Virus neutralization testing for different SARS-CoV-2 variants

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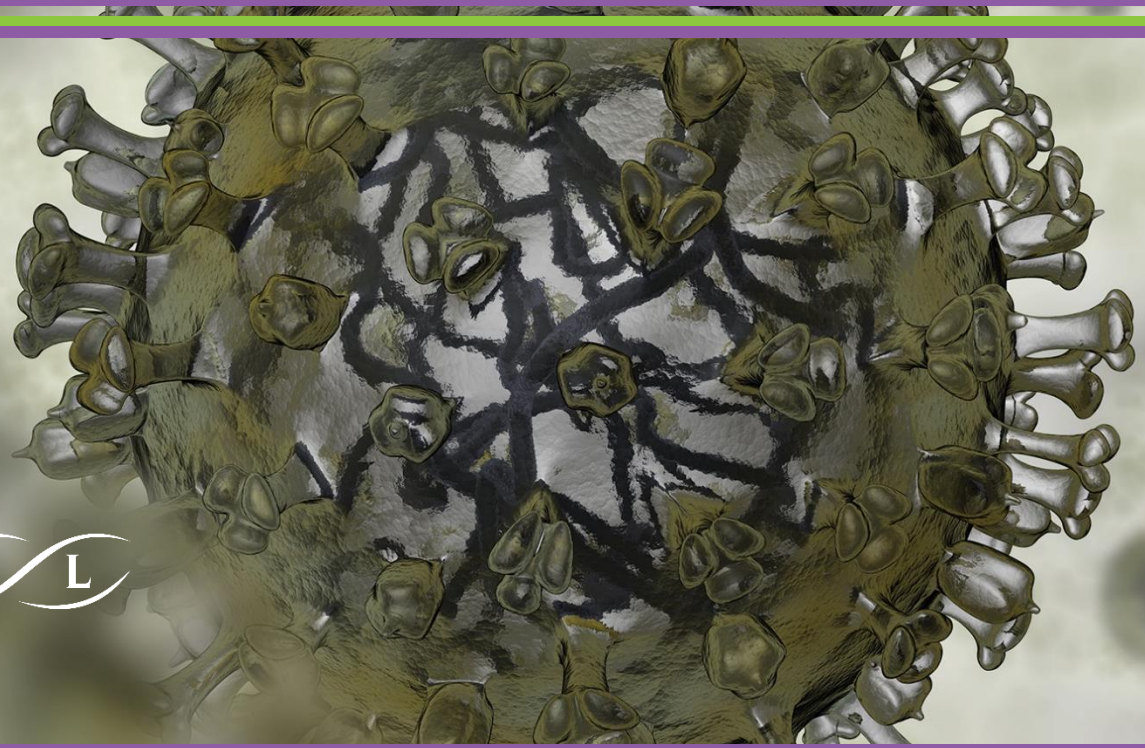


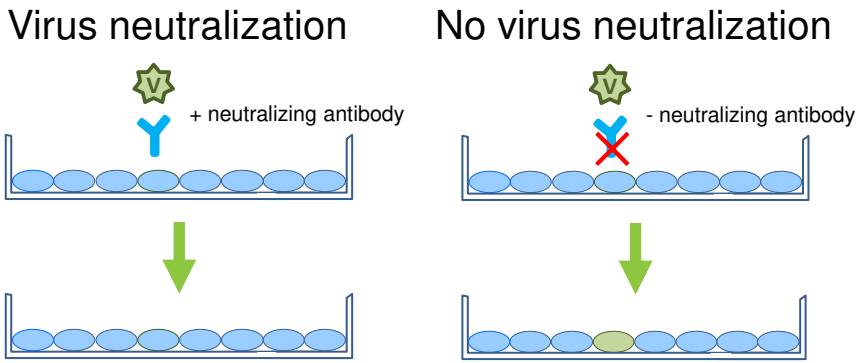
Viroclinics DDL
A Cerba Research Company

Measuring nAb titers in serum

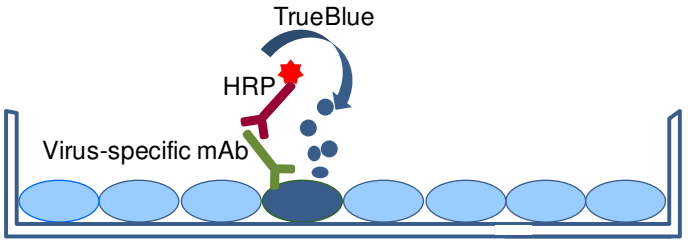
Assay format:

- Prepare serial 2-fold serum dilutions: 8 – 1,024 (vertical plate layout), or 8 – 8,192 (horizontal plate layout)
- Incubate serum dilutions with standardized amount of virus (1 hour; neutralization phase)
- Inoculate Vero-E6 cell monolayer with virus serum mixtures (1 hour; infection phase)
- Remove inoculum and incubate cells with fresh medium containing 1.6% CMC (Carboxymethyl cellulose; 22 hours; replication phase)
- Formalin fixation of the cells
- Perform virus-specific **ViroSpot immunostaining** of cells using a nucleocpasid-specific mAb (conserved epitope present in all virus variants to date)
- Count spots (micro-plaques) using C.T.L. Immunospot Image Analyser
- Compute serum neutralization titer

ViroSpot immunostaining



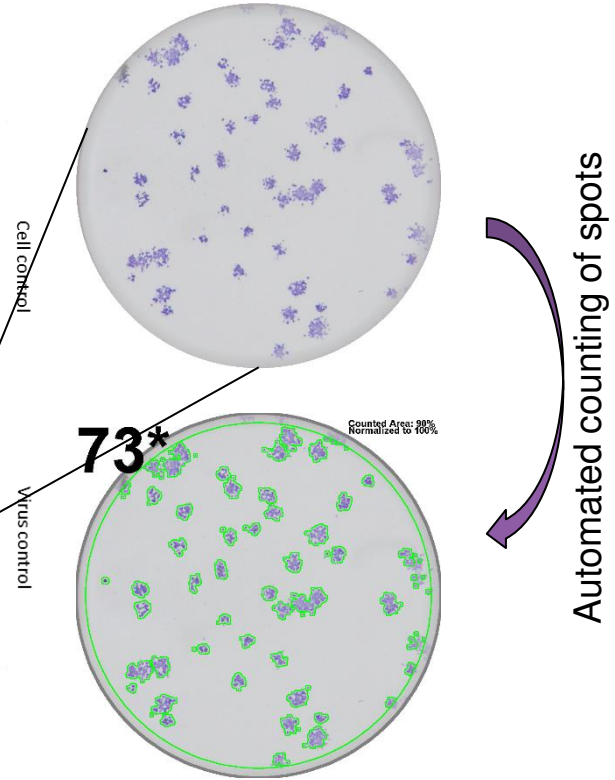
Immunostaining of
SARS-CoV-2-positive cells



The mAb targets the viral nucleocapsid protein

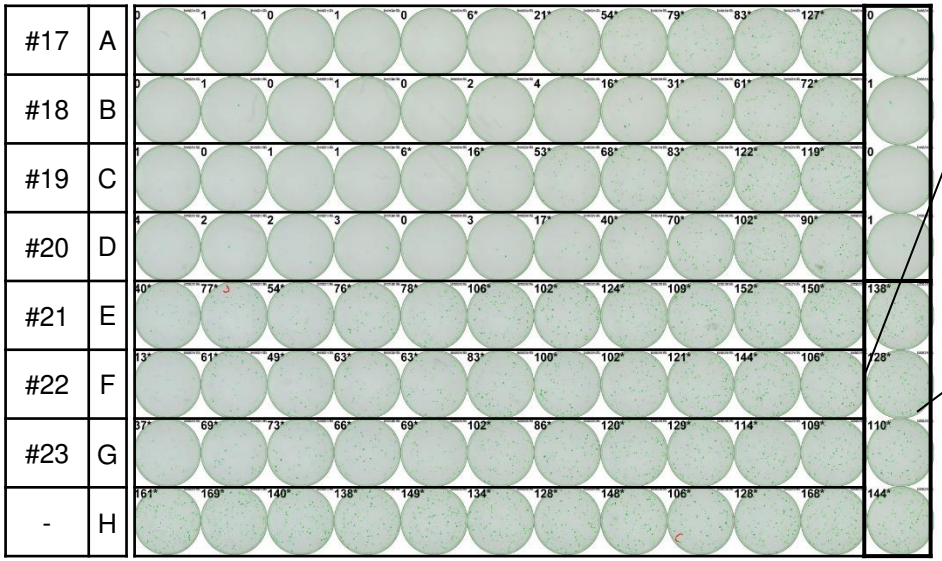
SARS-CoV-2 neutralization (D614G)

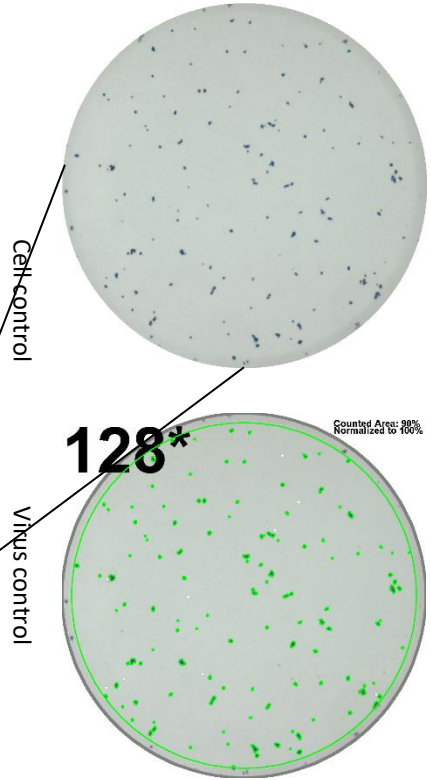
SARS-CoV-2 D614G		Plate 1A											
Dilution factor	Row	1	2	3	4	5	6	7	8	9	10	11	12
8	A												
16	B												
32	C												
64	D												
128	E												
256	F												
512	G												
1,024	H												
MN50		>1,024	>1,024	129	77	704	454	543	227	162	160	219	N/A
MN80		194	175	35	20	135	92	91	32	67	45	65	N/A
MN90		99	71	17	<8	28	52	25	19	42	27	32	N/A



SARS-CoV-2 neutralization (BA.1)

BA.1	Plate 24											
Sample	1	2	3	4	5	6	7	8	9	10	11	12



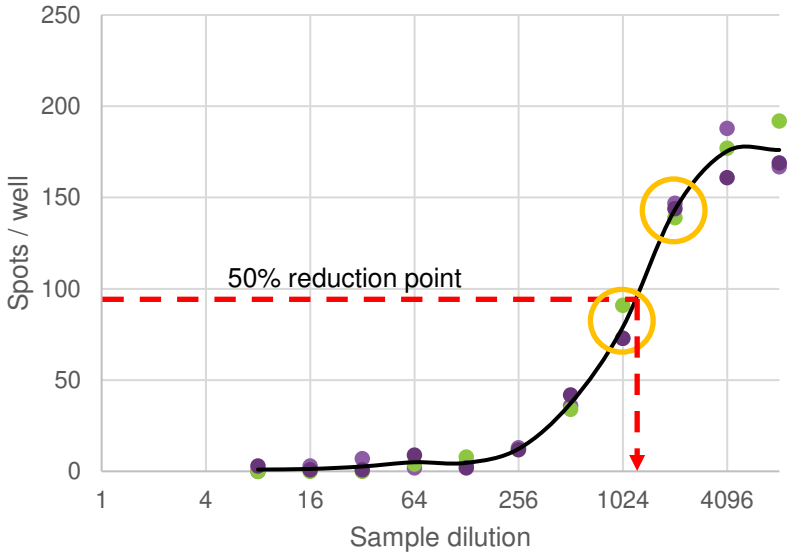


Automated counting of spots

BA.1 microplaques are notably smaller than D614G microplaques

Yet also the small microplaques can be readily distinguished and counted by the Biospot software of the C.T.L. immunospot Analyser

Calculation of nAb titer

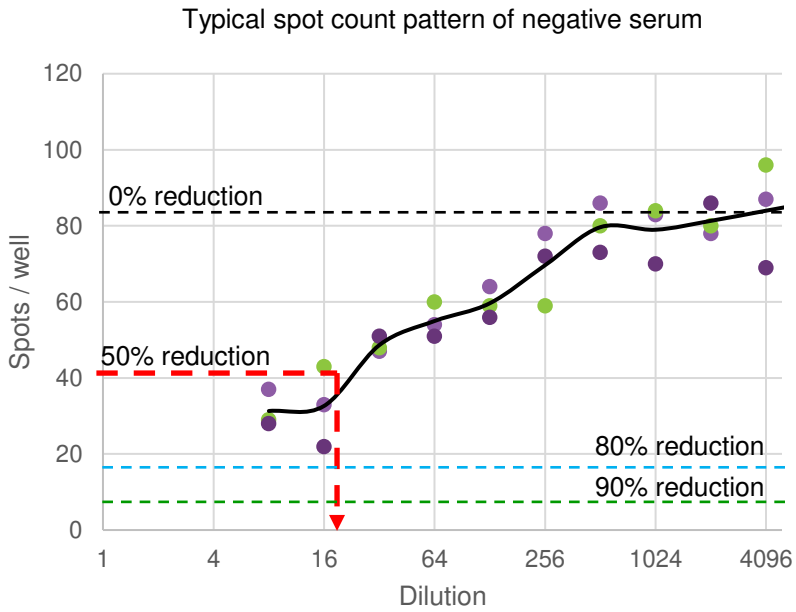


- The nAb titer (MN50 in this example) is calculated using the ICx formula from Zielinska et al 2005.
- This formula uses the sample dilutions directly above and below the reduction point (50%) AND the spot counts corresponding with those dilutions to calculate the MN50 titer
- For variant D614G, MN titers can be converted into international units per mL (IU/mL), since the positive control sample is calibrated against the WHO International Standard.
- Expressing results in IU/mL facilitates the comparability of results between different assays and different labs

Zielinska, Edyta et al. "Development of an improved microneutralization assay for respiratory syncytial virus by automated plaque counting using imaging analysis." *Virology journal* vol. 2 84. 9 Nov. 2005

Specificity

- Pre-SARS-CoV-2 sera (collected <2019) were included in the validation to assess specificity, as these sera should be negative for SARS-CoV-2 nAbs. Two were tested for binding antibodies using a MSD kit. The BAU for S, RBD and N were <LLOQ.
- Testing eight of these sera on three occasions in the MN assay showed that **at low serum dilutions** spot count were **sometimes** reduced just below 50% reduction point, but **never** below the 80% reduction point.
- These observations suggest that non-specific inhibition of infection may lead to false-positive MN50 titers on some occasions when testing human sera at dilutions as low as 1:8 and 1:16.
- To limit the number of false-positive MN50 results, we now compute MN50 titer only if the 1:8 dilution shows ≥75% reduction.



Specificity

Sample number	Omicron BA.1		
	MN50*	MN80	MN90
21	32 (<8)	<8	<8
	62 (<8)	<8	<8
	<8	<8	<8
22	<8	<8	<8
	162 (<8)	<8	<8
	50 (<8)	<8	<8
23	<8	<8	<8
	97 (<8)	<8	<8
	<8	<8	<8
24	<8	<8	<8
	<8	<8	<8
	24 (<8)	<8	<8
25	25 (<8)	<8	<8
	<8	<8	<8
	20 (<8)	<8	<8
26	<8	<8	<8
	<8	<8	<8
	<8	<8	<8
27	66 (<8)	<8	<8
	<8	<8	<8
	16 (<8)	<8	<8
28	16 (<8)	<8	<8
	<8	<8	<8
	<8	<8	<8

Sample number	Delta		
	MN50*	MN80	MN90
21	20 (<8)	<8	<8
	28 (<8)	<8	<8
	34 (<8)	<8	<8
22	<8	<8	<8
	<8	<8	<8
	32 (<8)	<8	<8
23	<8	<8	<8
	<8	<8	<8
	<8	<8	<8
24	<8	<8	<8
	<8	<8	<8
	128 (<8)	<8	<8
25	21 (<8)	<8	<8
	26 (<8)	<8	<8
	32 (<8)	<8	<8
26	<8	<8	<8
	<8	<8	<8
	<8	<8	<8
27	22 (<8)	<8	<8
	22 (<8)	<8	<8
	106 (<8)	<8	<8
28	<8	<8	<8
	<8	<8	<8
	50 (<8)	<8	<8

Sample number	D614G		
	MN50*	MN80	MN90
21	44 (<8)	<8	<8
	34 (<8)	<8	<8
	34 (<8)	<8	<8
22	72 (<8)	<8	<8
	44 (<8)	<8	<8
	14 (<8)	<8	<8
23	19 (<8)	<8	<8
	29 (<8)	<8	<8
	19 (<8)	<8	<8
24	23 (<8)	<8	<8
	<8	<8	<8
	9 (<8)	<8	<8
25	44 (<8)	<8	<8
	28 (<8)	<8	<8
	19 (<8)	<8	<8
26	<8	<8	<8
	<8	<8	<8
	<8	<8	<8
27	30 (<8)	<8	<8
	37 (<8)	<8	<8
	<8	<8	<8
28	35	<8	<8
	<8	<8	<8
	<8	<8	<8

*Values in brackets are results after applying the ≥75.0% reduction rule to 1:8 dilutions.

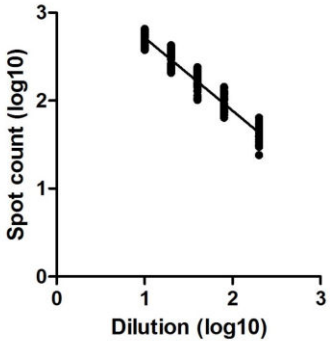
None of the pre-2019 sera showed reduction ≥ 80% or ≥ 90% at the lowest (1:8) dilution

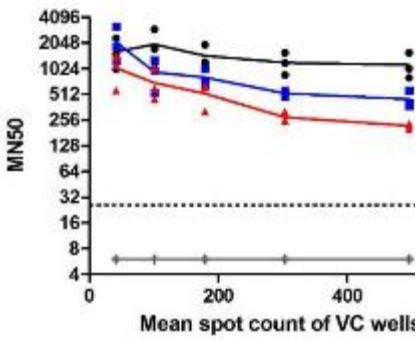
Sample #23 and #27:
S, RBD and N BAU <LLOQ

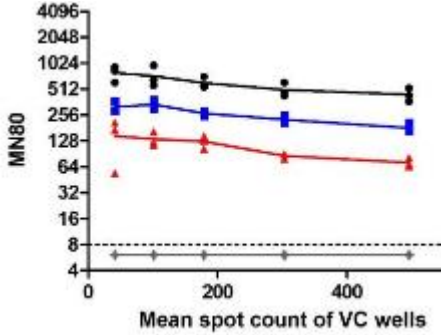
Note:
The possible cause(s) of inhibition that appears to be non-specific requires further investigation.

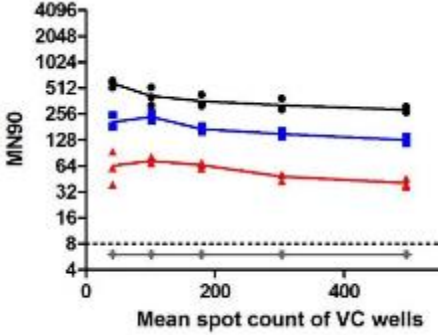
Robustness (BA.1 virus input vs MN titer)

Linear decrease of virus spot counts in virus stock dilutions









Dilution #	*Dilution (fold)*	*Mean spot count (n)*	*Mean ± 1SD (68%)*
1	10	496	411 – 581
2	20	301	237 – 365
3	40	181	139 – 223
4	80	103	81 – 125
5	200	41	32 – 50

Sample #13
Sample #14
Sample #15
} Sars-CoV-2 positive in January 2022

Sample #21
Sample #22
Sample #23
} Sera collected before 2019

(#23: S, RBD and N BAU <LLOQ in MSD)

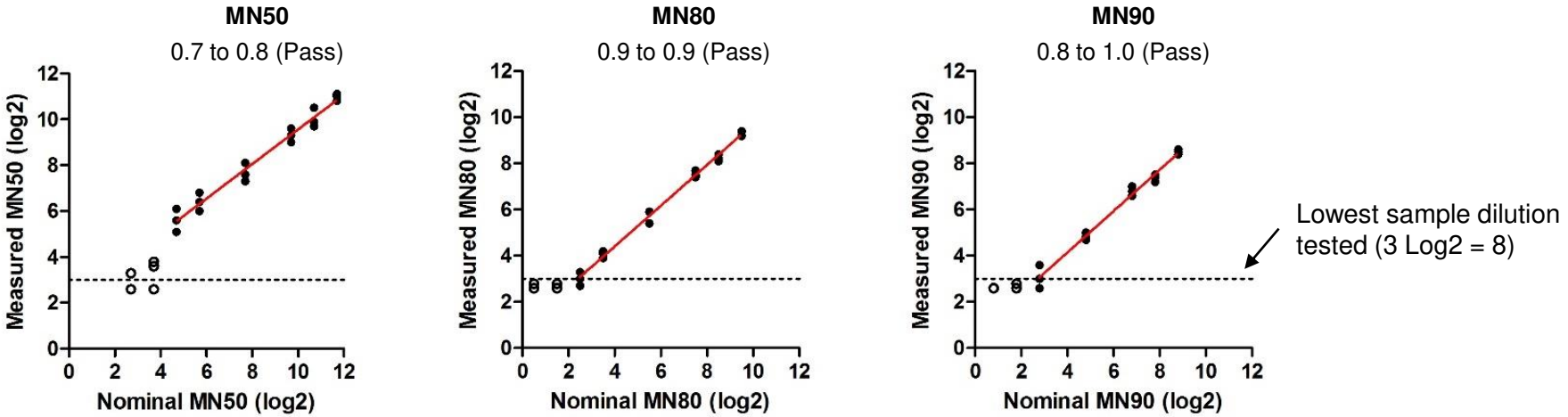
Below a mean spot count of 103 ± 1 SD (81-125), MN titers tended to be more variable and overestimate.

MN titer were stable across a large range of virus concentrations, here up to a mean spot count of 496 ± 1 SD (411-581)

Linearity (BA.1)

A high titer SARS-CoV-2 neutralizing antibody-positive serum sample was serially diluted in negative serum matrix to obtain samples with different antibody levels. These were tested three times per assay run on three occasions.

90% confidence interval of the slope (0.7 – 1.3)



Symbols are the mean log2 titer per sample per run.
Closed symbols: acceptance criteria for precision and relative accuracy were met.
Open symbols: GMT<8 or intermediate precision criteria not met.

Summary Omicron BA.1 MN validation

Validation parameter	Acceptance criteria	MN50	MN80	MN90
Specificity	90% of the determinations on samples qualified as negative should have a MN titer <LLOQ	Pass	Pass	Pass
Relative accuracy	Mean measured log2-transformed MN titers should differ no more than 1.0 log2 from the nominal log2-transformed MN titers.	Within assay range the difference was ≤0.9log2	Within assay range the difference was ≤0.5log2	Within assay range the difference was ≤0.4log2
Repeatability	The $GCVr$ should be ≤94.7%*	Within assay range the $GCVr$ was ≤55.3% Overall $GCVr$ was 37.6%	Within assay range the $GCVr$ was ≤31.0% Overall $GCVr$ was 20.3%	Within assay range the $GCVr$ was ≤32.3% Overall $GCVr$ was 18.3%
Intermediate precision	The $GCVi$ should be ≤94.7%*	Within assay range the $GCVi$ was ≤64.6% Overall $GCVi$ was 41.8%	Within assay range the $GCVi$ was ≤31.2% Overall $GCVi$ was 22.5%	Within assay range the $GCVi$ was ≤43.1% Overall $GCVi$ was 22.0%
Linearity	The 90% confidence interval of the slope is between 0.7 and 1.3	0.7 to 0.8	0.9	0.8 to 1.0
Dilutional Linearity	The mean measured log2-transformed MN titer of a sample diluted in IM, corrected for the dilution factor, differs no more than 1.0 log2 from the mean log2-transformed MN titer of the undiluted sample	Maximum allowed lowest starting dilution: 256-fold**	Maximum allowed lowest starting dilution: 256-fold**	Maximum allowed lowest starting dilution: 64-fold**

*These $GCVr$ and $GCVi$ acceptance criteria correspond to ≤ 1.0 log2 difference between MN titer results of each determination and the mean of all determinations per sample

**Higher starting dilutions may prove to be valid when more potent samples become available.

Summary Omicron BA.1 MN validation (Cont'd)

Validation parameter	Acceptance criteria	MN50	MN80	MN90
Lower limit of quantification (LLOQ)	Lowest MN titer in linear range meeting acceptance criteria of specificity, accuracy, repeatability and intermediate precision	26, using 8-fold starting dilution	8, using 8-fold starting dilution	8, using 8-fold starting dilution
Upper limit of quantification (ULOQ)	Highest MN titer in linear range meeting acceptance criteria of accuracy, repeatability and intermediate precision	3,291, using 8-fold starting dilution 105,320, using 256-fold starting dilution	743, using 8-fold starting dilution 23,770, using 256-fold starting dilution	446, using 8-fold starting dilution 3,565, using 64-fold starting dilution
Range	All measurements within the assay range should meet acceptance criteria of linearity. Range is confined by the LLOQ and ULOQ	26 – 105,320	8 – 23,770	8 – 3,565
Robustness: Virus input	The virus spot count should be proportional to the virus stock dilution factor. Difference in MN titer results between acceptable lowest and highest virus input should not be >fourfold. Results of negative samples should be <LLOQ	81 – 581 spots per well		

Conclusions

- The SARS-CoV-2 MN assays on the ViroSpot immunostaining platform employ mAb's directed against conserved epitopes, which detected all SARS-CoV-2 virus variants to date
- The specificity of MN50 results can be improved by computing titers only if the lowest serum dilution shows ≥75% reduction of infectivity.
- The assay has been validated for early and recent variants, including D614G, Delta and Omicron BA.1
- Validation for BA.2, BA.4 and BA.5 is underway

- For more information by e-mail:
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