp-2 IFA should be monitored by internal (IQC per run and periodic blinded reading of representative cases) and external quality assessment programs.
>
> Grade A

R19

98 % ≥ 8
99 % ≥ 7
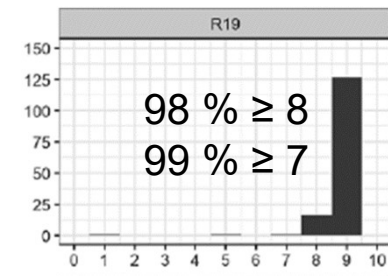
Everybody is involved in quality assurance

Topic	Number of responses	%
Performance of run IQC	323	96
Control inter-observer variation	277	79
Participation in EQC schemes	273	91

R19-R25: Quality approaches in HEp-2 IFA

R 21.
At least 2 IQC samples
(one negative and one low positive [with a target level matching a LR of 2-5 for ANA-associated rheumatic diseases]) should be included in each run
and judged semi-quantitatively (either by end-point titration or automated intensity scoring)

Grade A/B

85% ≥ 8
96 % ≥ 7
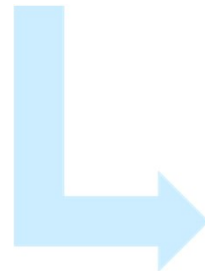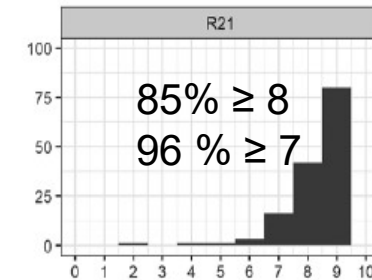
Titer 1/160 HEp-2000
LIU 552-910 Novaview CAD

Op De Beeck et al. Autoimm Rev 2011;10:801-8 and Claessens J et al. Autoimm Rev 2018;17:533-40

Quality approaches in HEp-2 IFA. Survey

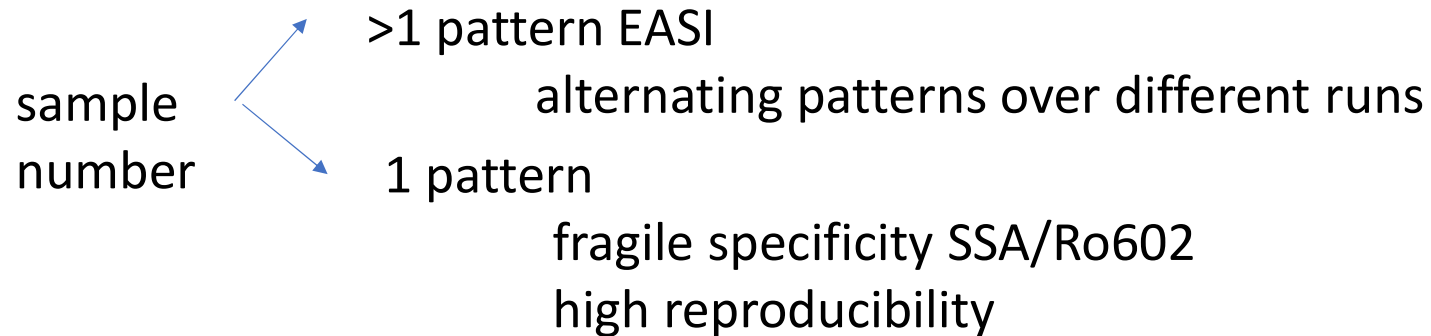Variability in approach towards RUN IQC > number and level of the IQC samples

Level of positive IQC samples (n=288)	%
High level	52
Medium level	19
Cut-off level	13
Different level	16

| Number IQC sample/run (n=300)	%
1 Positive and 1 negative IQC	62
> 1 Positive and 1 negative IQC	13
1 Positive and no negative IQC	21
> 1 Positive and no negative IQC	1,7

Bonroy et al CCLM, 61(7), 1167–1198. https://doi.org/10.1515/cclm-2023-0209 and *Adapted from Vercammen et al. CCLM*, 61(7), 1199–1208. https://doi.org/10.1515/

R19-R25: Quality approaches in HEp-2 IFA

Pattern defined antibody positivity

sample number

→ >1 pattern EASI
   alternating patterns over different runs

↘ 1 pattern
   fragile specificity SSA/Ro602
   high reproducibility

> **R 23** The preferred pattern of the positive control sample preferentially has a high reproducibility (e.g. isolated homogeneous or specked on CAD systems)
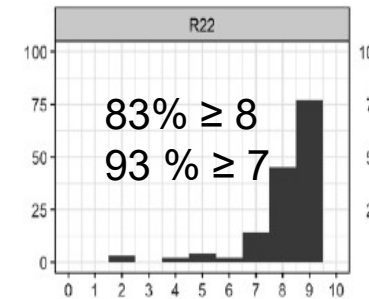>
> Grade A/B

R23

88% ≥ 8
98 % ≥ 7
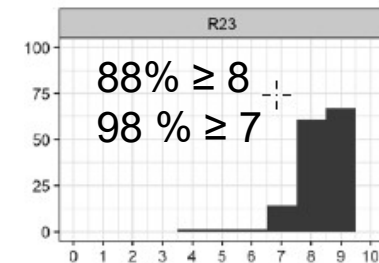
R19-R25: Quality approaches in HEp-2 IFA

R 22.

In addition to kit controls, it is advised
to run IQC samples from patient origin,
either pooled or unique samples
as they are processed as routine samples (thus allowing
monitoring of the whole assay procedure)

Grade A/B

83% ≥ 8
93 % ≥ 7

Acceptance criteria
+/- 1 (2) titers
Mean +/- 2 SD Probability index/FI (CAD)

Quality approaches in HEp-2 IFA. Survey

Variability in approach towards run IQC > origin and dilution of the IQC samples

Origin of the IQC samples (n=296)	%
Commercial origin only	58
Patient origin only	13
Commercial and patient origin	29

Dilution of the IQC samples (n=296)	%
Undiluted (different from patient samples)	63
Diluted (same dilution as patient samples)	30
Diluted (different dilution as patient samples)	7

Pooled or single patient IQC samples (n=128)	%
Pooled patient sample	11
Single patient samples	64
Pooled and single patient samples	24

IQC patient control CAD
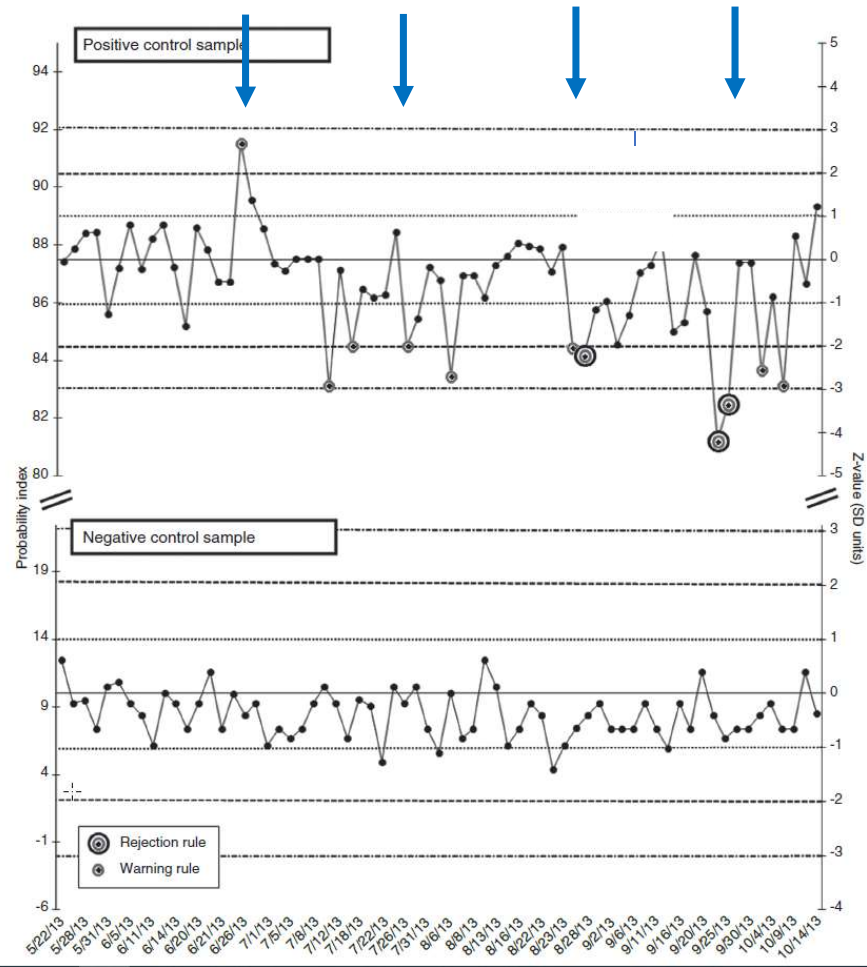
Data UZ Gent

IQC patient control CAD
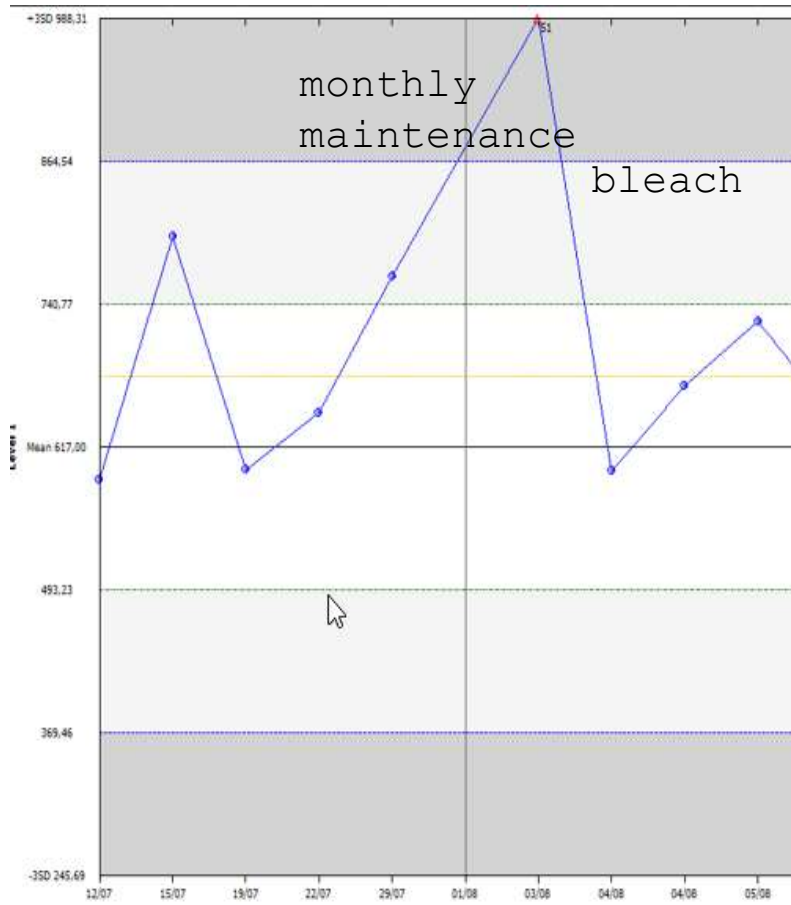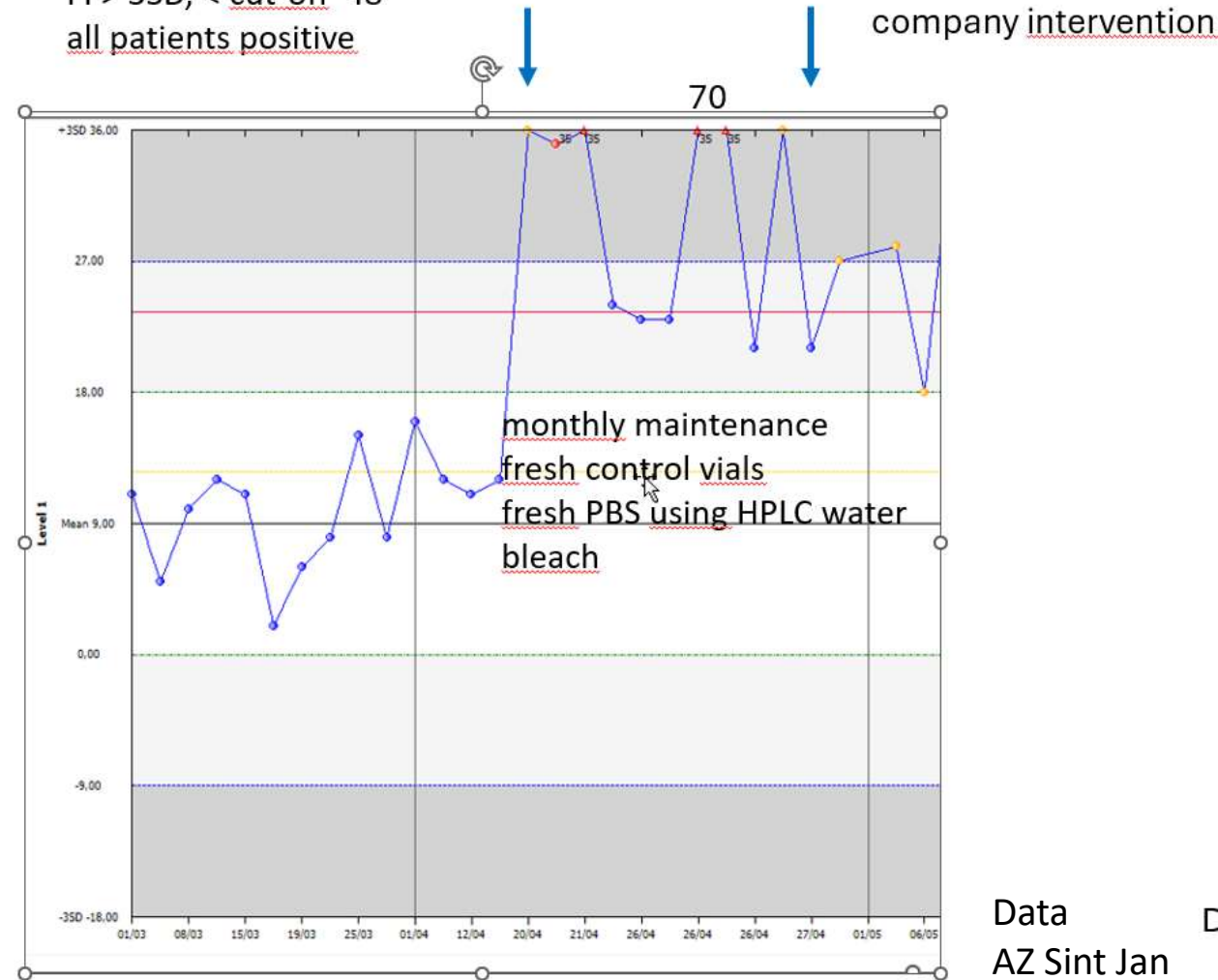
Positive patient IQC

Negative patient IQC

885

741

Post yearly maintenance

Change light guide
Supplementary wash
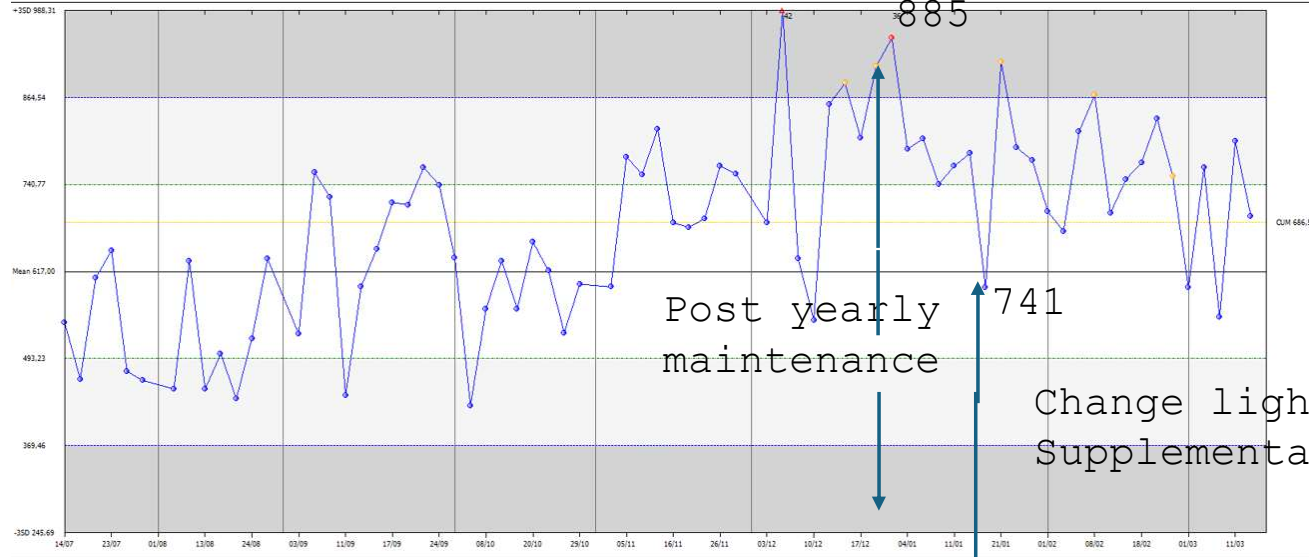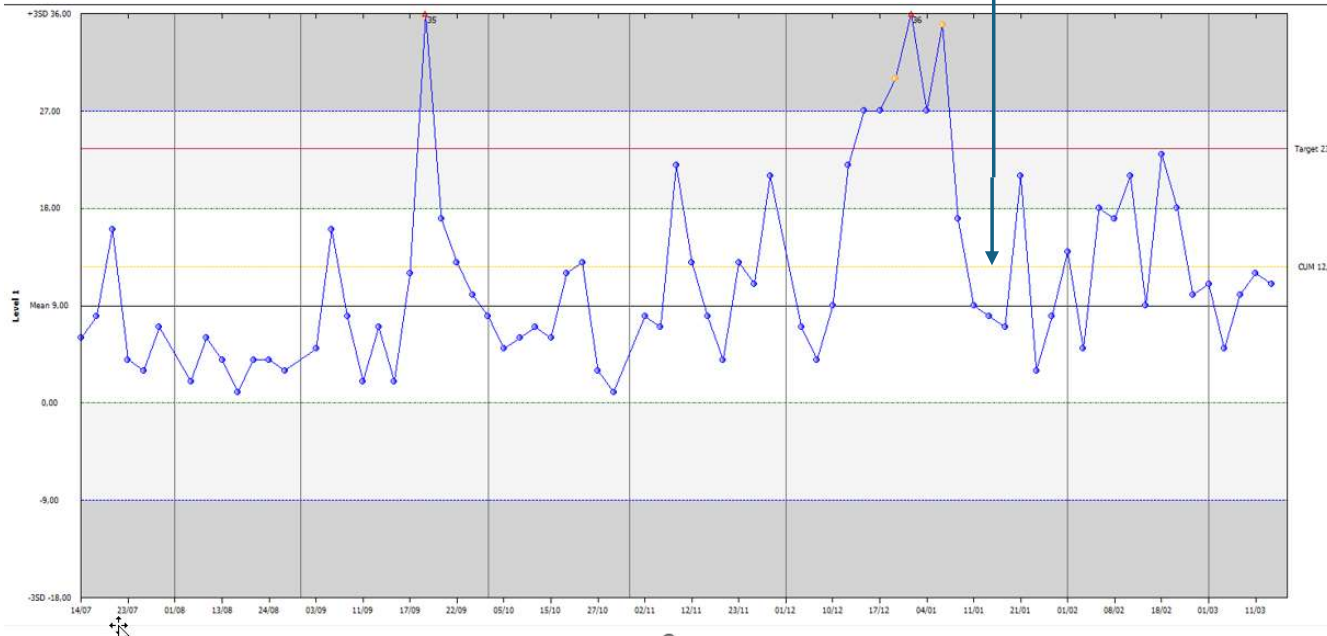
Artifically induced errors detectable by IQC and Quality indicators

**Table 3:** Impact of each error on the different quality indicators.

	Rescanning 5×				Slide incubation >3 h	Old conjugate 3 months	Needle contamination
	Scan 2	Scan 3	Scan 4	Scan 5			
LIU[a] positive kit iQC[b]	−11.1%	−13.3%	−19.7%	−26.2%	−9.4%	−1.8%	−0.8%
LIU[a] negative kit iQC[b]	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
LIU[a] positive sample iQC[b] speckled	−35.1%	−45.4%	−50.2%	−69.4%	−40.5%	−49.2%	194.7%
LIU[a] positive sample iQC[b] homogeneous	−17.7%	−49.6%	−39.5%	−63.2%	−25.6%	−56.6%	134.9%
LIU[a] negative sample iQC[b]	−16.7%	−11.1%	−33.3%	−47.2%	−11.1%	−44.4%	1276.0%
% positive ANA IIF[c] Patient samples/run	−8.6%	−4.0%	−10.0%	−10.0%	0.0%	−10.0%	50.0%
Median patient sample LIU[a]/run	−14.9%	−41.5%	−54.9%	−65.9%	−24.7%	−60.1%	166.6%

	Needle obstruction	Contrad dilution	PBS buffer dilution	Sample wash step	Conjugate wash step	Old buffer
LIU[a] positive kit iQC[b]	1.4%	6.2%	−2.4%	−10.5%	−2.5%	−4.2%
LIU[a] negative kit iQC[b]	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
LIU[a] positive sample iQC[b] speckled	−85.7%	−30.4%	105.9%	−43.8%	43.1%	−8.1%
LIU[a] positive sample iQC[b] homogeneous	−92.2%	−33.1%	123.6%	−39.2%	64.6%	−12.8%
LIU[a] negative sample iQC[b]	−100.0%	−25.0%	1430.6%	−22.2%	1.9%	1.9%
% positive ANA IIF[c] Patient samples/run	−62.5%	0.0%	60.0%	10.0%	25.0%	0.0%
Median patient sample LIU[a]/run	−86.0%	−33.2%	303.4%	−37.5%	89.1%	−9.1%

Results are expressed as relative deviation in LIU from the target values (iQC) or reference run (median LIU/run and % positive/run), with LIU changes exceeding the warning limits highlighted in italic and changes exceeding the stop limits in italic and bold. [a]LIU, light intensity units; [b]iQC, internal quality control; [c]ANA IIF, anti-nuclear antibodies indirect immunofluorescence test.

Bogaert et al. CCLM 2019;57(7):990-998

Data OLV Aalst

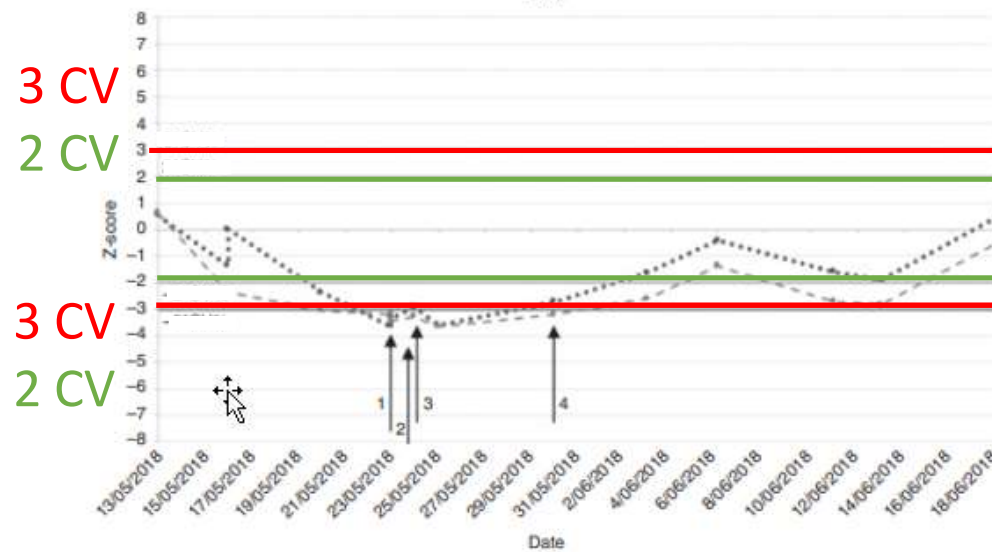# Routine use of IQC and Quality indicators

Z-score
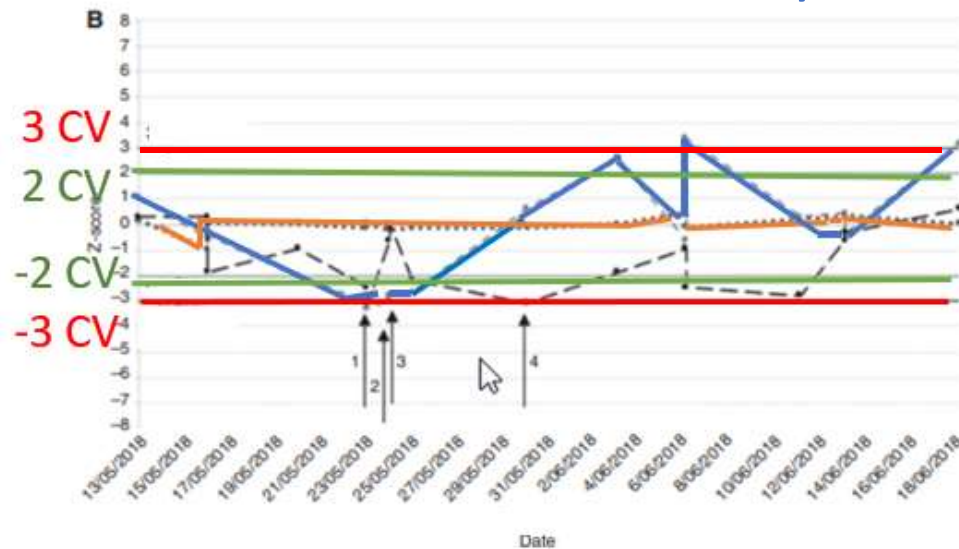
LIU positive kit IQC
Target LIU 2000

LIU positive patient IQC
Target LIU 210

LIU negative patient IQC
Target LIU 13

1 CV =25%

% Positive ANA samples/run
target 59%

Median patient sample LIU
Target 79

Adapted from Bogaert et al. CCLM 2019;57(7):990-998

Data OLV Aalst

Take home messages

Verification different cell compartments
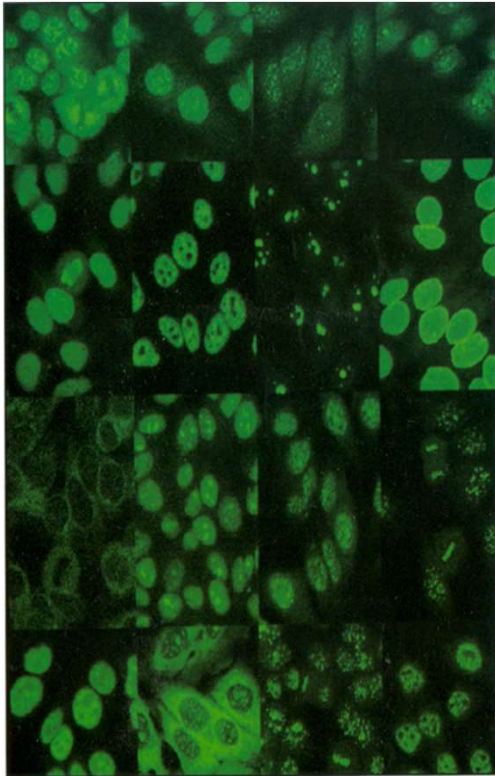Lot evaluation different antigens
titer/FI

Tools for IQC and quality performance

Titer/FI patient IQC % positive patients
median FI
% positive CTD/ENA

Thanks to all laboratory
technicians
and
colleagues !!!!!