

Development of Decentralized Livestock Pathogen POC Testing - Lessons Learned from the Pandemic

IB Marsh, RJ Barnewall, TM Williams, PMV Cusack,
N Sales, F Galea, AN Szentirmay, JM. Ruijter,
MJB. van den Hoff and JC Quinn

BIOSEARCH™
TECHNOLOGIES
GENOMIC ANALYSIS BY LGC



What is Bovine Respiratory Disease?

Characterised by

- Nasal and/or oral discharge
- Lethargy
- Inappetence
- Coughing
- Diarrhea
- Dehydration

Why is BRD important?

- Australia, approx. \$40 million per annum
- Globally, approx. \$3 billion per annum



Bovine Respiratory Disease (BRD)

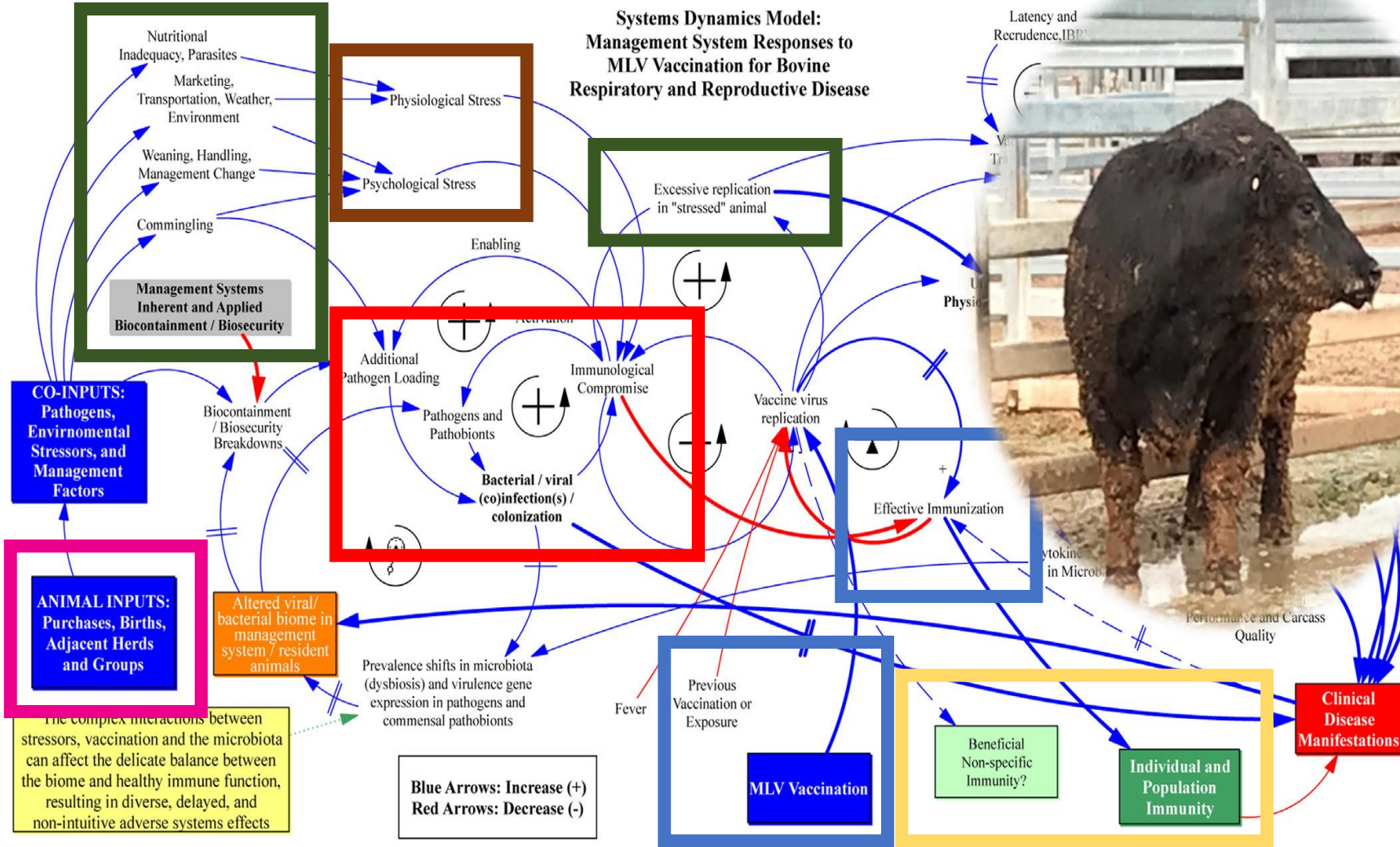
'Bovine Respiratory Disease (BRD) is arguably the most complicated mammalian disease that exists.'

Richardson and Falkner, 2020, Vet Clin Food Anim 36 (2020) 473–485



Bovine Respiratory Disease

- Animal
- Maturity
- Naivety
- Exposure
- Vaccination
- Treatment
- Management



Project Task

Develop a molecular capability to :

- Better understand BRD
- Better understand agents
- Better understand affected animals
- Used for mass screening
- Aid management in feedlot systems



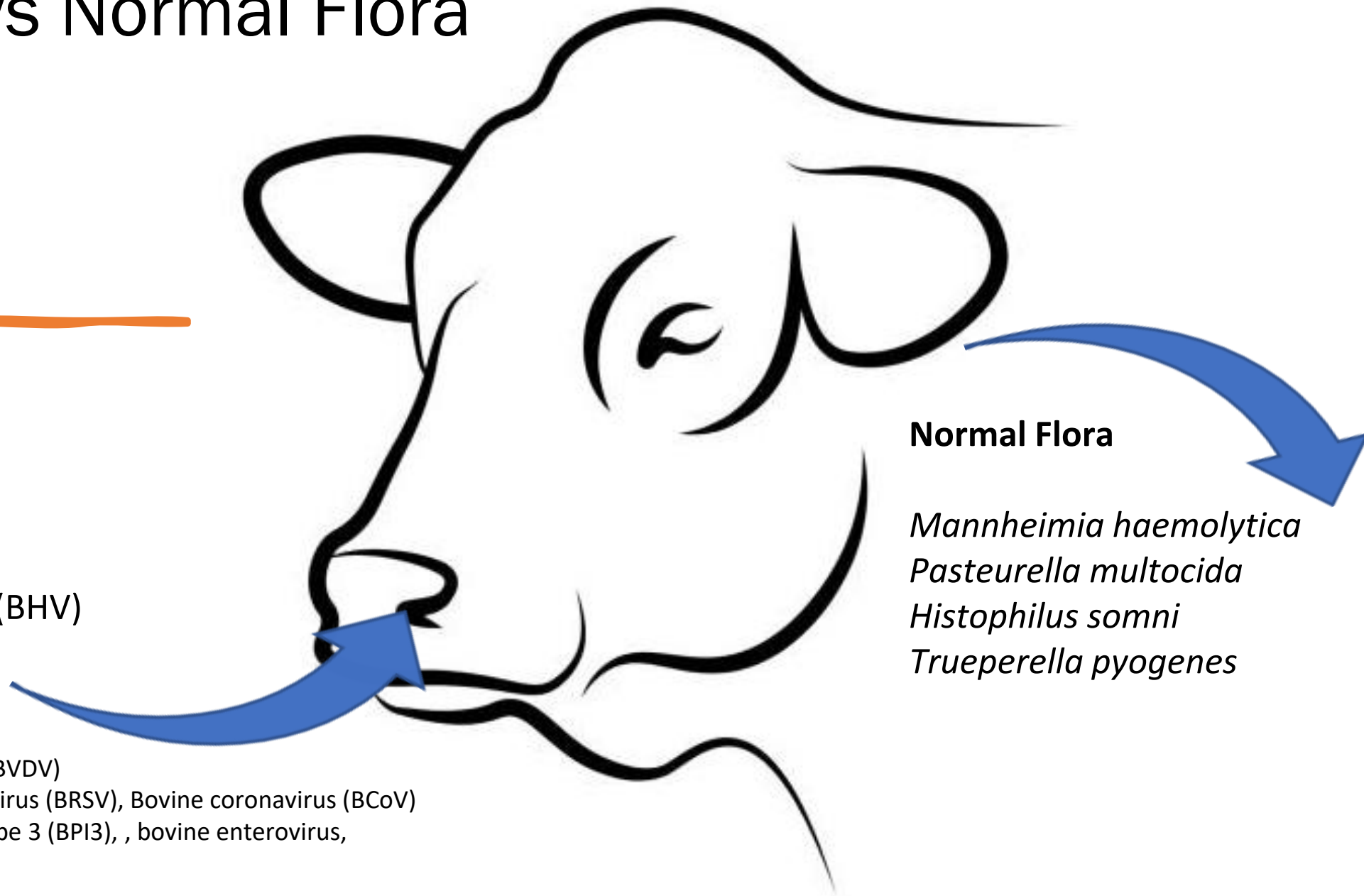
Pathogen vs Normal Flora



True Pathogens

Bovine herpes virus (BHV)
Mycoplasma bovis

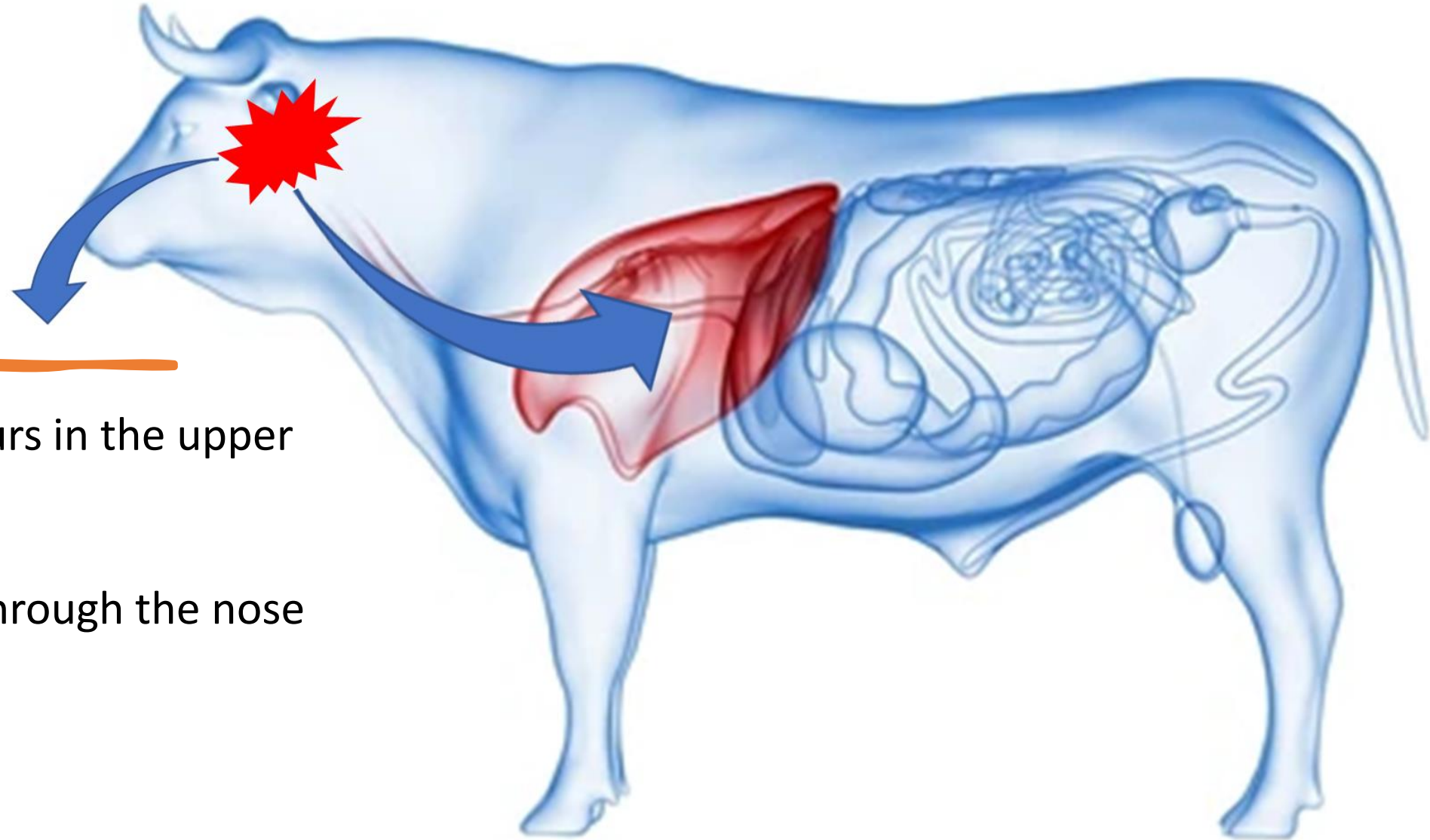
Plus additional risks from:
Bovine viral diarrhoea virus (BVDV)
Bovine respiratory syncytial virus (BRSV), Bovine coronavirus (BCoV)
Bovine parainfluenza virus type 3 (BPI3), , bovine enterovirus,
bovine adenovirus,



Normal Flora

Mannheimia haemolytica
Pasteurella multocida
Histophilus somni
Trueperella pyogenes

Proliferation



- Proliferations occurs in the upper respiratory tract
- Move to the lungs
- Agents excreted through the nose

The Test

- Sample using standard swab
 - Two true qPCR multiplex tests
-

- **Test 1**

- Bovine herpes virus (BHV)
- *Histophilus somni*
- *Trueperella pyogenes*
- *Bovine beta actin control*

- **Test 2**

- *Mycoplasma bovis*
- *Mannheimia haemolytica*
- *Pasteurella multocida*
- *Bovine beta actin control*



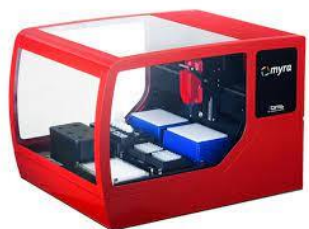
The Constraints

- Detect multiple agents in a single test
- Fast (same day or less) results
- Performed on site (no lab required)
- Easy to interpret
- Low cost PCR platform
- **<\$8 per animal (mob pricing)**
- **Extraction efficiency**
- **Accurate quantitation**



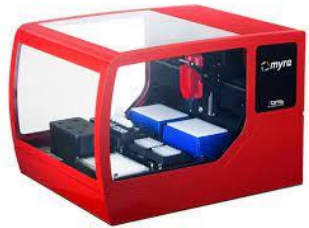
Our Answer


bms
bio molecular systems



Our Answer

bms
bio molecular systems



BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC

FAM™

CAL Fluor(R) Orange 560

CAL Fluor® Red 610

Quasar® 670


bio molecular systems

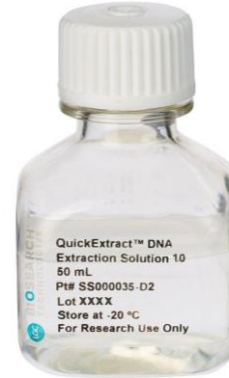


Dye	Excitation	Emission	Channel	Application
BEBO	468	492		Intercalating
LC Green®	455	495		HRM dye
SYTO® 9	483	503		HRM dye
FAM™ (best)	494	515		Conjugated label
SYBR® Green I	494	521		Intercalating
RiboGreen®	500	520		RNA label
PicoGreen®	502	523		ds DNA label
Eva Green®	503	527		HRM dye
TET™	521	536	suboptimal	Conjugated label
CAL Fluor® Gold 540	522	541	suboptimal	Conjugated label
JOE™	520	548	suboptimal	Conjugated label
VIC®	538	554		Conjugated label
HEX™	535	555		Conjugated label
CAL Fluor Orange 560 (best)	540	561		Conjugated label
Quasar® 570	548	566		Conjugated label
Cy™3	550	570		Conjugated label
NED™	546	575		Conjugated label
TAMRA™	555	576		Conjugated label
CAL Fluor® Red 590	565	588	X	Conjugated label
ROX™	573	602		Conjugated label
Texas Red®	583	603		Conjugated label
CAL Fluor® Red 610 (best)	590	610		Conjugated label
LC® Red 640	620	635	suboptimal	Conjugated label
Quasar® 670 (best)	647	667		Conjugated label
Cy™5	651	674		Conjugated label
Cy™5.5	675	694		Conjugated label
Quasar® 705	690	705	X	Conjugated label

BIOSEARCH[™]
TECHNOLOGIES

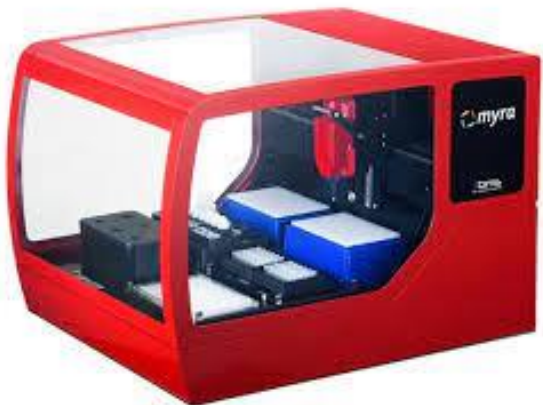
GENOMIC ANALYSIS BY LGC

QuickExtract[™] DNA Extraction Solution



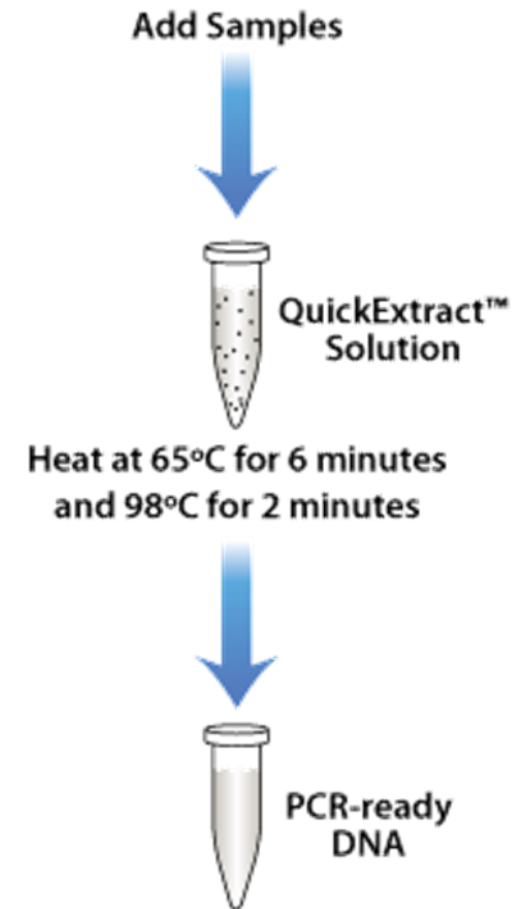
Extract DNA in 8 minutes or less

- Efficiently extract and store PCR-read
- DNA Ideally suited to automated liquid handling

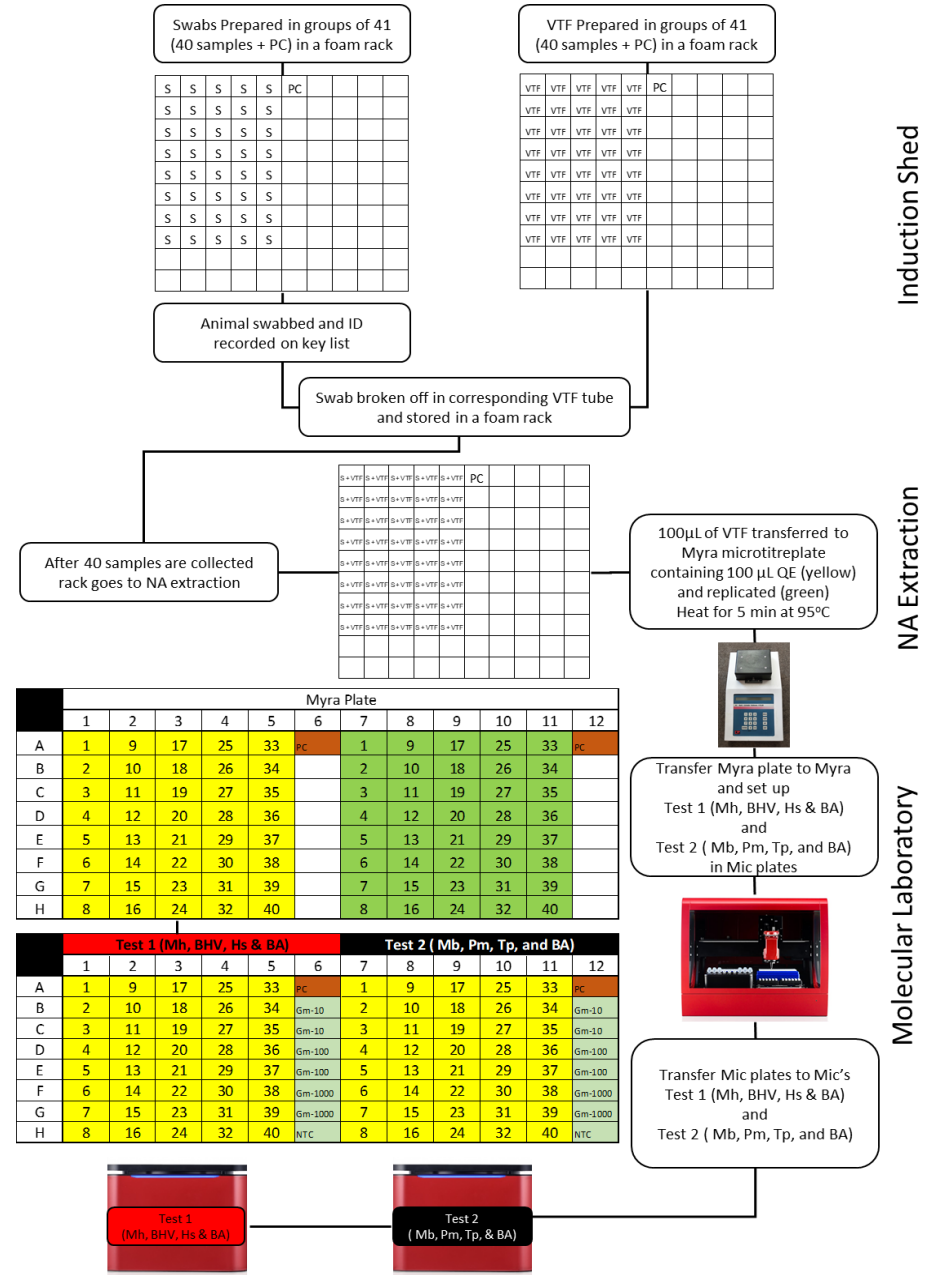


QuickExtract[™] DNA Extraction Solution

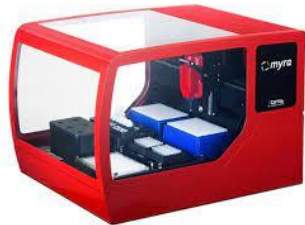
- Specifically optimised for fast, simple extraction of (q)PCR-ready DNA
- 3-8 minute protocol for most sample types
- No centrifugation steps, no spin columns, scalable based on sample size
- Automation-friendly
- Safe: uses only non-toxic reagents
- Can be stored at 4 °C for up to 1 month



The Approach

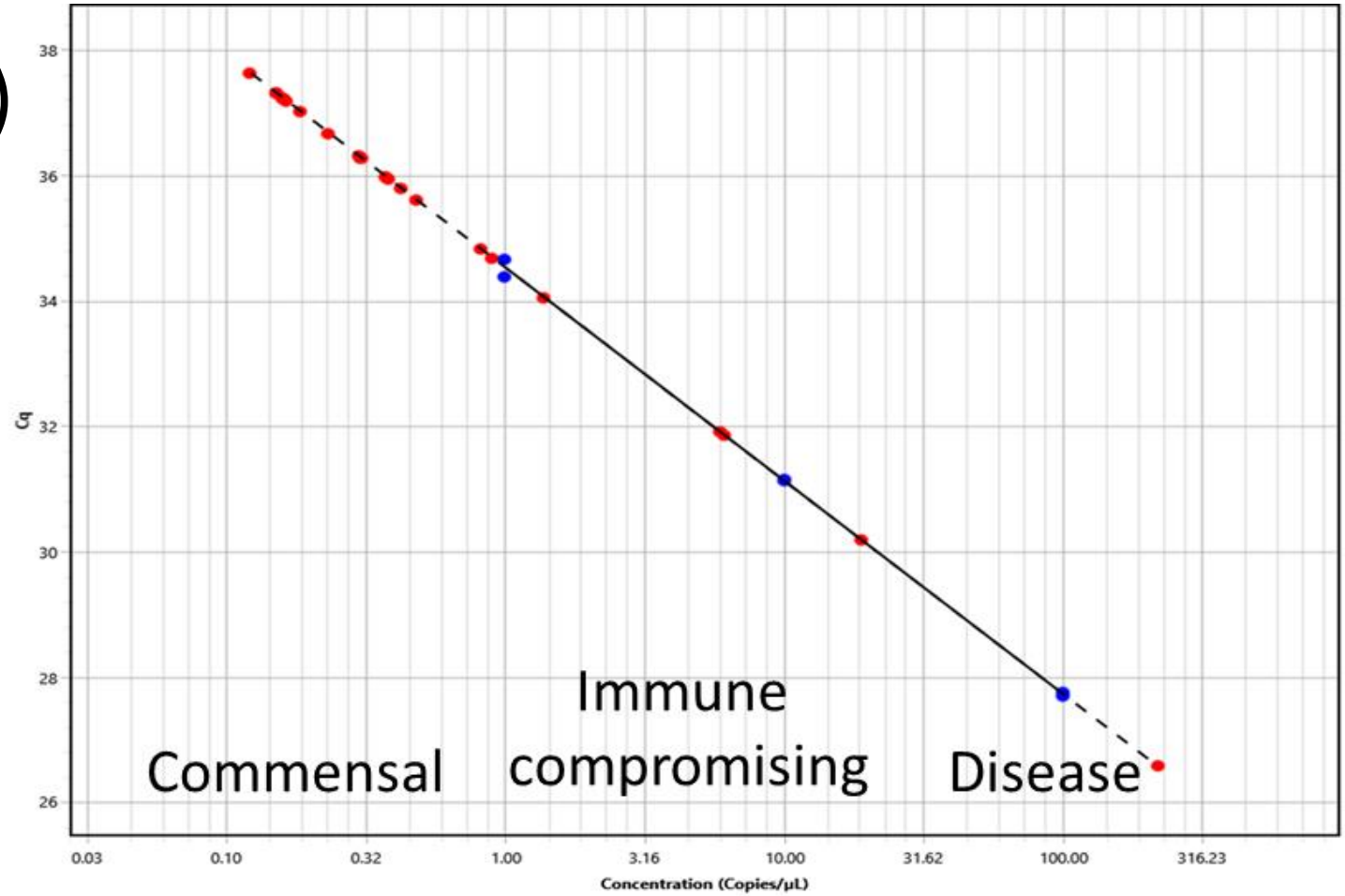


The Approach



Tests		Details	
No. of cattle tested		40 per run	
No. of tests per animal		2	
No. of PCR tubes per animal		2	
No. of pathogens tested		6	
Test 1 (Multiplex)		1 Viral, 2 bacterial and 1 endogenous control	
Test 2 (Multiplex)		3 bacterial and 1 endogenous control	
Test Protocol	Details	Operator time	
No. Operators	1		
DNA extraction	20 mins	20 mins	
PCR reaction preparation Automated reaction preparation Automated sample addition	20 mins	5 mins	
PCR	75 mins	5 mins	
Analysis		20-30 min	
Total time per run	2 hrs	1 hr	
Scalable	Details	Samples per 2 hours	
Option 1 (1 operator)	1 Liquid Handling station 2 Thermocyclers	First run – 40 Subsequent runs – 40	
Option 2 (1 operator)	1 Liquid Handling station 4 Thermocyclers	First run – 40 Subsequent runs – 80	
Option 3 (2 operators)	2 Liquid Handling station 4 Thermocyclers	First run – 80 Subsequent runs – 80	
Option 3 (2 operators)	2 Liquid Handling station 8 Thermocyclers	First run – 80 Subsequent runs – 160	
Deployable	Can entire protocol be deployed in field		
	Yes The entire protocol can be run identically in field or in the laboratory		
Results	Field	Laboratory	
Qualitative (presence absence)	Yes	Yes	
Semi-Quantitative (standard curve)	Yes	Yes	
True Quantitative (efficiency corrected single point calibration)	Yes (only platform at present to do this) Critical for accurate differentiation of disease or expression at high Cq values	Yes (only platform at present to do this) Critical for accurate differentiation of disease or expression at high Cq values	

The Outcome, (hopefully)



Quantitative PCR

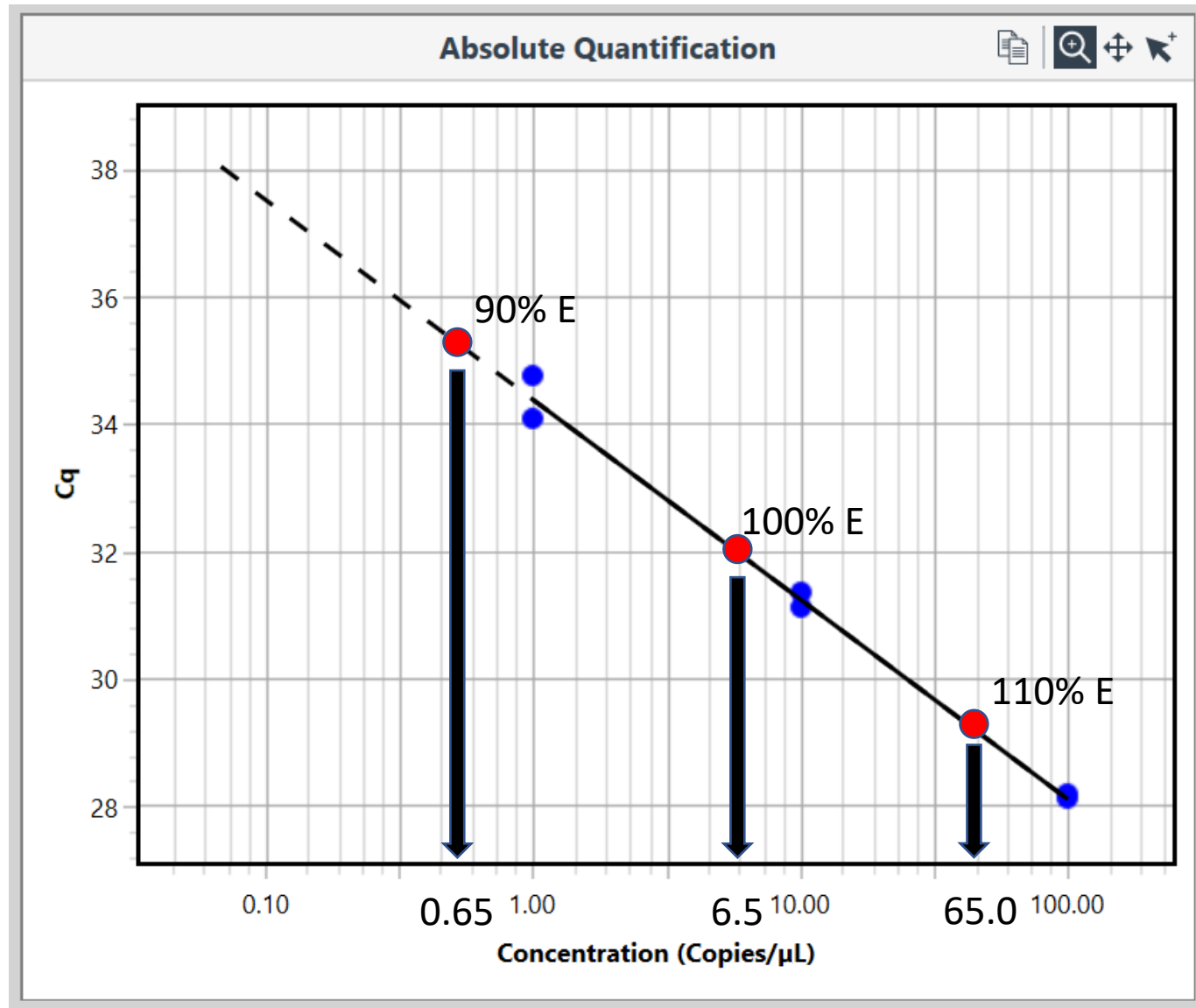
(Standard Curve)

Standard Curve Characteristics

Equation: $y = -3.137x + 34.4$

Efficiency: 1.083 $R^2: 0.9931$

Export...



Efficiency Corrected Quantitative PCR



Andrew Szentirmay



Jan Ruijter



Maurice van der Hoff



Rebecca Barnewall



The Best Covid lockdown Video Conference Meetings in 2020 😊

Quantitative PCR

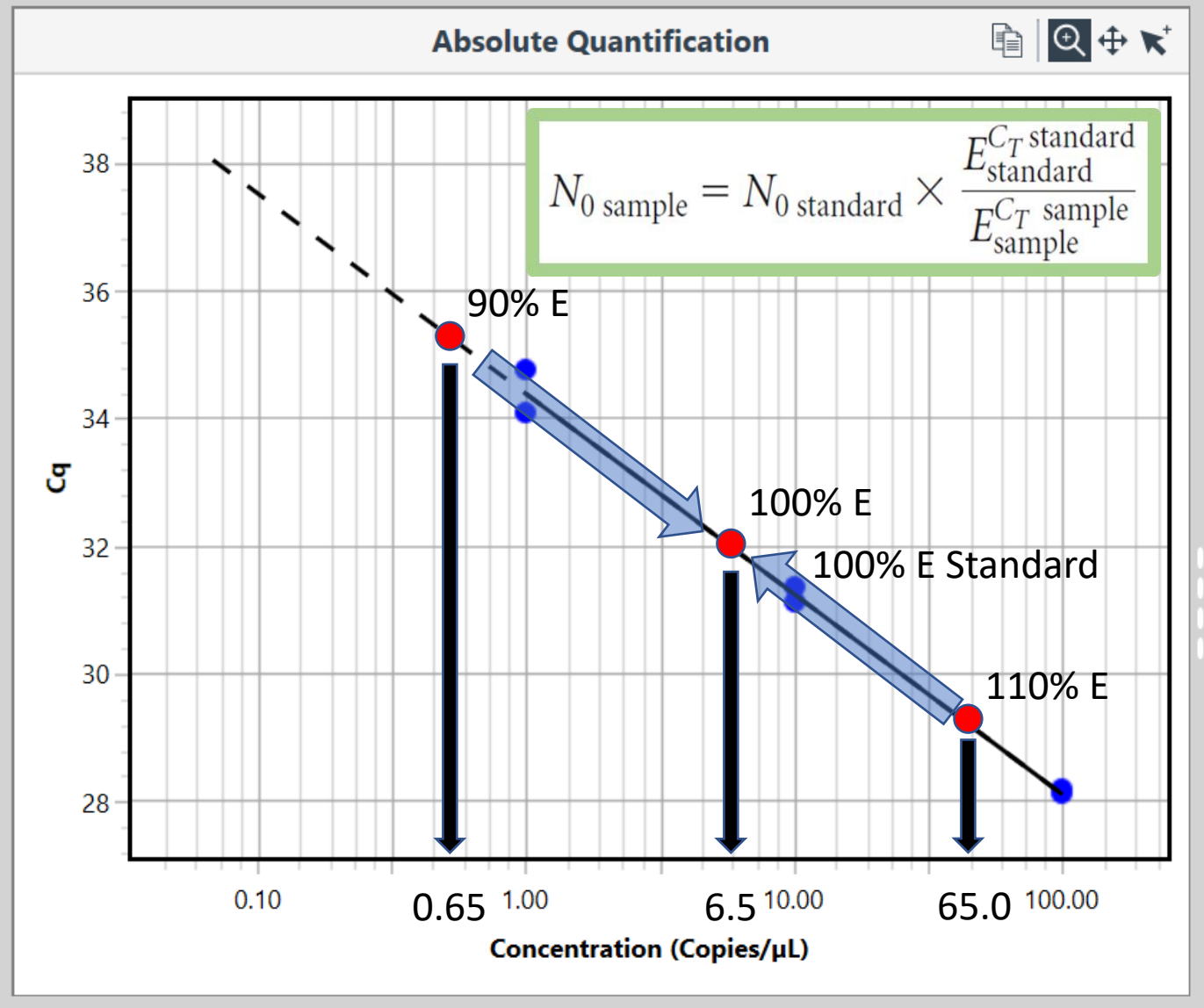
(Efficiency corrected)

Standard Curve Characteristics

Equation: $y = -3.137 x + 34.4$

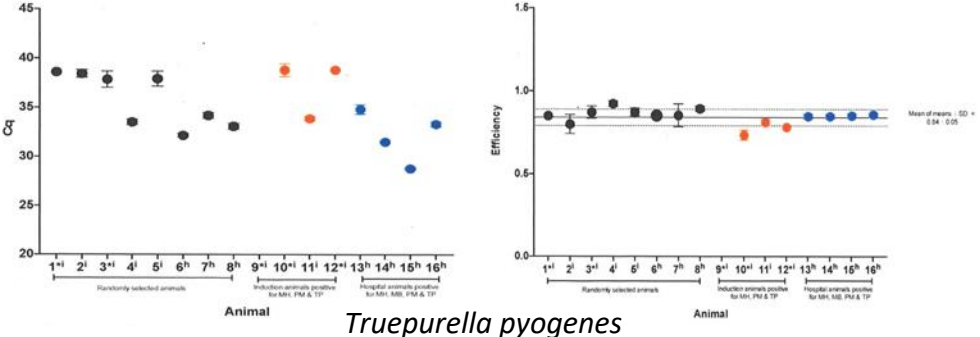
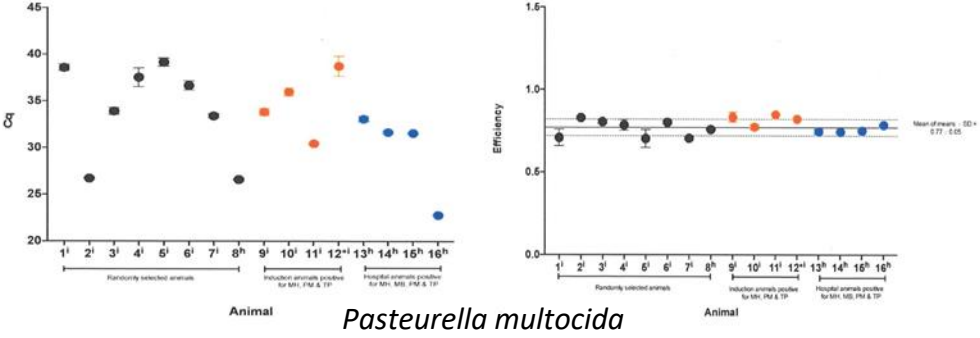
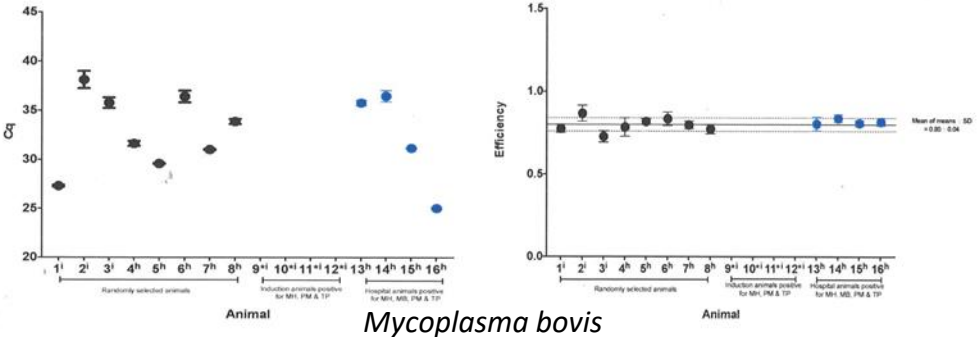
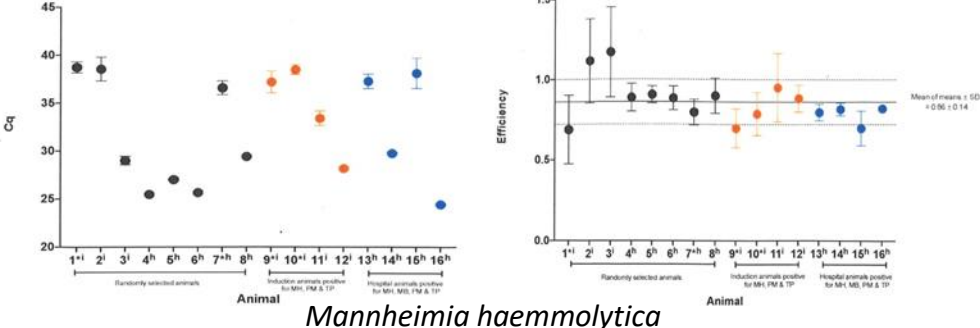
Efficiency: 1.083 R²: 0.9931

Export...



qPCR Efficiency Reproducibility

- 16 biologically distinct samples
 - 1-8 randomly selected animals
 - 9-12 induction animals
 - 13-16 hospital animals
- 6 technical replicates
- PCR for
 - *Mannheimia haemolytica*
 - *Mycoplasma bovis*
 - *Pasteurella multocida*
 - *Trueperella pyogenes*
- Cq values (left)
- Efficiency values (right)



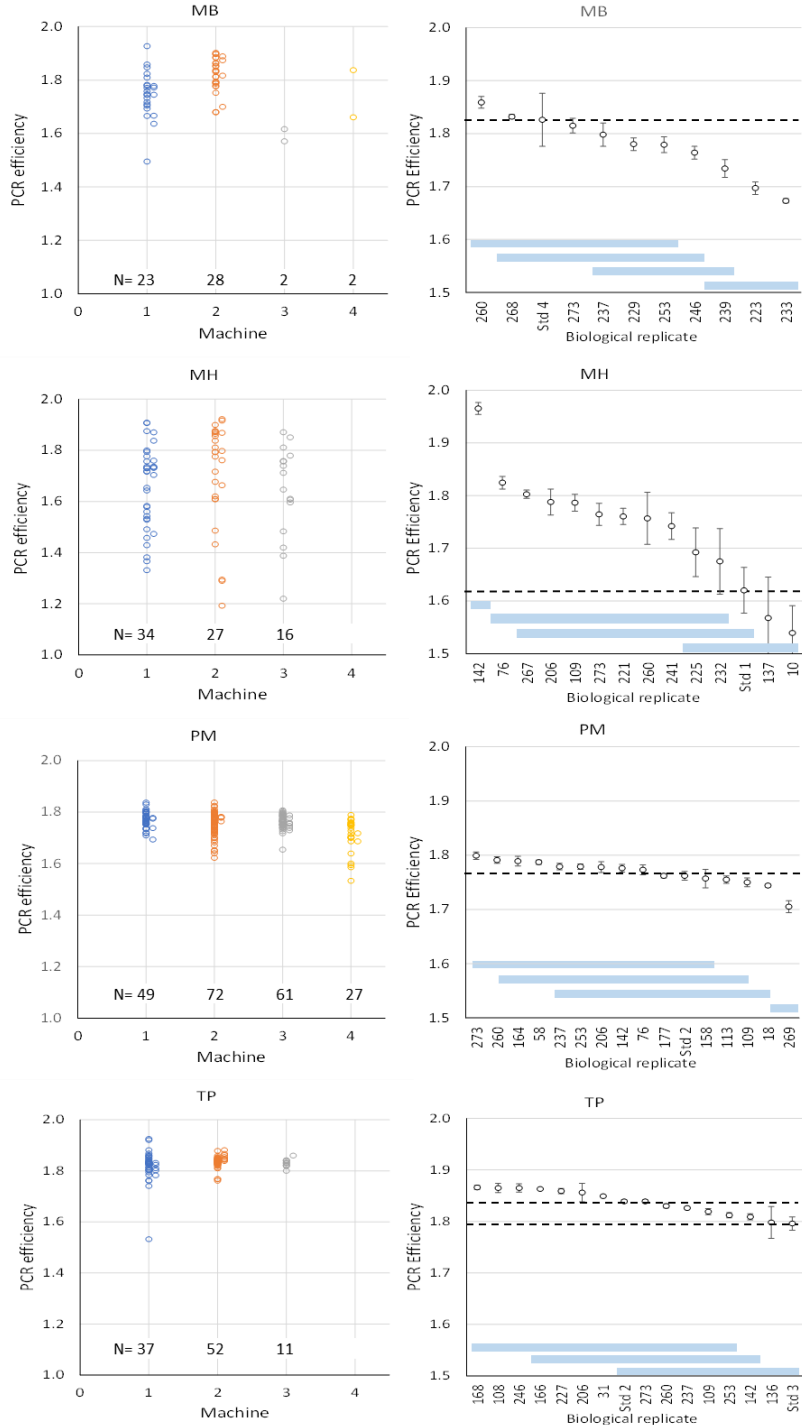
qPCR Efficiency Reproducibility Across individual PCR Mic thermocyclers

Left Figure

- Scatterplots of individual biological samples per qPCR machine (n=4)
- Offset data points represent the biological samples used to test the reproducibility of the observed PCR efficiency
- Number of observations per target and machine differs as each agent is not present in every subject

Right Figure

- PCR efficiencies for selected biological samples showing the mean of 6 technical replicates per sample.
- Dotted black lines indicate the standard sample.
- Blue horizontal bars indicate homogeneous subsets of samples that do not differ significantly from each other
- Non-overlapping parts of the blue bars indicate sample(s) that differ significantly at P=0.05.



Significant achievement /outcome from the project

Clinical Chemistry 67:6
829-842 (2021)

Review

Clinical Chemistry

Peer-reviewed journal

Clinical Chemistry is a peer-reviewed medical journal covering the field of clinical chemistry. It is the official journal of the American Association for Clinical Chemistry. [Wikipedia](#)

Impact Factor: 8.327 (2020)

History: 1955-present

ISSN: 0009-9147 (print); 1530-8561 (web)

Publisher: [American Association for Clinical Chemistry \(United States\)](#)

Indexing: Indexing;

OCLC number: 01554929

Disciplines: [Clinical chemistry](#), [Medical laboratory](#)

Efficiency Correction Is Required for Accurate Quantitative PCR Analysis and Reporting

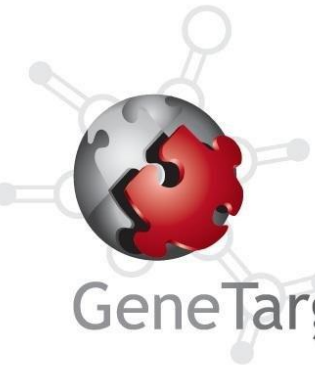
Jan M. Ruijter,^a Rebecca J. Barnewall,^{b,c} Ian B. Marsh,^d Andrew N. Szentirmay,^e Jane C. Quinn,^{b,c} Robin van Houdt,^f Quinn D. Gunst,^a and Maurice J.B. van den Hoff^{a,*}

^aDepartment of Medical Biology, Amsterdam University Medical Centres, Location Academic Medical Center, Amsterdam, the Netherlands; ^bSchool of Animal and Veterinary Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia; ^cNSW Department of Primary Industries), Graham Centre for Agricultural Innovation, Charles Sturt University, Wagga Wagga, NSW, Australia; ^dNew South Wales Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Narellan, NSW, Australia; ^eGene Target Solutions, Dural, NSW, Australia; ^fDepartment of Medical Microbiology and Infection Prevention, Amsterdam University Medical Centres, Amsterdam, the Netherlands.

Deployment



Andrew Szentirmay



GeneTargetSolutions



D'Central



Department of
Primary Industries

Alpha test



GeneTargetSolutions



D'Central



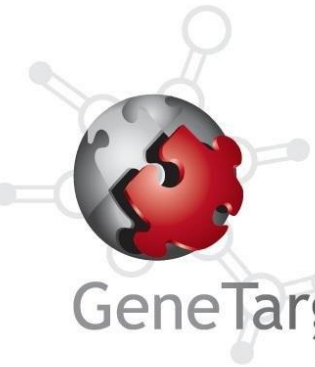
Department of Primary Industries

Alpha test



Department of
Primary Industries

Power



GeneTargetSolutions

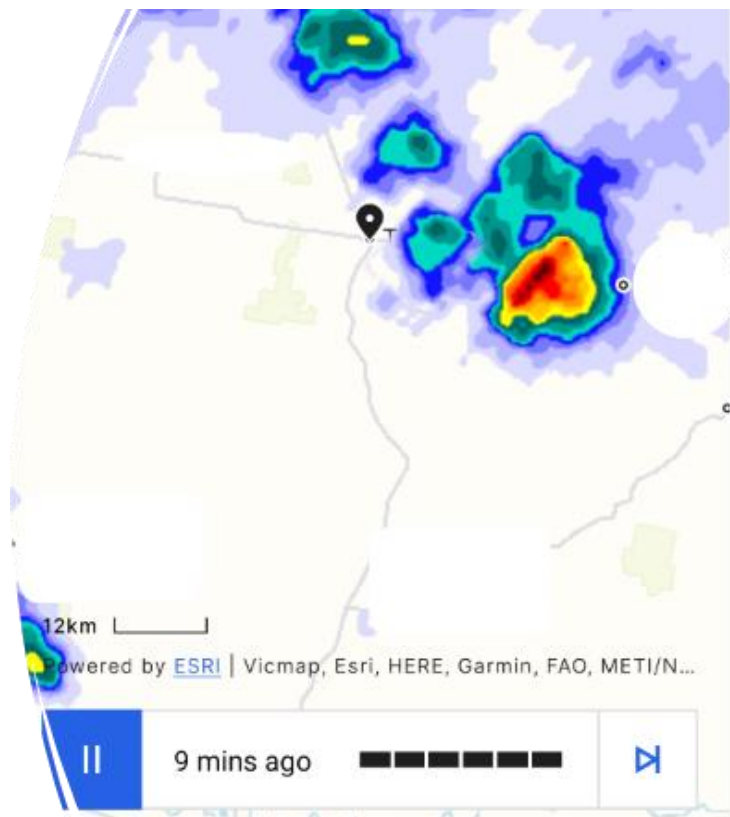


D'Central



Department of
Primary Industries

Storm



Primary study on 2 NSW feedlots

AUSTRALIAN
**VETERINARY
JOURNAL**





AUSTRALIA'S
PREMIER VETERINARY
SCIENCE TEXT



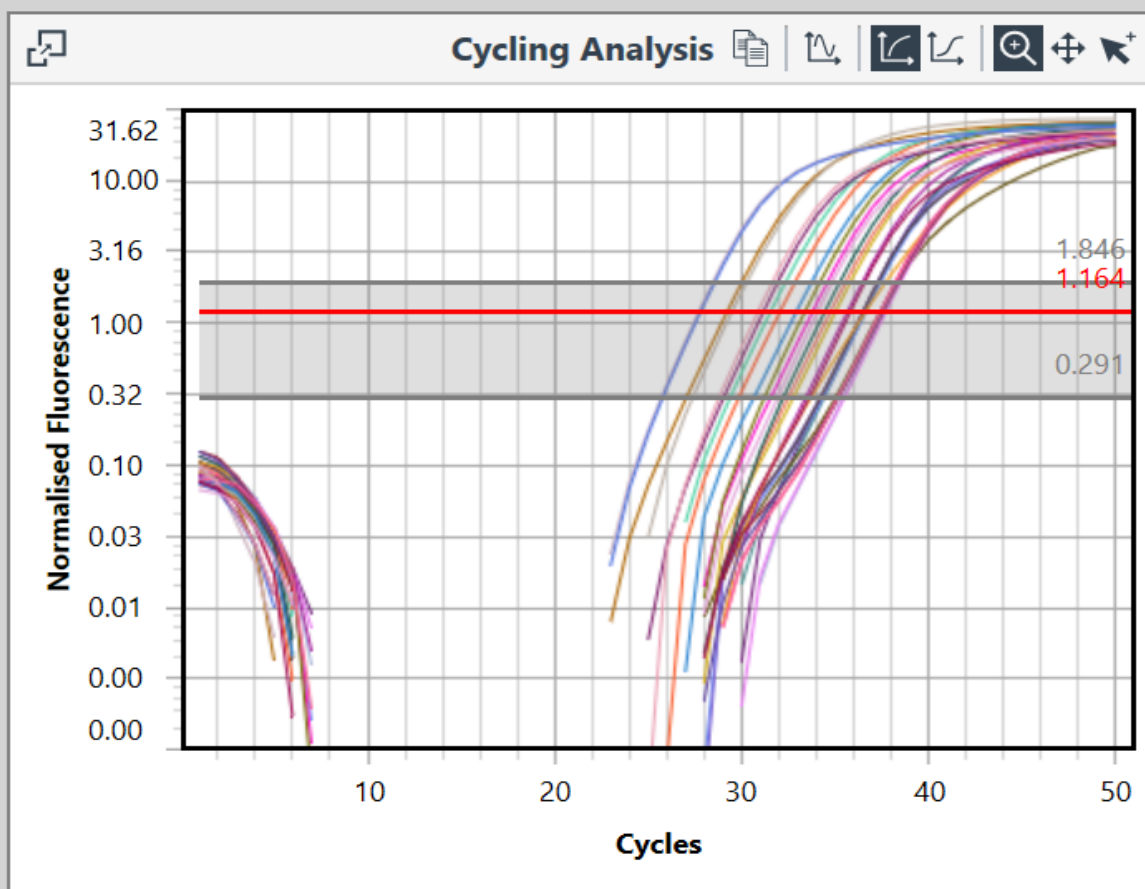
PRODUCTION ANIMALS

ORIGINAL ARTICLE

Efficiency-corrected PCR quantification for identification of prevalence and load of respiratory disease-causing agents in feedlot cattle

RJ Barnewall,^{a,b}  IB Marsh,^c TM Williams,^{a,b,†}  PMV Cusack,^{a,d} N Sales,^c  F Galea,^c AN Szentirmay^e and JC Quinn^{a,b,*} 

Results



Parameters

Target: EMAI HSV2 Source Data: Cycling Orange

Method: Dynamic Ignore Cycles Before: 0

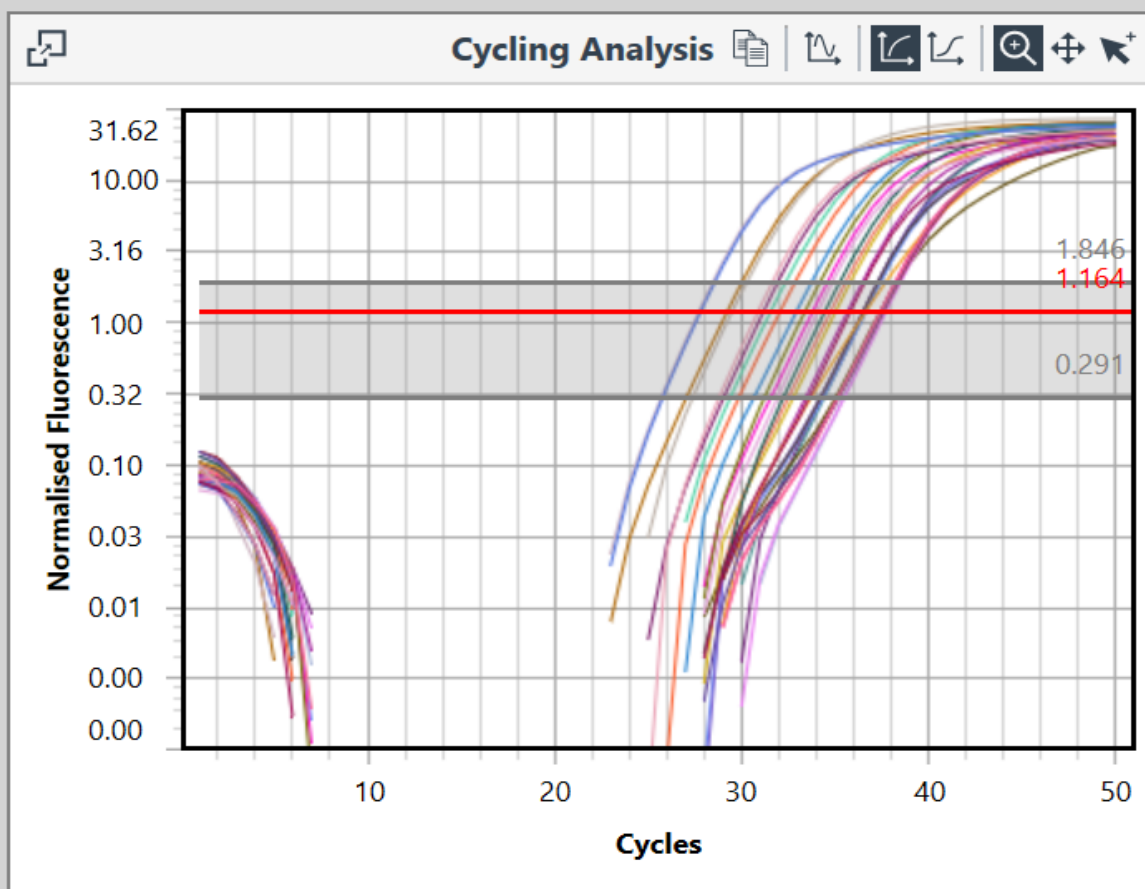
Threshold Start: 1.00 Auto Set Threshold:

Exclusion: Extensive Fluorescence Cutoff Level: 5.0%

Results

Well	Sample	Cq	Efficie...	R ²	Result
27	772B				Exclud...
28	773B	36.37	0.84	0.99990	
29	774B	33.27	0.88	0.99964	
30	775B				Exclud...
31	776B	35.54	0.85	0.99995	
32	777B	36.51	0.60	0.99968	
33	778B				Exclud...
34	779B	37.44	0.80	0.99970	
35	780B				Exclud...
36	781B	37.39	0.83	0.99983	
37	782B	32.84	0.84	0.99994	
38	783B	37.32	0.83	0.99996	
39	784B				Exclud...
40	785B				Exclud...
41	PC				Exclud...
42	Super...	27.59	0.97	0.99953	
43	Super...	27.70	0.96	0.99951	
44	Super...	31.09	0.91	0.99992	
45	Super...	30.78	0.93	0.99971	
46	Super...	35.71	0.88	0.99993	
47	Super...	34.02	0.90	0.99990	
48	Water				Exclud...

Results



Parameters

Target: EMAI HSV2 Source Data: Cycling Orange

Method: Ignore Cycles Before:

Threshold Start: Auto Set Threshold:

Exclusion: Fluorescence Cutoff Level:

Results

Well	Sample	Cq	Efficie...	R ²	Result
27	772B				Exclud...
28	773B	36.37	0.84	0.99990	
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34	779B	37.44	0.80	0.99970	
35	780B				Exclud...
36	781B	37.39	0.83	0.99983	
37	782B	32.84	0.84	0.99994	
38	783B	37.32	0.83	0.99996	
39	784B				Exclud...
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41	PC				Exclud...
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46	Super...	35.71	0.88	0.99993	
47	Super...	34.02	0.90	0.99990	
48	Water				Exclud...

PODD BRD Efficiency Corrected (EC) Data Sheet

Key

User input data
Drop down menu
Mic input data

Location

Day

Plate

TARGET =	Histophilus somni	
R Squared		
Acceptable limits	>	0.98
Standards Analysis		
100 copies	Cq	E
Average	26.40	0.98
Standard error	0.079	0.006
10 copies	Cq	E
Average	29.93	0.97
Standard error	0.123	0.010
1 copy	Cq	E
Average	33.72	0.92
Standard error	0.271	0.026

Samples (unknowns)		
R Squared		
Acceptable limits	>	0.98 Based on BMS Recommendations
E		
Average of unknowns	0.86	E(sample)
Standard error	0.018	
Quantitation thresholds		
E (average+0.1)	<	0.96
E (average-0.1)	>	0.76
All samples outside of these E limits for quantitation must be considered as qualitative results only		
Cycles excluded (minimise impact of background noise on analysis) =		
Mic Run Number		

Run Acceptance

Control	Result
PC (Process Control)	-1
100 Copies/μL	26
10 Copies/μL	30
1 Copies/μL	34
Water	-1

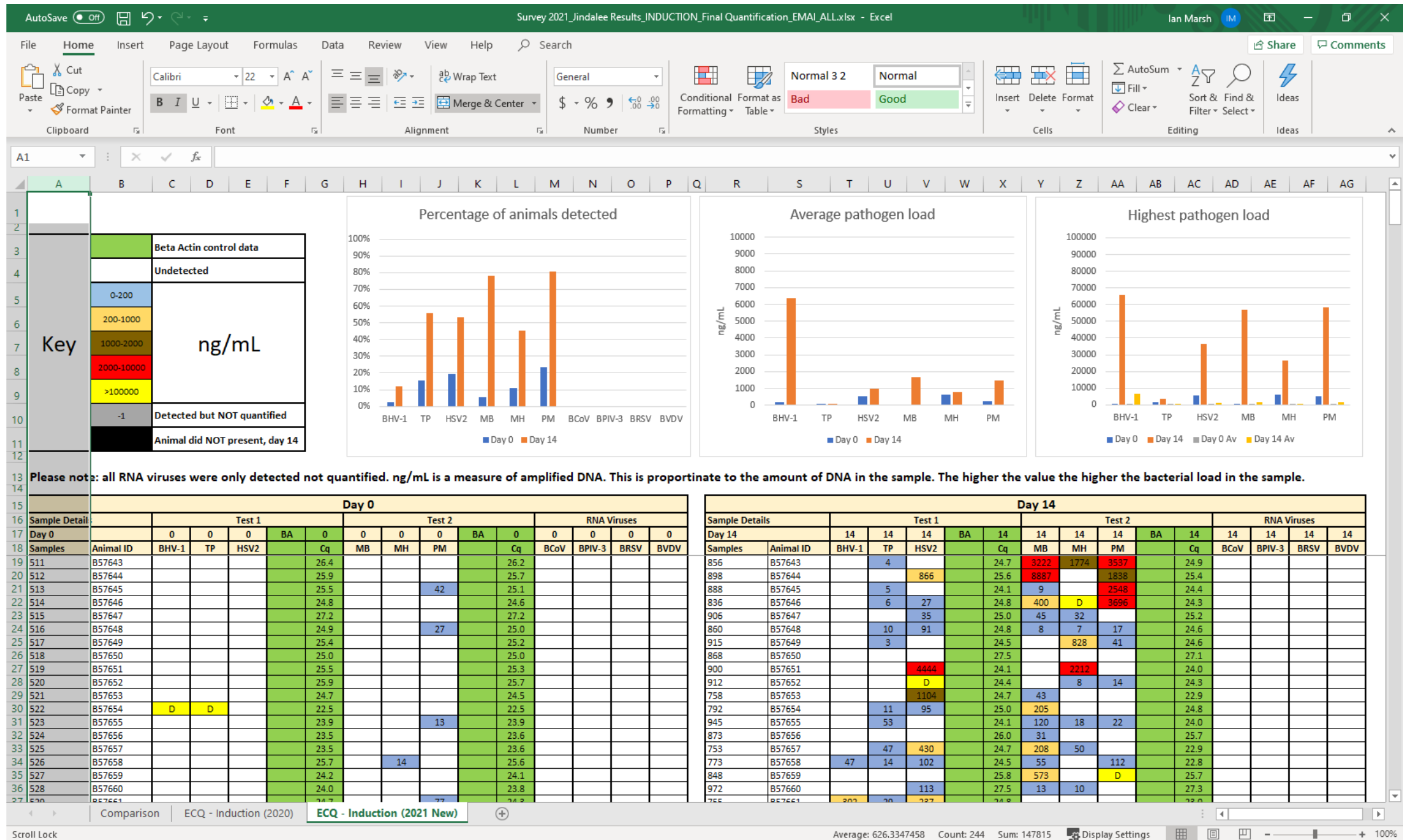
$$N_{0 \text{ sample}} = N_{0 \text{ standard}} \times \frac{E_{CT \text{ standard}}}{E_{CT \text{ sample}}}$$

RAW EXPORTED DATA							Select
COLUMN (Title)	Well	Sample	Cq	Efficiency	R ²	Result	(ng/mL)
SAMPLE		1 780A		-1	-1	Excluded	3580
SAMPLE		2 781A	23.6869382	1.113140235		0.99864	
SAMPLE		3 782A	25.1127775	1.058921045		0.99841	
SAMPLE		4 783A	36.3112219	0.781542325		0.99959	
SAMPLE		5 784A	35.3150237	0.773329916		0.99992	
SAMPLE		6 785A	30.566147	0.998161832		0.99921	
SAMPLE		7 786A		-1	-1	Excluded	BHV 100 = 3780
SAMPLE		8 787A	33.8687528	0.821840257		0.99956	HsV2 100 = 3580
SAMPLE		9 788A	41.056857	0.66825519		0.99629	Mb 100 = 3500
SAMPLE		10 789A	36.2507419	0.764154871		0.99979	Mh 100 = 3470
SAMPLE		11 790A	35.857637	0.838069756		0.99988	Pm 100 = 3400
SAMPLE		12 791A	34.9027699	0.81292209		0.99997	Tp 100 = 3560
SAMPLE		13 792A		-1	-1	Excluded	
SAMPLE		14 793A		-1	-1	Excluded	
SAMPLE		15 794A		-1	-1	Excluded	
SAMPLE		16 795A		-1	-1	Excluded	
SAMPLE		17 796A	35.2418209	0.759134819		0.99962	
SAMPLE		18 797A		-1	-1	Excluded	

ROUNDED DATA			
Cq (sample)	Rounded E	Rounded R2	
0.00	0.00	0.00	
23.69	1.11	1.00	
25.11	1.06	1.00	
36.31	0.78	1.00	
35.32	0.77	1.00	
30.57	1.00	1.00	
0.00	0.00	0.00	
33.87	0.82	1.00	
41.06	0.00	1.00	
36.25	0.76	1.00	
35.86	0.84	1.00	
34.90	0.81	1.00	
0.00	0.00	0.00	
0.00	0.00	0.00	
0.00	0.00	0.00	
0.00	0.00	0.00	
35.24	0.76	1.00	
0.00	0.00	0.00	

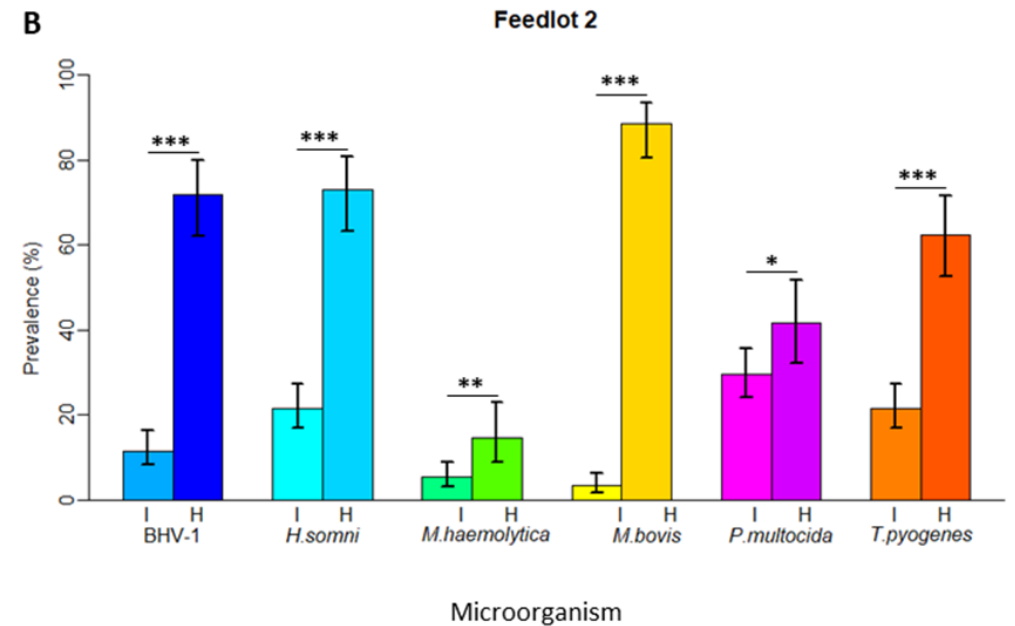
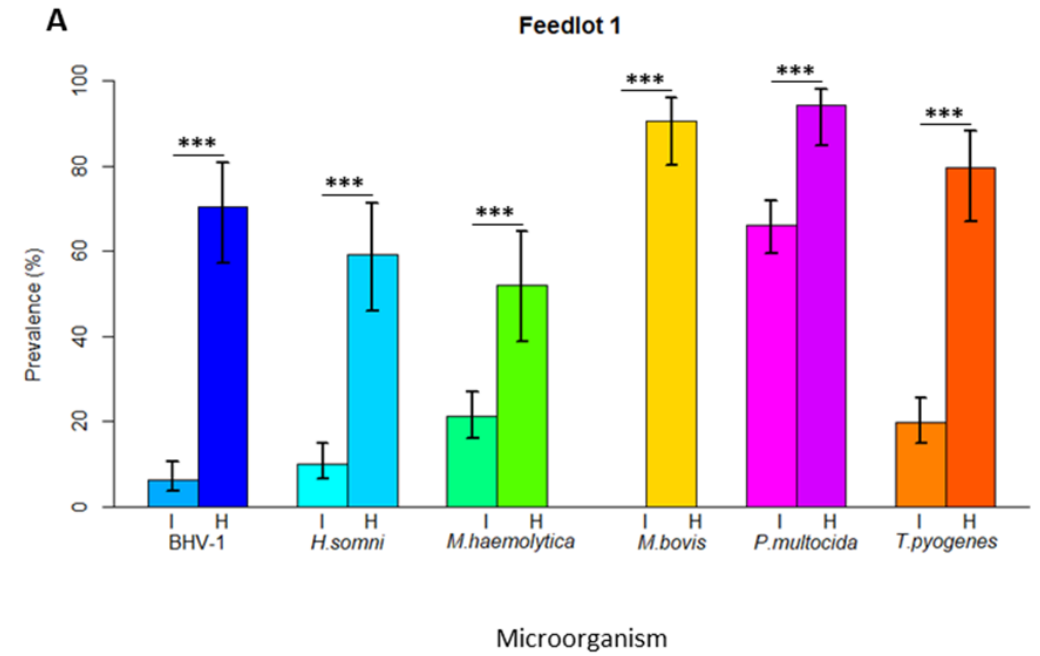
EC QUANTIFICATION	FINAL RESULTS
ng/mL	ng/mL
undetected	
4870.22816691	D
3219.15979165	D
189.40519271	189
396.36730053	396
156.66985153	D
undetected	
363.87110990	364
242162193406.20100000	D
279.87736695	280
80.29898982	80
232.25156184	232
undetected	
undetected	
undetected	
undetected	
548.67921910	D

Data



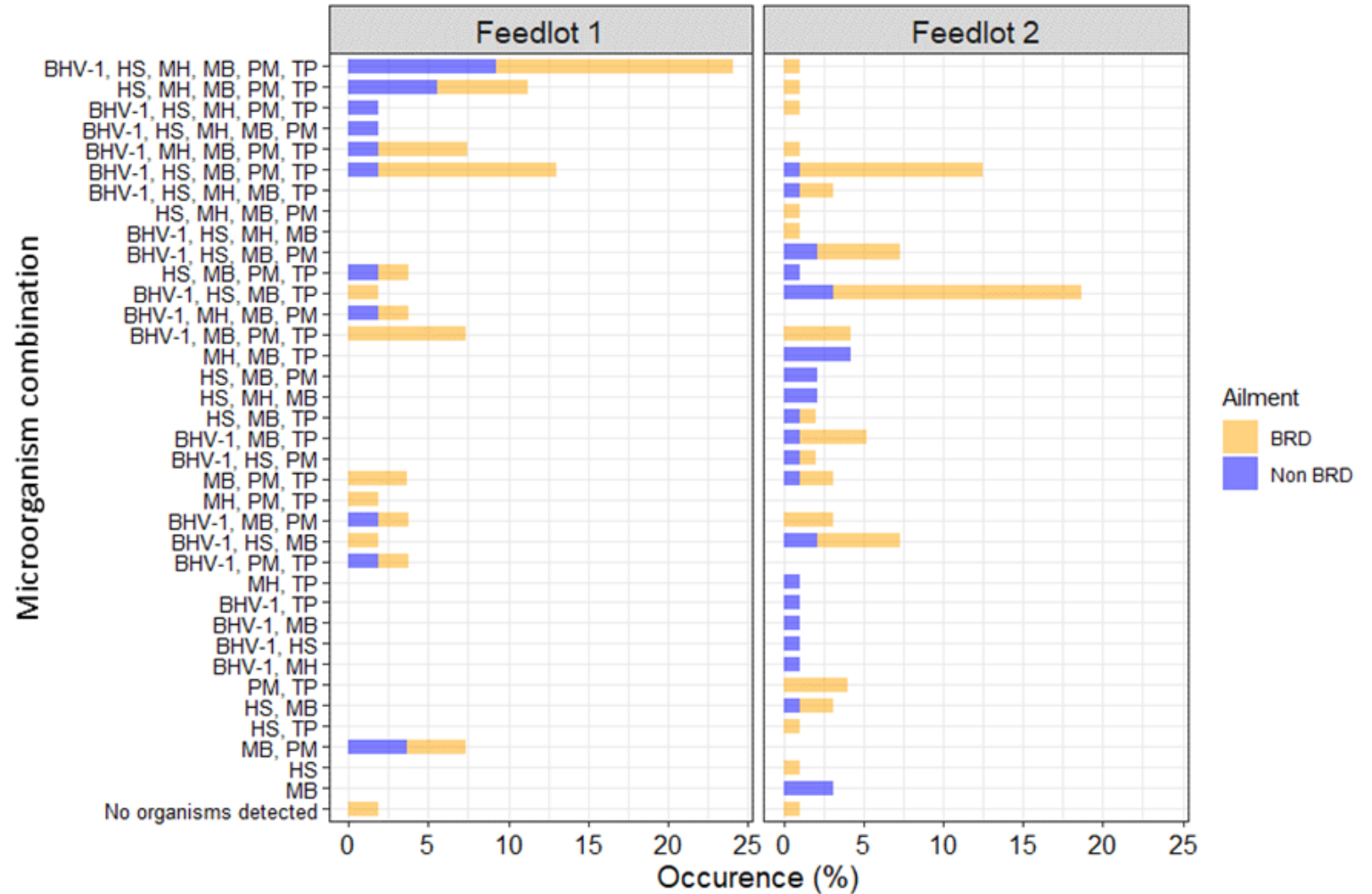
Prevalence

- Prevalence (% +/- CI) of the BRD agents at 2 feedlots induction
- Induction (I) and hospital (H) cohorts
- Fischer's exact test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$,



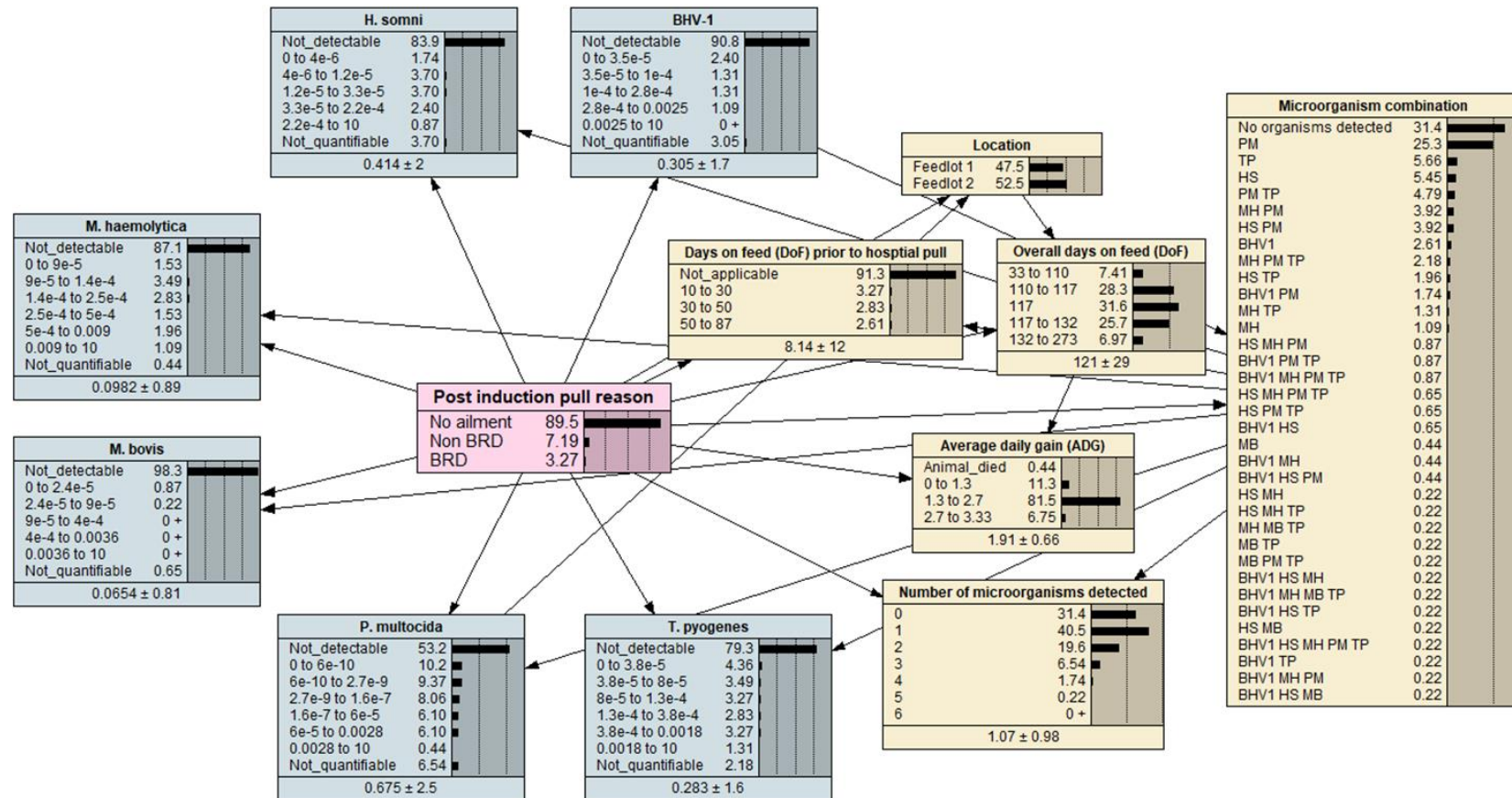
Agent Combination

- Occurrence of combinations of PCR detectable agents
- Hospital animals only
- Feedlot 1 n=54, Feedlot 2 n=96



Bayesian Network Modelling

- Predicting hospital treatment (pull) reason of induction cohorts
- 2 Australian feedlots, with no post induction pull reasons selected.



Survey 2021



- 4 Feedlots and different in distinct geographic locations in NSW
- Sampling animals at
 - Day 0 (induction)
 - Day 14 (Re-vaccination)
 - Hospital
- Approximately 500 cattle/feedlot
- 2000 cattle twice (4000 samples)
- 8000 qPCR tests

RESULTS – being analysed right now

- qPCR + clinical + environmental data

Bovine Respiratory Disease (BRD)

'Bovine Respiratory Disease (BRD) is arguably the most complicated mammalian disease that exists.'

Richardson and Falkner, 2020, Vet Clin Food Anim 36 (2020) 473–485



I think we discovered its even more complicated

Our Project Team

Dr Ian Marsh (NSW DPI EMAI lead)

Andrew Szentirmay (GTS)

Dr Jan Ruijter (Amsterdam UMC)

Dr Maurice van der Hoff (Amsterdam UMC)

Ms Narelle Sales (NSW DPI EMAI)

Ms Francesca Galea (NSW DPI EMAI)

Professor Jane Quinn (CSU lead)

Ms Rebecca Barnewall (PhD candidate CSU, MLA scholarship recipient)

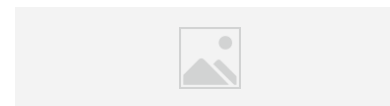
Dr Thomas Williams (CSU Post-doc)

Ms Nancy Saji (CSU Post-doc)

Ms Veronika Vicic (PhD candidate CSU, MLA scholarship recipient)

Dr Michael Campbell (CSU)

Professor Paul Cusack (Adjunct CSU, ALPS)



Thank you

Joy Kang

Andrew Szentirmay

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