CELL-BASED TOXICITY ASSAYS

REPLACING ANIMAL TESTS FOR CLOSTRIDIAL ANTIGENS

Mohammad Daas
Silvia Fragoeiro
Imke Kross
MSD Animal Health, Milton Keynes, UK

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MSD Animal Health

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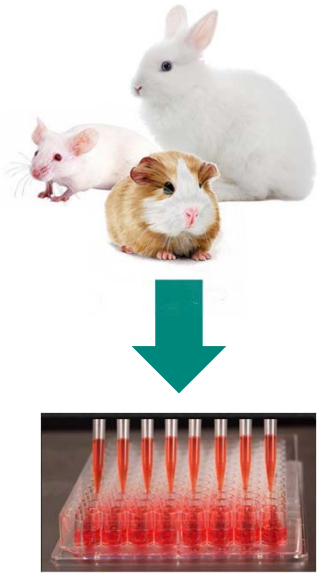

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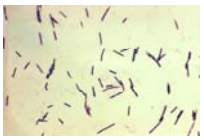
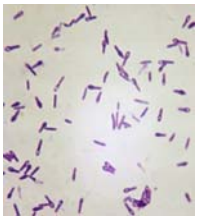
Introduction – Clostridial antigens and vaccines

- *Clostridial* bacteria are ubiquitously found in soil and the intestines of animals.
- Infection can be triggered by a variety of conditions ranging from a change in diet through to shearing cuts.
- Toxins produced by clostridia are typically responsible for pathology.
- Most clostridial toxins are pore forming, creating perforations in cell membranes, leading to cell death.
- Disease progression is usually rapid making treatment with antibiotics impractical.
- Vaccination is the best strategy.





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Introduction – High level manufacturing process

Initial cultures

Fermentations

Centrifugation for bacterial cell removal

Addition of formaldehyde to supernatant containing toxin

Incubation until toxin has been converted into toxoid

Antigen testing methods

Vaccine formulation

Vaccine testing methods

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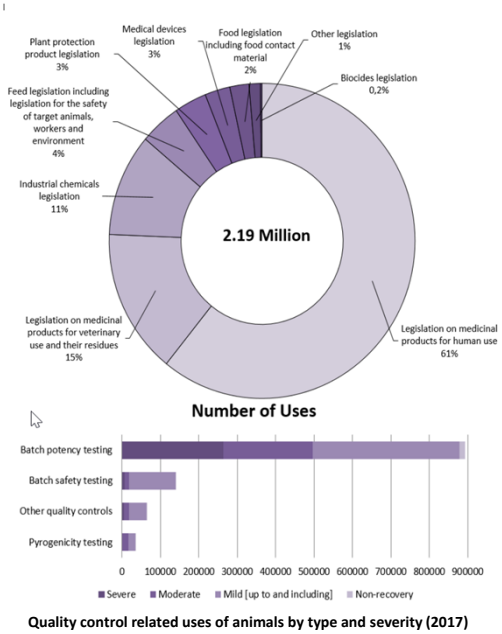
Introduction – Animal usage for regulatory testing

- Regulatory animal testing accounts for ~25% of all animal testing in the EU.
- Potency testing accounts for the greatest proportion of severe tests performed on animals.
- Animal toxicity assays are often incorrectly perceived as the 'gold standard'.
- Regulatory acceptance is one of the main barriers to adoption of alternative *in vitro* models.



Quality control related uses of animals by type and severity (2017)

Source: European commission 2019 report
Statistics on the use of animals for scientific purposes in the Member States of the European Union in 2015-2017.

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Introduction – Animal alternative cell line cytotoxicity assays

- *in vitro* toxicity assays offer substantial advantages over their *in vivo* counterparts.
- Cell viability and proliferation are key indicators of cell health.
- Physical and chemical stress can affect cell viability and metabolism.
- Cytotoxicity can be caused via various mechanisms:
  - Cell membrane damage
  - Seizing protein synthesis
  - Irreversible receptor binding
  - Inhibition of biochemical reactions within the cell.
- Cell-based *in vitro* assays are developed to quantify these changes and the choice of assay should depend on factors such as the assay sensitivity, running cost and assay duration.

Review: Sultan Aslanturk (2018) *in vitro* cytotoxicity and cell viability assays: Principles, advantages, and disadvantages, *IntechOpen.*

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Scope of the project

Overall aim: To develop, validate and implement toxicity assays replacing the control animal tests routinely performed within QC laboratories.

C. perfringens D, ***C. chauvoe***i, ***C. septicum*** and ***C. novyi*** antigens

Project milestones

- Identify a suitable cell line for toxicity testing of *C. chauvoei* antigens.
- Confirm sensitivity of Vero cell line to *C. novyi* and *C. septicum antigens.*
- Overcome the technical challenges associated with *C. perfringens D* assay (previously abandoned in QC).
- Conduct validation and correlation studies.
- Submit dossier variation to regulatory authority.

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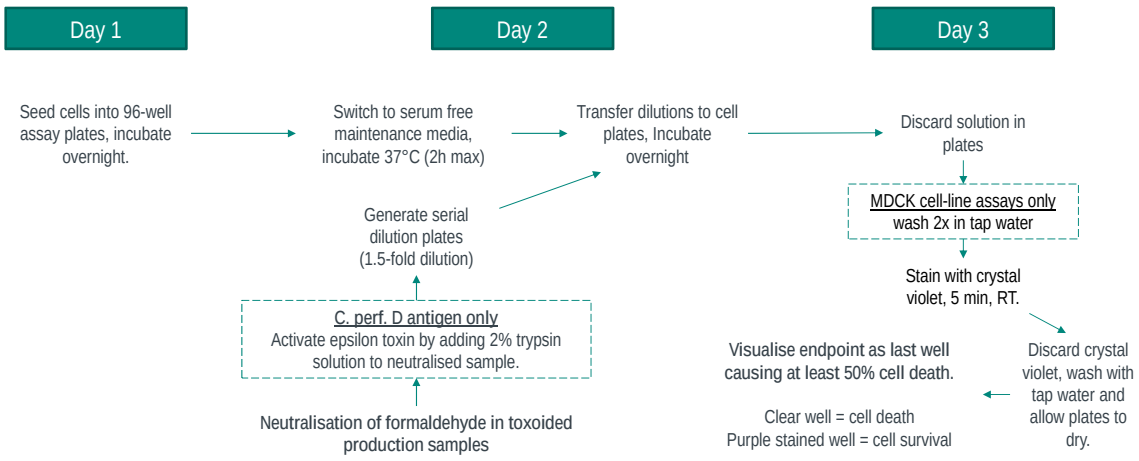
Assay Overview



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Cell assay final plate layout

Plate 1

		1	2	3	4	5	6	7	8	9	10	11	12
Sample 1	A	1	1.5	2	3	5	8	11	17	26	38	58	NC
	B												
Sample 2	C	1	1.5	2	3	5	8	11	17	26	38	58	NC
	D												
Sample 3	E	1	1.5	2	3	5	8	11	17	26	38	58	NC
	F												
Sample 4	G	1	1.5	2	3	5	8	11	17	26	38	58	NC
	H												

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	86	130	195	292	438	657	985	1478	2217	3325	4988	NC
B												
C	86	130	195	292	438	657	985	1478	2217	3325	4988	NC
D												
E	86	130	195	292	438	657	985	1478	2217	3325	4988	NC
F												
G	86	130	195	292	438	657	985	1478	2217	3325	4988	NC
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Plate 2

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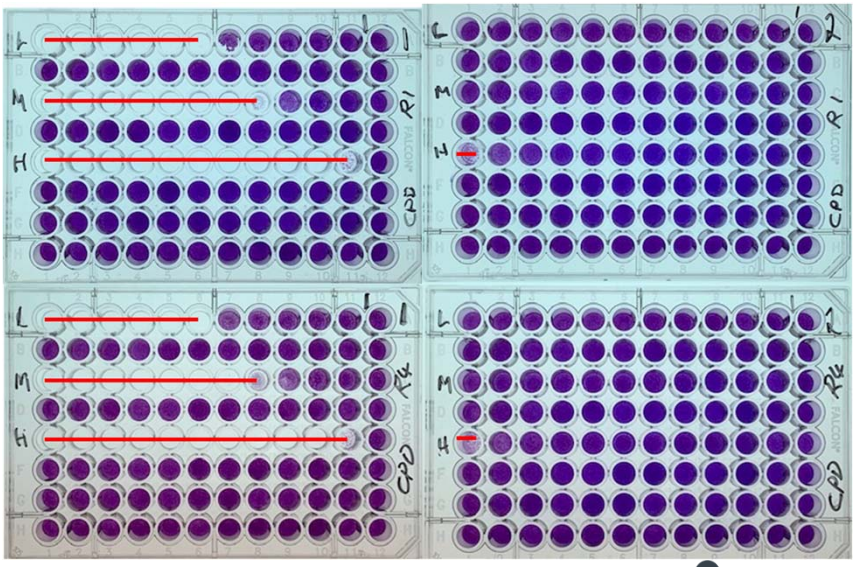
Cell assay final plate layout



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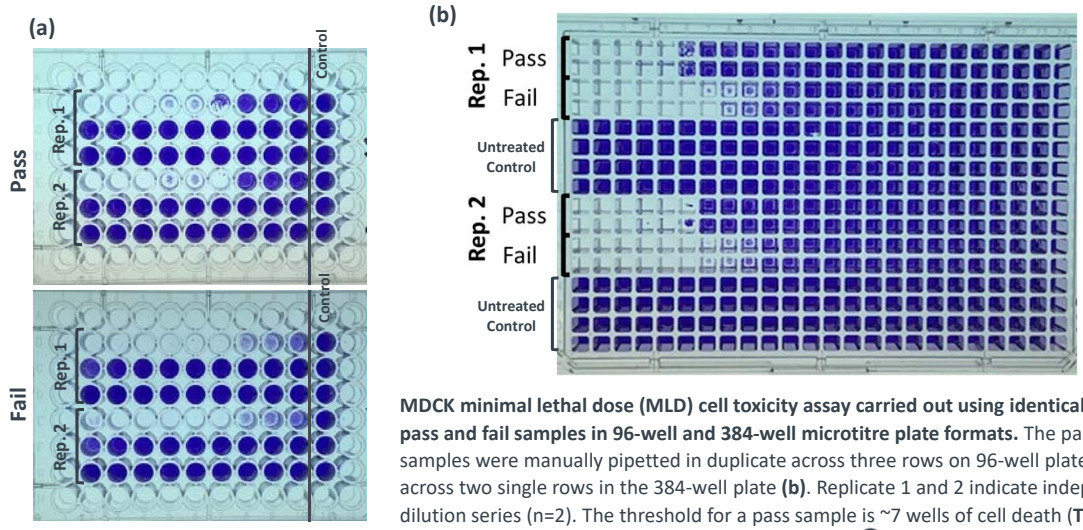
C.perf. D MLD assay demonstrates equivalence across both microtitre plate formats.



MDCK minimal lethal dose (MLD) cell toxicity assay carried out using identical CpD toxoid pass and fail samples in 96-well and 384-well microtitre plate formats. The pass and fail samples were manually pipetted in duplicate across three rows on 96-well plates **(a)**, and across two single rows in the 384-well plate **(b)**. Replicate 1 and 2 indicate independent dilution series (n=2). The threshold for a pass sample is ~7 wells of cell death (**TBD**).

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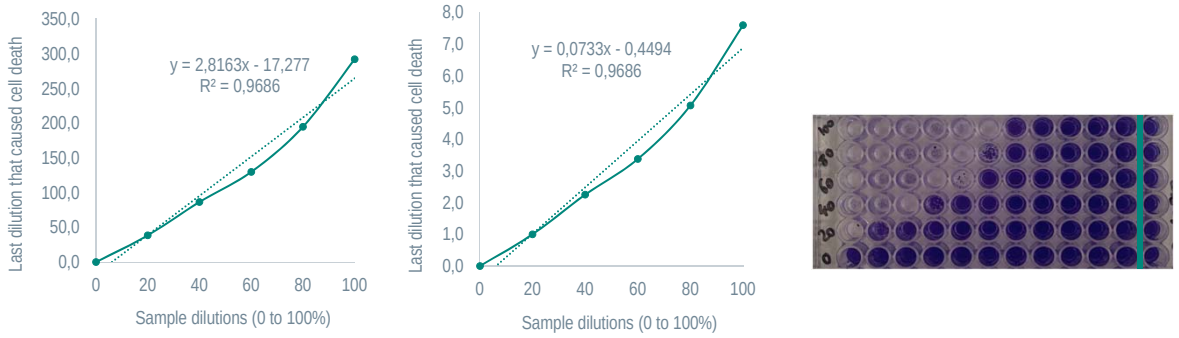
Both MDCK and Vero cell line assays demonstrate linearity



C. septicum
(Vero assay)

C. perfringens D
(MDCK assay)

Assay linearity was demonstrated for both cell lines through the dilution of toxoid samples

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Characterisation of cell line sensitivity towards *C. chauvoei* antigens

- MDCK cells are widely established to be sensitive to epsilon toxin, from *C. Perf. D.*
- Vero cells previously demonstrate sensitivity to *C. septicum* and *C. novyi* antigens.
- A sensitive cell line for *C. chauvoei* had not yet been established.
- Current *C. chauvoei* animal tests require the use of many mice and Guinea pigs.

Aims:

- Screen and compare the suitability of several cell lines for use with *C. chauvoei* cell-based toxicity assays.
- Assess which cell line is most suitable for bacterin and toxoid samples.
  - Both are used as components of the vaccine.

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MDCK cell line demonstrates toxicity to *C. chauvoei* antigens

	Samples from manufacturing to estimate toxicity of chauvoei antigens on cell lines (active toxin)			
	End of fermentation	Cell pellet	Supernatant	Supernatant Conc.
MDCK	7	3	6	9
VERO	4 – 5	2	5 – 6	-

Simulated concentration of QC samples

Sensitivity of cell lines to ***C. chauvoei*** antigens
These samples are not tested on animals
We should aim for a cell line that is sensitive in order to detect any residual toxin in the toxicity samples.

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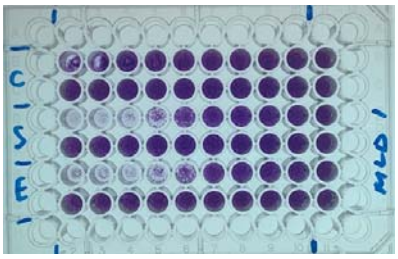
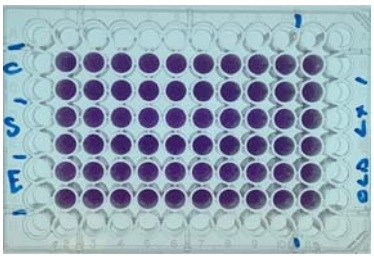
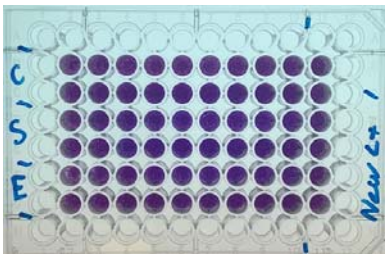
Neutralisation of toxicity using anti-toxin from rabbit Heptavac sera (Clostridial cocktail) and Guinea pig sera (*C. chauvoei* specific)



Fermentation samples MLD



Fermentation samples L+ rabbit Heptavac PP® sera



Fermentation samples L+ GP (*C. chauvoei* specific) sera

Both the rabbit and GP sera (diluted 1:50) effectively neutralised sample toxicity.

Note: Fermentation samples are not concentrated to production levels.

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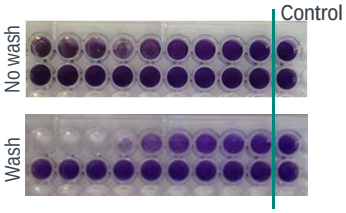
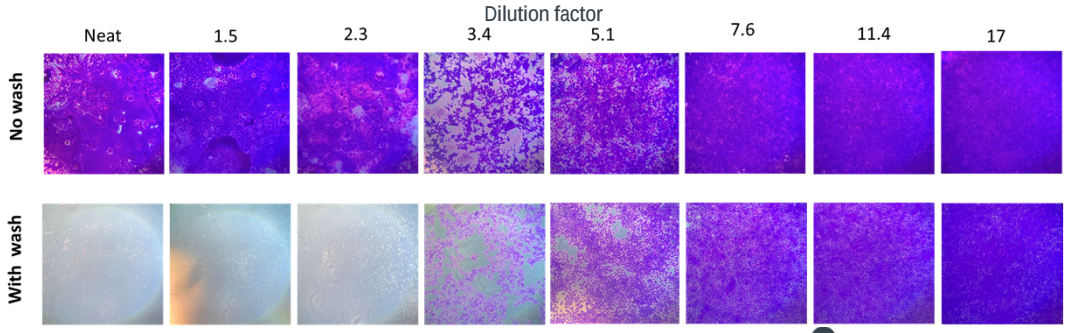
Technical challenges: Alleviating the impact of formaldehyde on cell monolayers

- The formaldehyde content of toxoid samples lead to false positive staining of initial wells containing highest concentrations of sample.
- *C. Perf. D* toxicity assay was previously abandoned from QC due to these non-specific readings.

Solution: The inclusion of a pre-stain wash step in tap water completely removes false positive that appear within initial wells.





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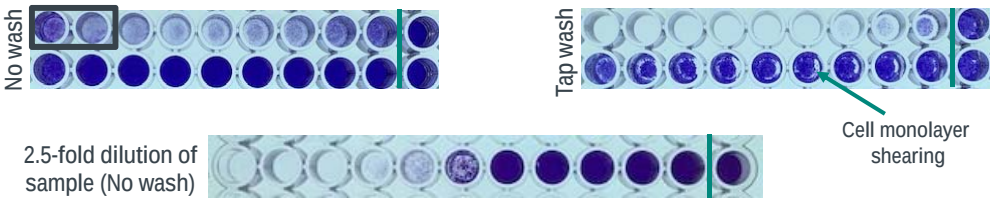
Technical challenges: Alleviating the impact of formaldehyde on cell monolayers

- Pre-stain washing of Vero cell assays was not possible due to shearing of cell monolayers.
- Between the two Vero cell line assays septicum assays demonstrated stronger non-specific staining within initial wells (correlates with higher formaldehyde content).

Solution: *C. septicum* and *C. novyi* samples were initially diluted 2.5-fold in order to overcome the non-specific staining observed within initial wells.



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Validation studies: Robustness and intermediate precision

Validation studies were performed to determine assay robustness, repeatability and intermediate precision.

Robustness parameters assessed:

- Overnight incubation time (**15h**, **18h** and **21h**)
- Cell seeding density (**1 x10^5**, **3 x10^5** and **5 x10^5** cells/ml)
- Cell refrigeration (4°C) time post-detachment (**fresh cells**, **2 days** and **4 days old**)

Repeatability and intermediate precision studies

- Performed in duplicate on three independent days by two operators using high, medium and low toxicity samples.
  - Low, medium and high sample toxicity was determined through pilot toxicity assays.
  - Samples were diluted or spiked with toxin in an attempt to reduce or increase toxicity, respectively.

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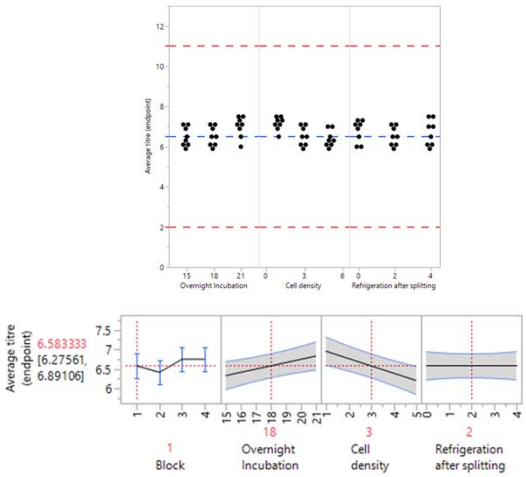
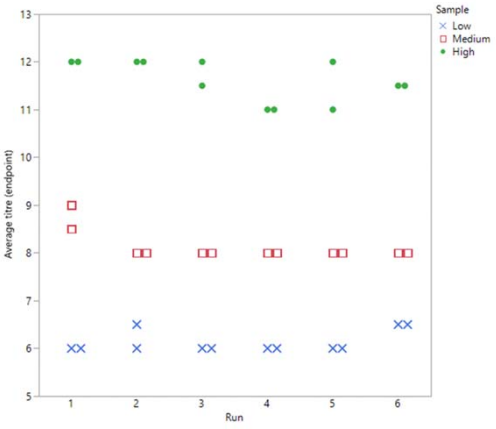
C. perfringens D validation studies: Robustness and intermediate precision



Robustness



Precision

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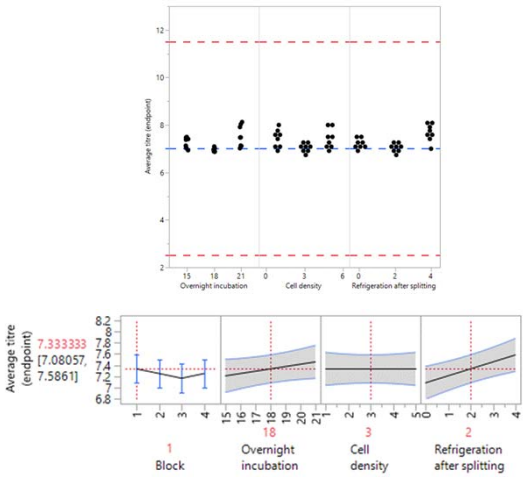
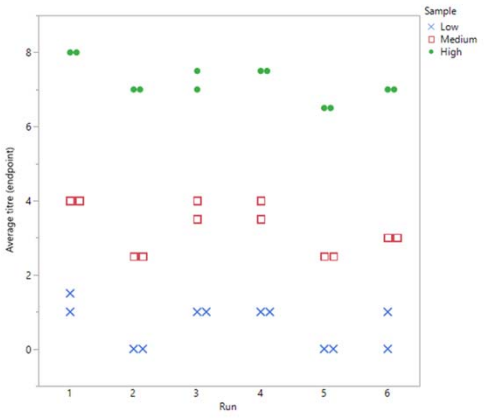
C. chauvoei (cell antigen) validation studies: Robustness and intermediate precision



Robustness



Precision

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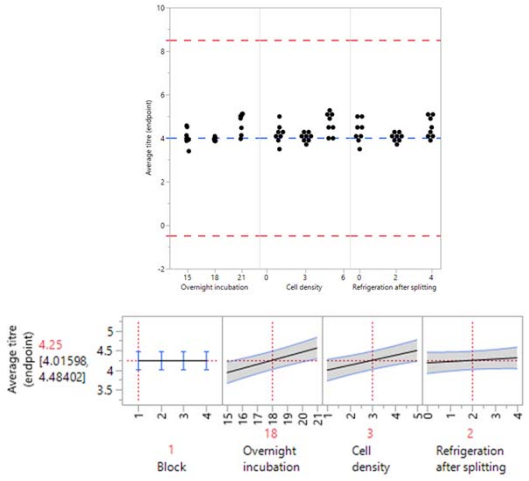
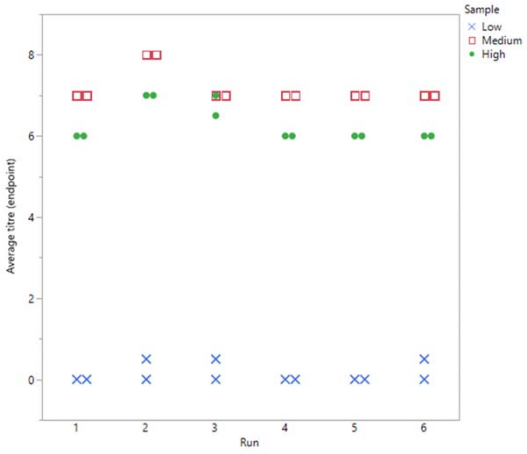
C. chauvoei (toxoid antigen) validation studies: Robustness and intermediate precision

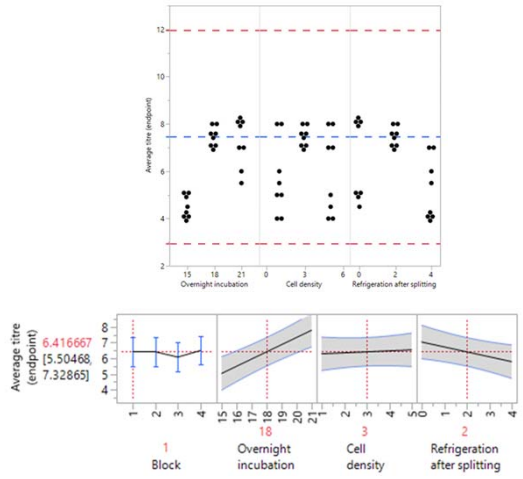


Robustness

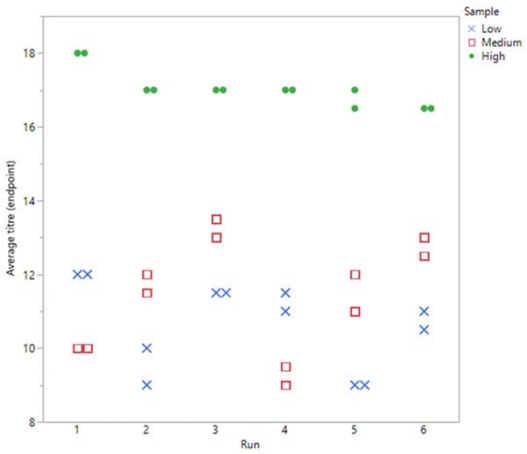


Precision

C. septicum validation studies: Robustness and intermediate precision

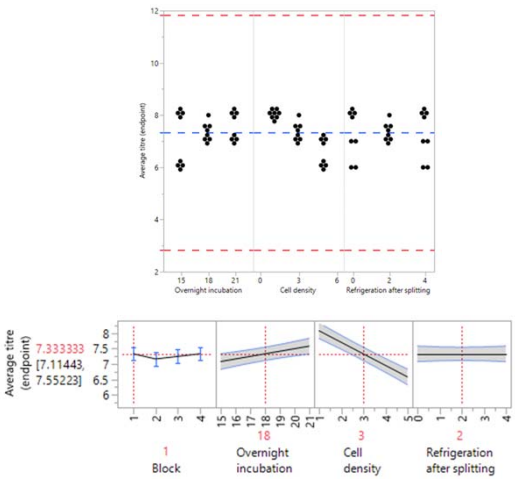


Robustness

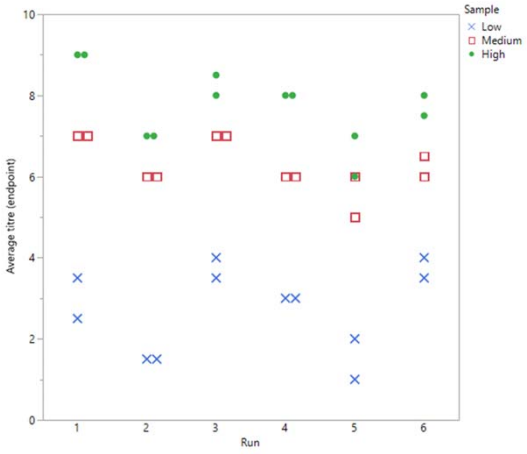


Precision

C. novyi validation studies: Robustness and intermediate precision



Robustness



Precision

Clostridial non-toxicity assays summarised

Strain	Project status	Cell line	Initial sample dilution
C. perfringens D	Assay robustness and precision (re)validated. Correlation studies ongoing.	MDCK	Neat
C. chauvoei (bacterin)	Assay robustness and precision validated. Correlation studies ongoing.	MDCK	2.5-fold
C. chauvoei (toxoid)	Assay robustness and precision validated. Correlation studies ongoing.	MDCK	Neat
C. septicum	Assay robustness and precision validated. Correlation studies ongoing.	Vero	2.5-fold
C. novyi	Assay robustness and precision validated. Correlation studies ongoing.	Vero	Neat

MDCK: Madin-Darby canine kidney
Vero: African green monkey kidney epithelial cells.

Conclusions

- Inclusion of a pre-stain wash step remediated the false positive absorbance readings that lead to the CPD non-toxicity assay being abandoned within the QC environment.
- All four cell line assays were found to be robust, reproducible and precise and met the acceptance criteria.

Ongoing work

- Concurrent *in vitro* testing of QC samples then correlation of cell assay results to *in vivo* pass/ fail result.
- Determine the titre endpoints that represent the pass/ fail threshold of each assay.

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