Rotavirus potency assay by $CCID_{50}$ method

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Rotavirus assay ($CCID_{50}$)

- Objectives
- Titration method
- Validation data
- Batch release testing
- Additional comments

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Rotavirus assay ($CCID_{50}$)

Objectives

1. To determine the viral titre in rotavirus in the batch submitted for lot release
2. To determine the identity G1 of the vaccine strain

Rotavirus assay ($CCID_{50}$)

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Rotavirus assay ($CCID_{50}$)

Titration method principle

To determine the potency of Rotavirus by assessing the dose infecting 50% of MA-104 cell monolayer (monkey kidney –by end-point dilution) followed by revelation with MoAb 2C9 (anti-VP7) and immunoperoxydase staining or indirect immuno-fluorescence

Rotavirus assay ($CCID_{50}$)

1. Preparation of cell substrate
2. Virus activation step (30min) dil 1/10 & Successive dilutions in activation medium
3. Inoculation virus suspension & incubation at 37± 1° C for 7 ± 1 days

Rotavirus assay ($CCID_{50}$)

4. Revelation: MoAb + immunoperoxydase or indirect immunofluorescence staining
5. Reading & Calculation of titres by Spearkman-Kärber method (or Reed-Muench)($logCCID_{50}/ml$)
6. Validity of the assay (criteria)
7. Acceptance of the batch (criteria)

1. Preparation of cell substrate

- MA-104 foetal monkey kidney cells
- cell growth medium:
  EMEM – 1% L-Glu – 10% FCS
- 50.000 ± 10.000 cells/ml
- 200 µl/well (microplates 96-wells)
- incubation 4 days at 37 ± 1C° - 5 ± 1 % CO_2
- monolayer confluent (100%)

2. Virus activation step & dilutions

- Reconstitution of vaccines (3 vials + 3 "stability" and reference preparation (1 vial in triplicate) with 1ml WFI
- dilution 1/10 in activation medium
  EMEM – 1% L-Glu -1% Ab – 0,32% trypsine
- 20-30 min at RT
- successive dilutions (1/10 – 1/4) - same medium

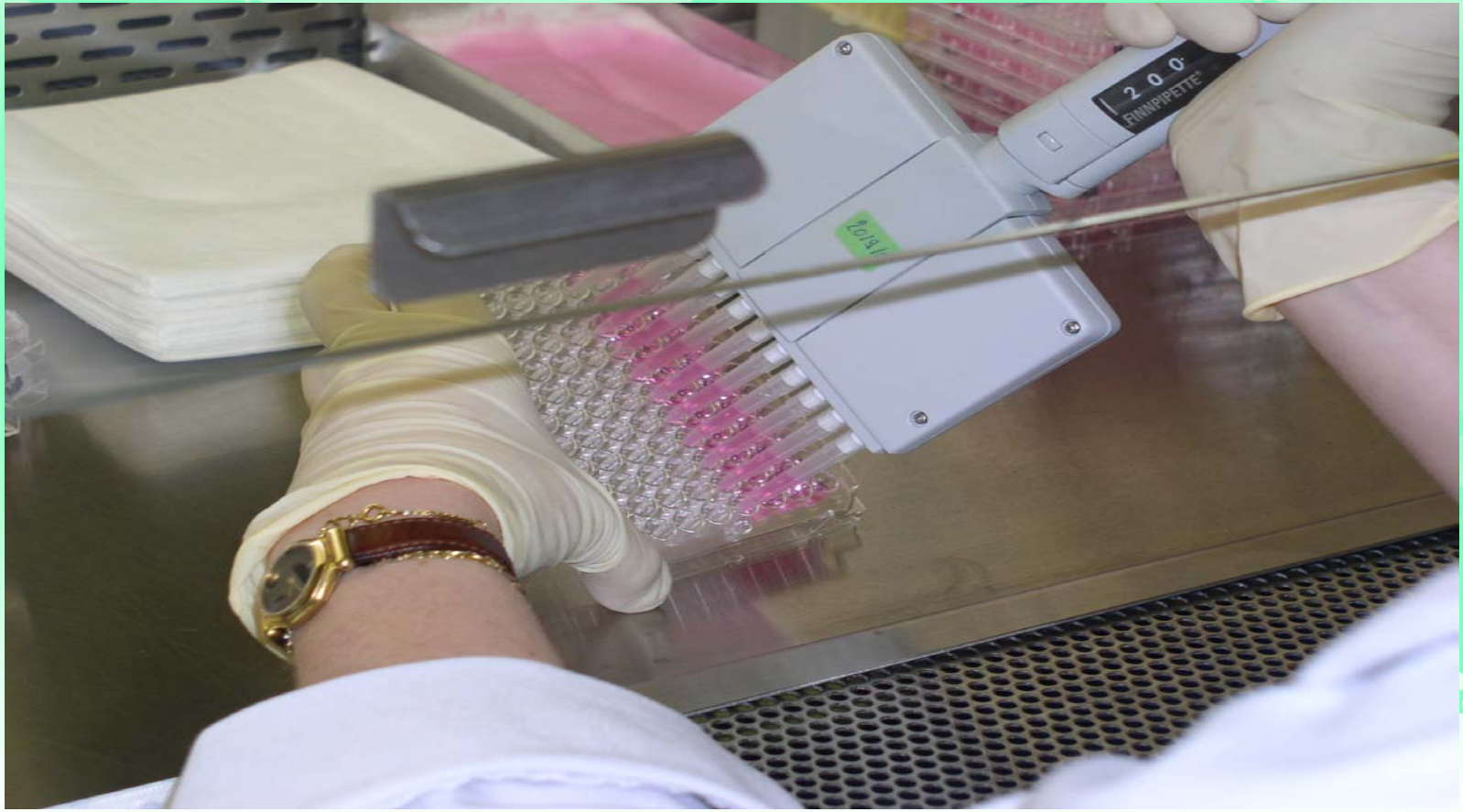
3. Inoculation of virus suspensions

- Microplates washed 2 times (150 µl/well)
- Carefully – multichannels
- Inoculation : 100µl/well - 10 wells/dilution – x dilutions/vial

1	2	3	4	5	6	7	8	9	10	11	12
4,6	4,6	4,6	4,6	4,6	4,6	4,6	4,6	4,6	4,6		T
5,2	5,2	5,2	5,2	5,2	5,2	5,2	5,2	5,2	5,2		T
5,8	5,8	5,8	5,8	5,8	5,8	5,8	5,8	5,8	5,8		T
6,4	6,4	6,4	6,4	6,4	6,4	6,4	6,4	6,4	6,4		T

- Incubation 7 days - 37± 1°C - 5 ± 1 % CO_2- HR

Washing before virus inoculation



4. Revelation

- To wash microplates (150 µl/well)
- Fixation : iced acetone solution (80%) – 20 min at -20°C - 150µl/well
- Acetone discarded (kleenex) and let dry
- 100µl MoAb 2C9/well (1/250 in PBS w/o Ca^{++} and Mg^{++} with 5% skimmed milk ="blotto")
- Incubation 1 hour at 37± 1°C
- To wash µplates 4 times with PBS w/o Ca^{++} and Mg^{++}

Distribution of MoAb anti-VP7

4a. Revelation by immunoperoxydase

- Ab Anti-mouse IgG conjugated to peroxydase (1/400 in blotto) – 50µl/well - incubation 1 h - 37°C ± 1°C.
- To empty µplates & wash 4 times /PBSw/o
- Fresh solution : 1 tablet DAB + 1 tablet urea in 30 ml distillated water
- 50 µl/well incubation 10 min at RT (25± 5°C).
- To wash µpls 3 times in tap water
- To let dry (kleenex wypall)

4b. Revelation by indirect immunofluorescence staining

- anti-mouse IgG conjugated to FITC (1/200 in blotto + Evans Blue 1/300)
- 100µl/well – Incubation 1 hour at 37± 1°C
- To wash µpls 4 times in tap water
- To let dry (kleenex wypall)

5. Reading & calculations

- reading : wells with stained cells = rotavirus positive
- calculation of titre by Reed-Muench or Spearman-Kärber
- estimation of the precision by Irwin-Cheesman

VACCIN

Frais

Date du test: -2004

	na	**na**	**na**
	na	**na**	**na**
7,2			
6,6			
6			
5,4			
4,8			
4,2			
	na	**na**	**na**

Spécification : ? 6,2 logCCID $_{50}$ /1,0 ml

Frais

Titre moyen: **=>** **#DIV/0!**

95%: **=>** **#DIV/0!**

Limites: **=>** **#DIV/0!** - **#DIV/0!**

37°C

	na	**na**	**na**
	na	**na**	**na**
7,2			
6,6			
6			
5,4			
4,8			
4,2			
	na	**na**	**na**

Stabilité

Titre moyen: **=>** **#DIV/0!**

95%: **=>** **#DIV/0!**

Limites: **=>** **#DIV/0!** - **#DIV/0!**

Perte de titre : **=>** **#DIV/0!**

Spécification: ?0,5 logCCID $_{50}$ /1,0 ml

REFERENCE

Gsk

RVC021A44/Q

6,4			
5,8			
5,2			
4,6			
	na	**na**	**na**
	na	**na**	**na**
	na	**na**	**na**

Gsk *Titre : 6,5 logCCID $_{50}$ /ml*

Titre moyen: **=>** ########

95%: **=>** ########

Limites: **=>** ######## - ########

n*= 0

Spearman-Kärber

$-\log CCID_{50} = -\log_{10}(d1) - [\log_{10}(d) \times (Sp-0,5)]$

Where

d1 = highest dilution with 100% CPE

d = dilution step (i.e. 0,6log)

Sp = sum of proportion of all positive wells

Spearman-Kärber

N° lot	Dilution	Positive recorded
	-4,6	10
	-5,2	8
	-5,8	2
	-6,4	0

- log $CCID_{50}$ = - 4,6 - [0,6 X ((10 + 8 + 2 + 0) / 10 - 0,5)]
- log $CCID_{50}$ = - 4,6 – 0,9
- log $CCID_{50}$ = - 5,5
+ log $CCID_{50}$ = + 5,5

Correction factor: $\log_{10}$ 1000 µl / 100µl = **1,0**
Vaccine titre : 5,5 + 1,0 = 6,5 logCCID50/ml

Precision by Irwin-Cheesman

$$s = \log d \times \sqrt{\sum [(p \times (1-p)) / n - 1]}$$

s = std deviation

d = dilution step

$\sum$ = sum of all dilutions

p = proportion of infected (positive) wells for one specific dilution

1 - p = proportion of non-infected (negative)wells

n = nr of inoculated well/dilution

Precision by Irwin-Cheesman

Dilution	Positive recorded	p	1-p	p x (1-p)
-4,6	10	10/10	0/10	0
-5,2	8	8/10	2/10	16/100
-5,8	2	2/10	8/10	16/100
-6,4	0	0/10	10/10	0

$s = 0{,}6 \times \sqrt{(32/100) / 9}$

$= 0{,}6 \times 0{,}1886$

$= 0{,}1132$

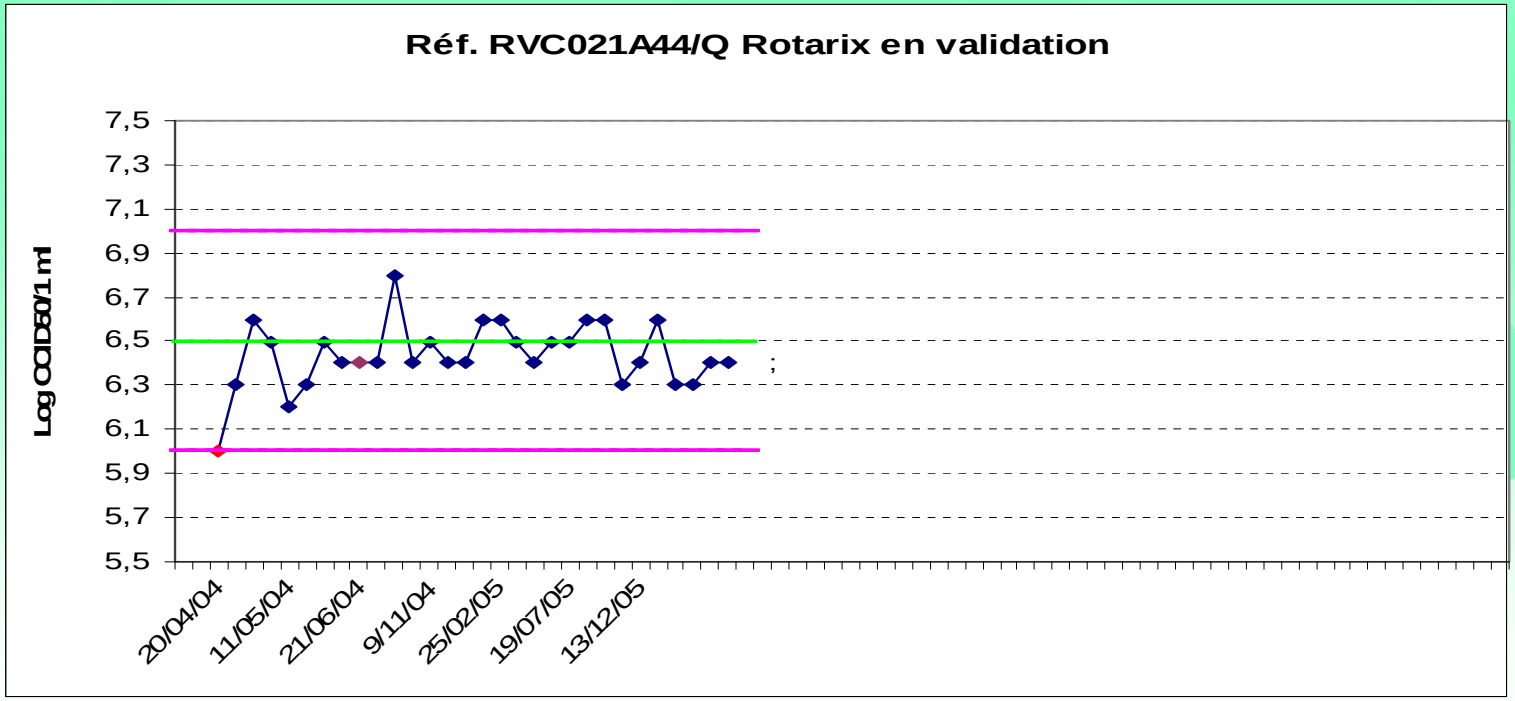
Confidence interval (P=0,95) : 0,1132 X 1,96 = 0,22= 0,2

Virus titre : 6,5 ± 0,2 log $CCID_{50}$/ dose

6. Validity of the assay

- negative control cells : free of toxicity or abnormalities and do not show any fluorescent/coloured cells
- Homogeneous distribution of CPE vs dilutions (10-90 % of CPE on 3 dilutions)
- the confidence interval (P=0,95) of the vaccine virus titre is ≤ 0,3log (*)
- max diff. 0,5log between 2 vials/3 (*)

- reference titre: Observed reference titre within 0,5 log of its established mean titre - Company: 6,5 ± 0,5 logCCID50/ml **or** IPH titre: 6.4/1ml - LCL: 6.0/1ml - UCL: 6.9/1ml – n=30



7. Acceptance of the batch (criteria)

- the estimated titre of the batch meets the release specification : ≥ 6,2 log$CCID_{50}$/ml
- the maximum loss of titre is 0,5 log$CCID_{50}$/ml

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Rotavirus assay ($CCID_{50}$)

- Validation data

1. The test specificity
2. The range of titration
3. The limit of detection
4. The precision (intra-assay repeatability)
5. The reproducibility (inter-assay repeatability)

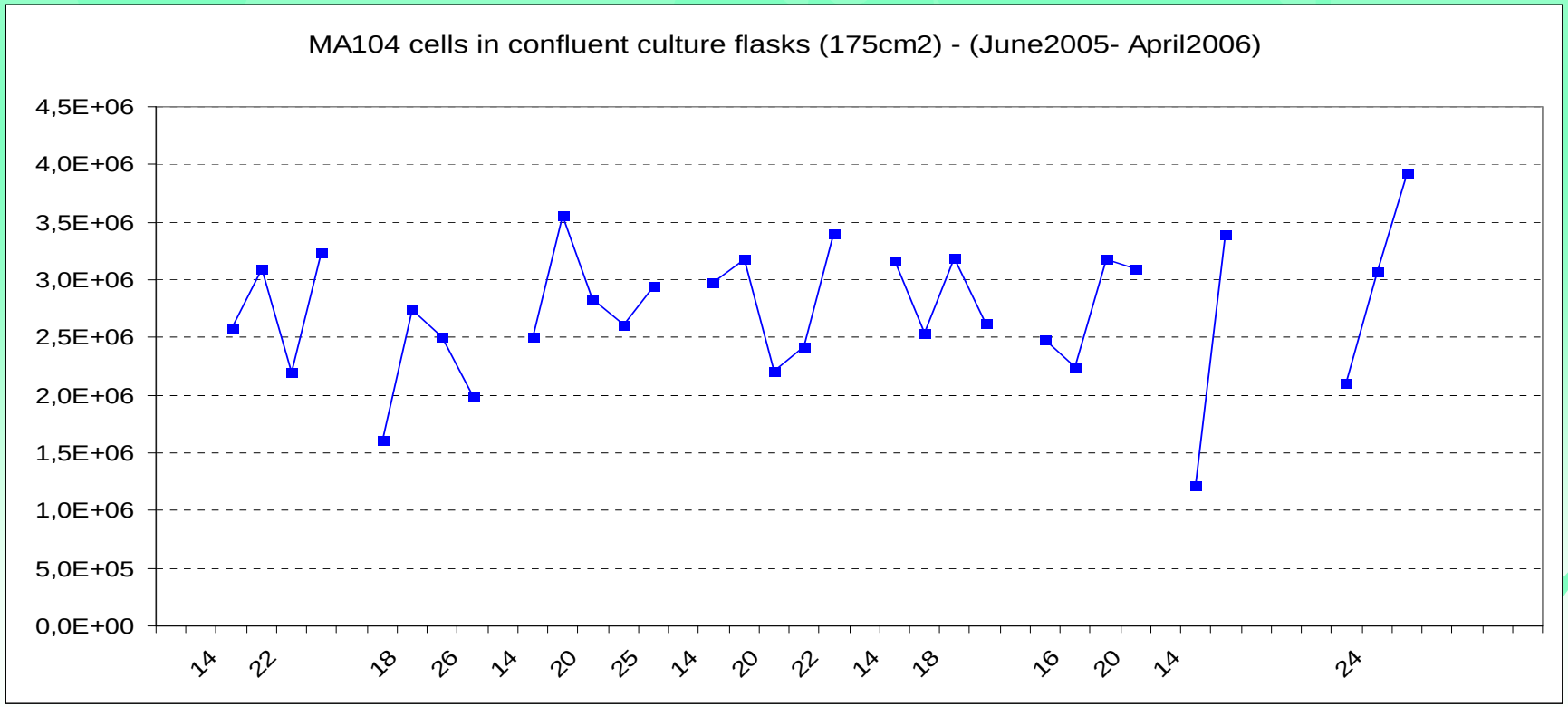
Rotavirus assay ($CCID_{50}$)

1. The test specificity

- MA-104 cells
- Trypsin activation step
- MoAb anti-VP7 & Validation data provided by Manufacturer (each new MoAb batch)
- File: demonstrated in identity test performed by seroneutralisation

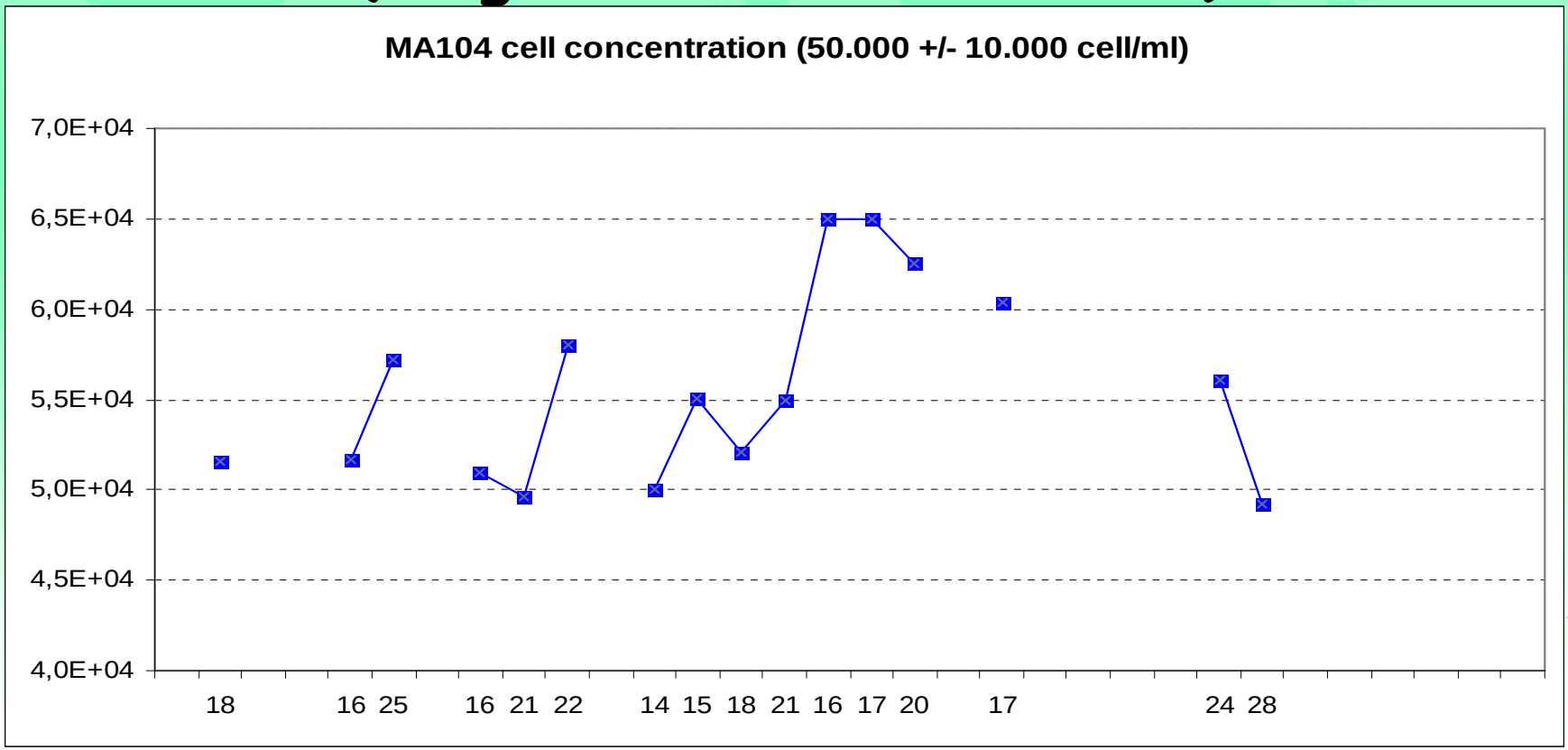
Rotavirus assay ($CCID_{50}$)

- Validation on MA-104 cells
  - cell bank (nr of passages)
  - cell concentration in culture flasks



Rotavirus assay ($CCID_{50}$)

- MA-104 cells
  - cell concentration inoculated (target 50.000+/-10.000)



Rotavirus assay ($CCID_{50}$)

2. The range of titration

   In practice: choice of range of dilution

3. The limit of detection

   End-point dilution method (0-100% CPE)

Rotavirus assay ($CCID_{50}$)

4. The precision (intra-assay repeatability)

one sample – x replicates – same day

Date	replicate 1	replicate 2	replicate 3	mean	max diff	stdev
14/03/2006	6,2	6,5	6,3	6,3	0,3	0,15
28/03/2006	6,2	6,3	6,3	6,3	0,1	0,06
4/04/2006	6,6	6,6	6,7	6,6	0,1	0,06
			Mean	6,4	0,2	0,09

Rotavirus assay ($CCID_{50}$)

5. The reproducibility (inter-assay repeatability) – time - operators

RVC021A44/Q in-house ref.		
Nr	30	
Mean	6,4	
Stdev	0,2	
cv (%):	2,4%	
min	6,0	
max	6,8	
	Lower Limit	Upper limit
m± 2s = alert limits	6,1	6,7
m± 3s = action limits	6,0	6,9

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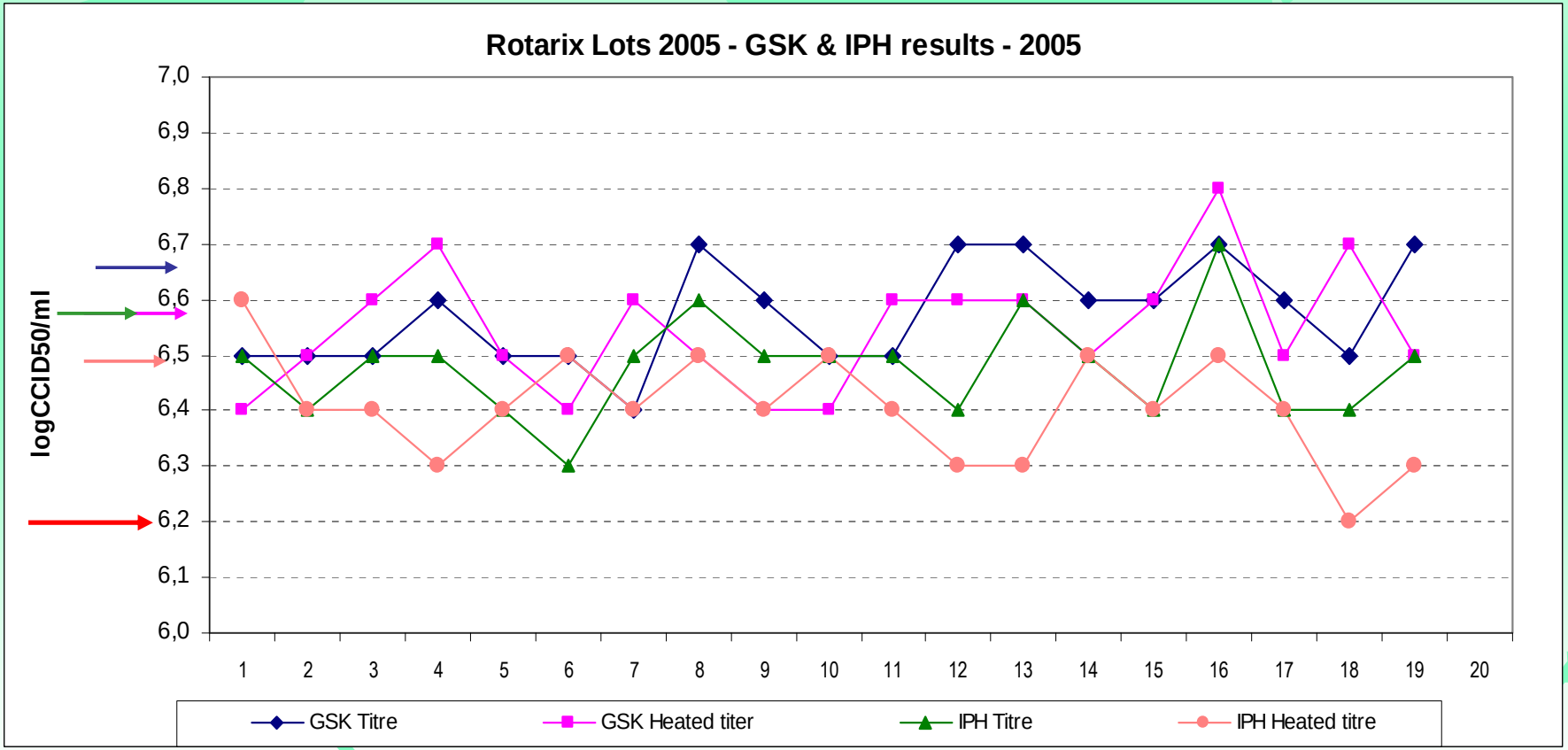
Rotavirus batch release testing

1. Appearance
2. pH
3. Identity (by titration)
4. Potency
5. Thermal stability (7d - 37±1°C)

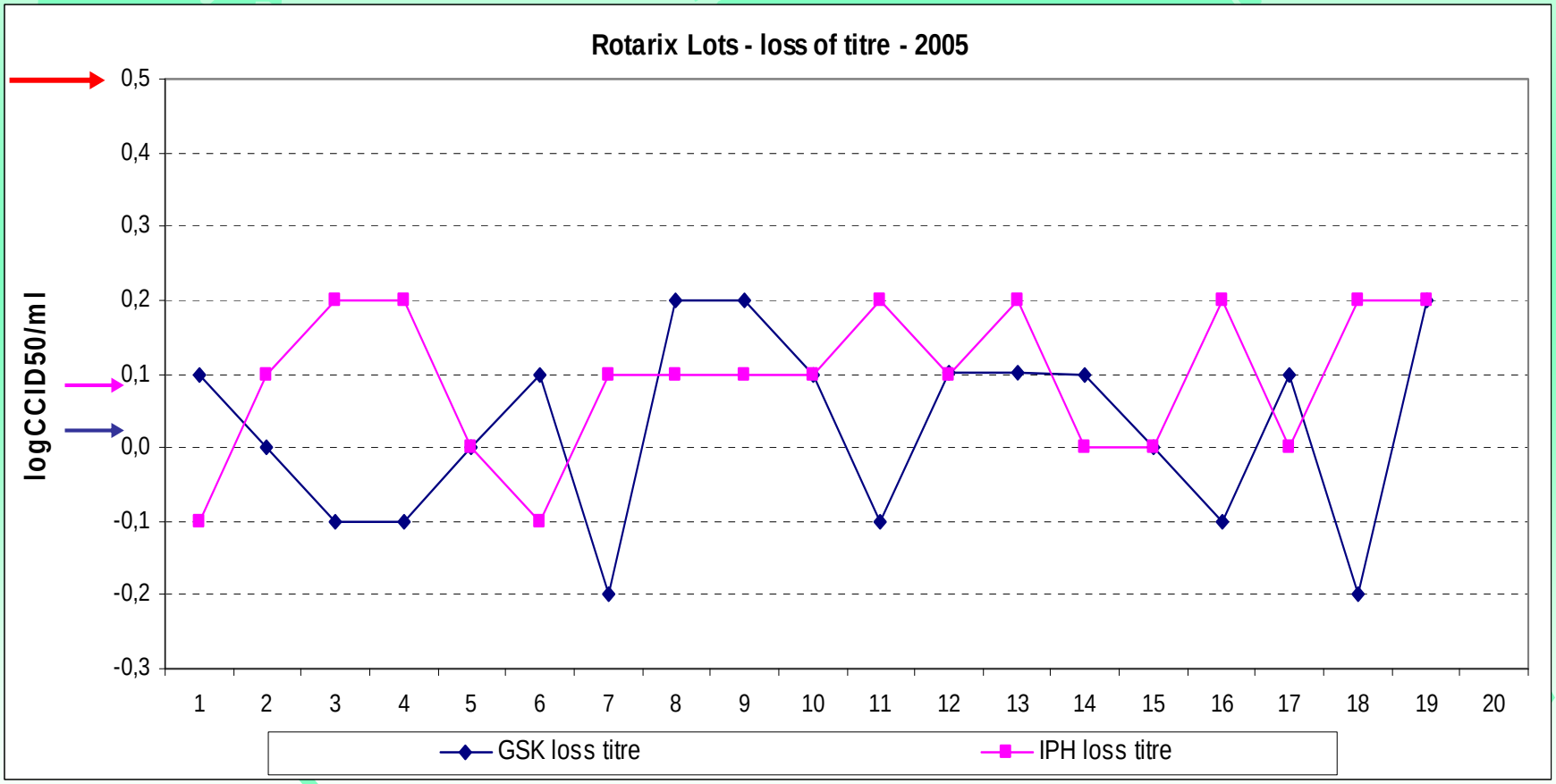
Rotavirus batch release testing

Test	Specification
Appearance before reconstitution	Whitish cake or powder
Appearance after reconstitution	Clear colourless solution
pH after reconstitution with WFI	Between 7,5 and 8,5
Identity	Positive by titration
Titre	≥ 6,2 logCCID50/ml
Heated titre (7d, 37°C)	To be determined (logCCID50/ml)
Titre loss	Not more than 0.5 $\log_{10}$ $CCID_{50}$/vial from the release titre

Rotavirus batch release testing



Rotavirus batch release testing



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Additional comments

- Availability of qualified
  - ✓ MA-104 cell bank
  - ✓ Stock of MoAb 2C9
- Expensive equipment (Epi-fluorescence)
- Questions ?

Rotavirus assay ($CCID_{50}$)

MUCHAS GRACIAS POR SU ATTENCION