Detection of Bovine Viral Diarrhea (BVD) Virus in Ear Skin Tissue Samples: Evaluation of a Combination of QIAGEN MagAttract 96 cador Pathogen Extraction and *virotype* BVDV RT-PCR-based Amplification.

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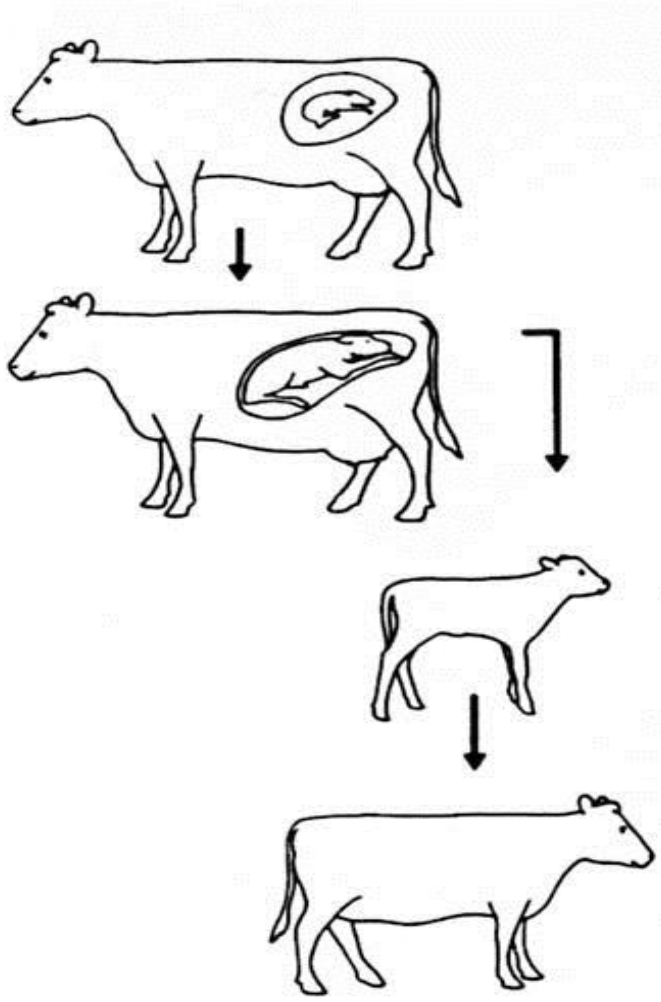
The BVD Challenge: PI Animals



Cow and fetus infected with non-cytopathic BVDV in early pregnancy (40-120 days gestation).

Cow becomes immune. Fetus is immunotolerant to infecting BVDV strain.

Calf remains persistently infected (PI) for life.

Mutation to, or super-infection with, cytopathic virus may occur. PI animal succumbs to acute or chronic mucosal disease.

Samples for BVDV Detection

- **Acute infection:**

  Lymphoid tissues (spleen, mesenteric lymph nodes, Peyer's patches, thymus)

  WBC fraction of whole blood

  Feces, nasal exudates

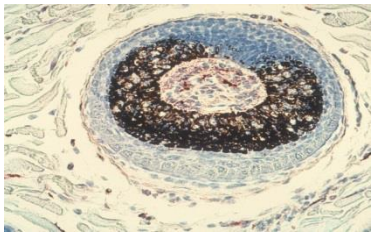
- **PI animals:**

  Serum, WBC fraction of whole blood, bulk milk

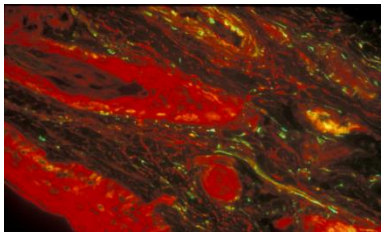
  Skin tissue (**ear notch**)

Methods to Diagnose BVDV Infection:

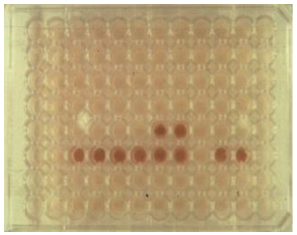
Immunohistochemistry (ICH)

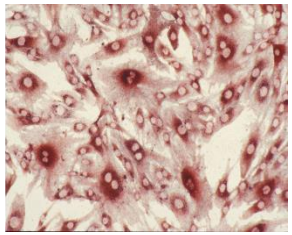


Direct FA test

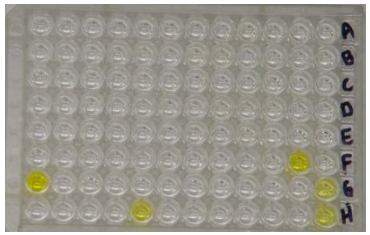


Immunoperoxidase Monolayer Assay (IPMA)





Antigen Capture ELISA (ACE)



In-Lab BVDV Real-Time RT-PCR

- **Duplex real-time RT-PCR assay**

  Detecting a BVDV conserved target sequence within the 5'UTR

  Includes an *in vitro* transcribed RNA internal control (IC) to monitor PCR inhibition

- **PCR template**

  RNA extracted from an aliquot of the supernatant from 10 pooled ear skin pieces that are mechanically disrupted in lysis buffer

In-Lab BVDV Real-time RT-PCR

- **Primers and probe**:

  5'UTR of BVDV genome

  Forward: 5' cca tgc cct tag tag gac ta 3' (+)

  Reverse: 5' caa ctc cat gtg cca tgt ac 3' (-)

  Probe: FAM-5' ctc tgc tac acc cta tca ggc t 3'-BHQ (-)

- **PCR kit**

  QIAGEN One-Step RT-PCR Reagent

- **Amplifies BVDV types 1 and 2**

  NADC validation panel-tested

In-Lab BVDV Real-Time RT-PCR: Internal Control

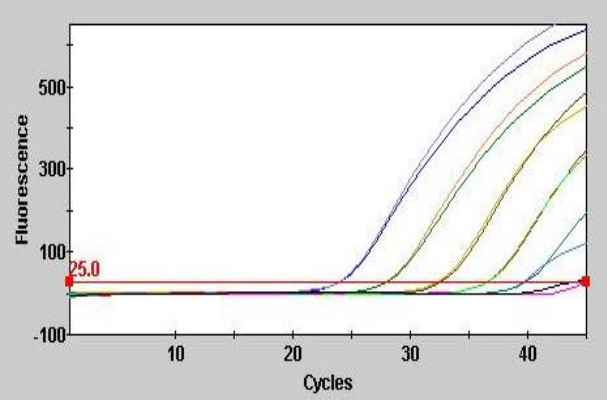
- Designed according to published method *S.B. Kleiboeker, JCM 42: 2055-2061, 2003*
- Mimic RNA: 322 bp pUC19 sequence flanked by BVDV primers

- *In vitro*-transcribed from PCR product:
- Probe for mimic: pUC19-specific

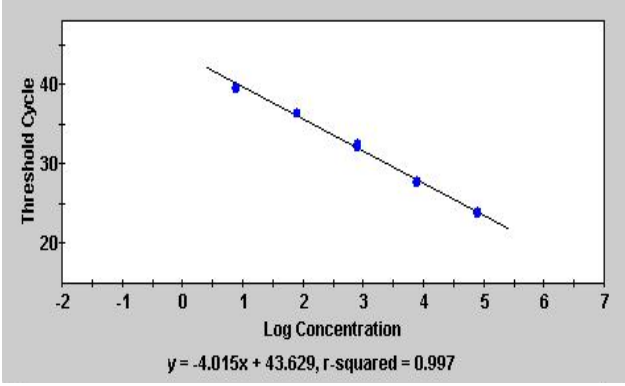
  5' label: Texas Red

  3' label: BHQ-2

In-Lab BVDV Real-Time RT-PCR: Analytical Validation



Detection limit: 0.8 $TCID_{50}$



Linear assay with 89% efficiency

In-Lab BVDV Real-Time RT-PCR: Laboratory Validation

- Commercial magnetic-bead based extraction kit
- Tested tissue pools: 40 known BVDV + ear notch samples by ACE and FA; previously tested BVDV - ear notches
- Pool: 1 positive tissue + 9 negative tissues
- Tissue pool lysed in 1 ml of commercial lysis buffer; 50 µl of lysed supernatant processed
- RT-PCR Result: 40 positive for BVDV
  0 negative
  0 inhibited reactions

In-Lab Assay: Work Flow

- **Pooling**

10 ear notch tissue pieces (2 mm x 2 mm) placed in a tube containing 1 ml of commercial lysis buffer

- **TissueLyser II (homogenization step)**
- **Centrifugation**
- **RNA extraction**

MagMax Thermo Fisher

- **RT-qPCR**

QIAGEN One-Step RT-PCR

QIAGEN Assay: Work Flow

- **PBS soak**

Ear notches (6mm diameter) were individually soaked in PBS for 1-2 hrs

- **Pooling**

Combining aliquots from 10 individual samples

- **RNA extraction**

Aliquot of the pooled fluids

QIAGEN MagAttract 96 cador Pathogen Kit

- **Amplification**

QIAGEN *virotype* BVDV Reagents

Performance of In-Lab Assay versus QIAGEN Assay :
Combinations of Extraction and RT-qPCR

- Complete in-lab workflow **(L-L)**
- In-lab extraction and QIAGEN RT-qPCR **(L-Q)**
- QIAGEN extraction and in-lab RT-qPCR **(Q-L)**
- Complete QIAGEN workflow **(Q-Q)**

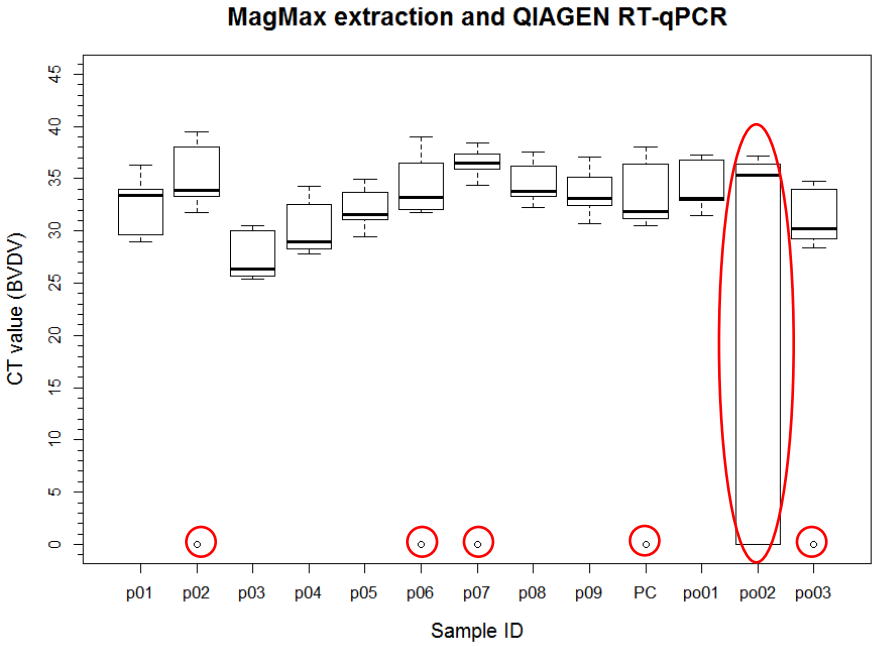
Performance of In-Lab Assay versus QIAGEN Assay :

- Each sample was extracted three times
- Each extract was also tested in triplicate
- For initial overview, all results were treated as individual experiments
- Mean and Standard Deviation (SD) for each sample were calculated
- All results (n=9) were analyzed and plotted as boxplots, using R (statistical software)

Results:
BVDV Amplification

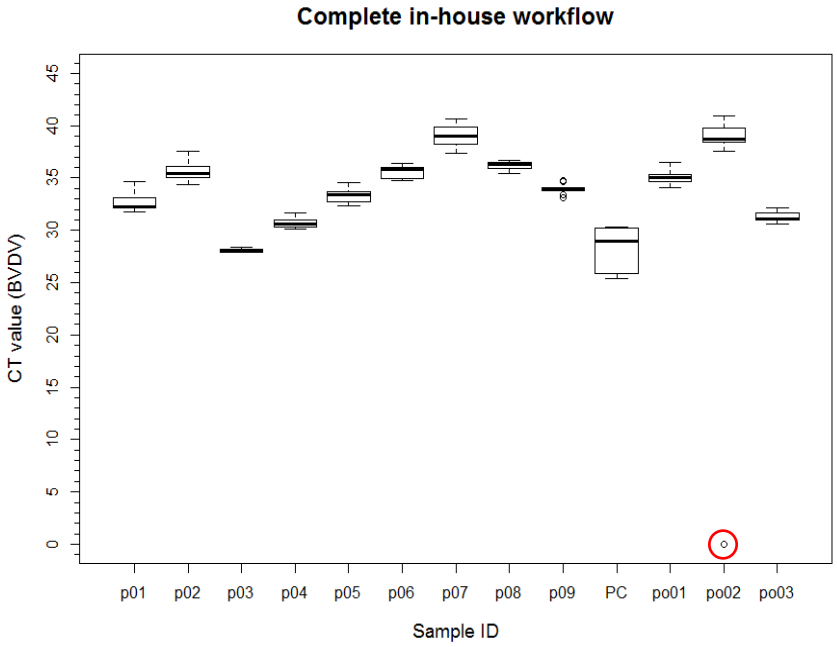
BVDV										
Sample	Q-Q Mean	Q-L Mean	L-Q Mean (all)	L-Q Mean (-Rep1)	L-L Mean	Q-Q SD	Q-L SD	L-Q SD (all)	L-Q SD (-Rep1)	L-L SD
Positive 1	**28.74**	30.54	32.13	30.82	32.78	**0.46**	0.10	2.57	2.04	0.95
Positive 2	**33.08**	35.15	35.62	35.02	35.73	**0.40**	0.33	11.48	2.75	0.92
Positive 3	**26.06**	27.60	27.44	26.02	28.09	**0.51**	0.26	2.08	0.65	0.17
Positive 4	**28.86**	29.71	30.06	28.43	30.67	**0.52**	0.22	2.36	0.43	0.43
Positive 5	**29.09**	31.49	32.18	31.03	33.26	**0.28**	0.21	1.81	0.87	0.68
Positive 6	**32.44**	34.65	34.66	32.74	35.62	**0.39**	0.33	11.17	12.22	0.57
Positive 7	**34.06**	37.75	36.67	36.25	39.03	**0.44**	0.55	11.57	0.96	1.06
Positive 8	**33.21**	35.92	34.47	33.35	36.19	**0.28**	0.32	1.69	0.53	0.39
Positive 9	**31.42**	33.59	33.64	32.58	33.97	**0.38**	0.23	1.96	1.38	0.49
Negative 1	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 2	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 3	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 4*	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 5	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 6	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 7	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 8	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 9	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 10*	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 11	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 12	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 13	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Negative 14	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Pool 1	**31.01**	33.08	34.16	32.69	35.06	**0.29**	0.20	2.15	0.65	0.67
Pool 2	**36.07**	38.69	36.28	36.28	39.13	**0.27**	0.97	18.03	13.53	12.33
Pool 3	**28.64**	30.22	31.52	29.78	31.27	**0.37**	0.39	10.16	11.14	0.47
NTCe	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
PC	**31.51**	28.49	33.65	31.48	28.26	**0.32**	2.11	10.92	11.75	2.00
NTCq	**0.00**	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00
Neg4,10 Q-Q was adjusted to 0.00					**avg sd**	**0.38**	0.48	6.76	4.53	1.62
neg 4 SD Q-Q:11.08					**min sd**	**0.27**	0.10	1.69	0.43	0.17
neg10 SD Q-Q: 7.04					**max sd**	**0.52**	2.11	18.03	13.53	12.33

BVDV Detection:
MagMax Extraction and Qiagen virotype RT-qPCR (L-Q)
Complete in-Lab Work Flow (L-L)



SD: 4.53 (0.43 – 13.53)

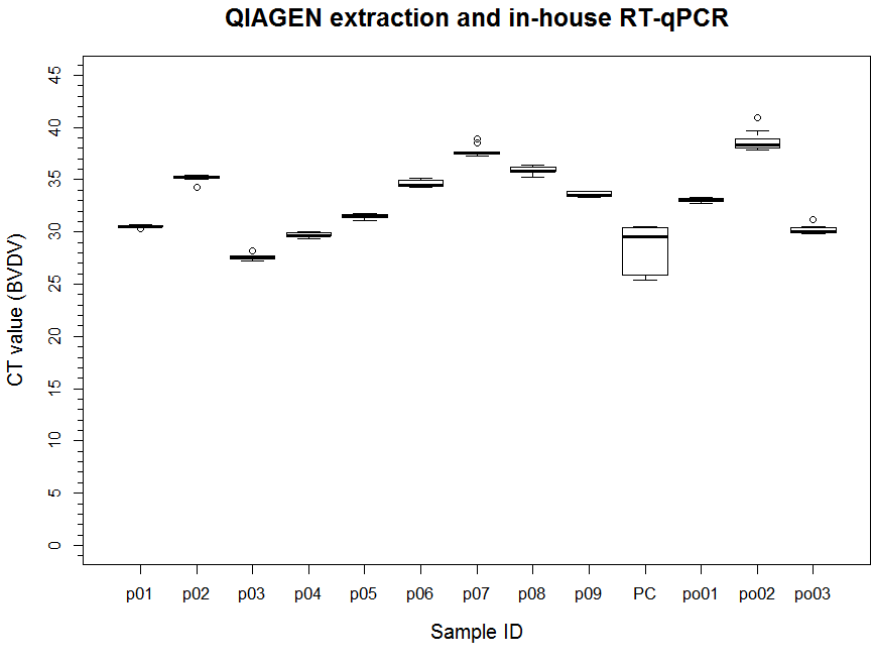
High standard deviation between triplicates and six false-negative samples



SD: 1.62 (0.17 – 12.33)

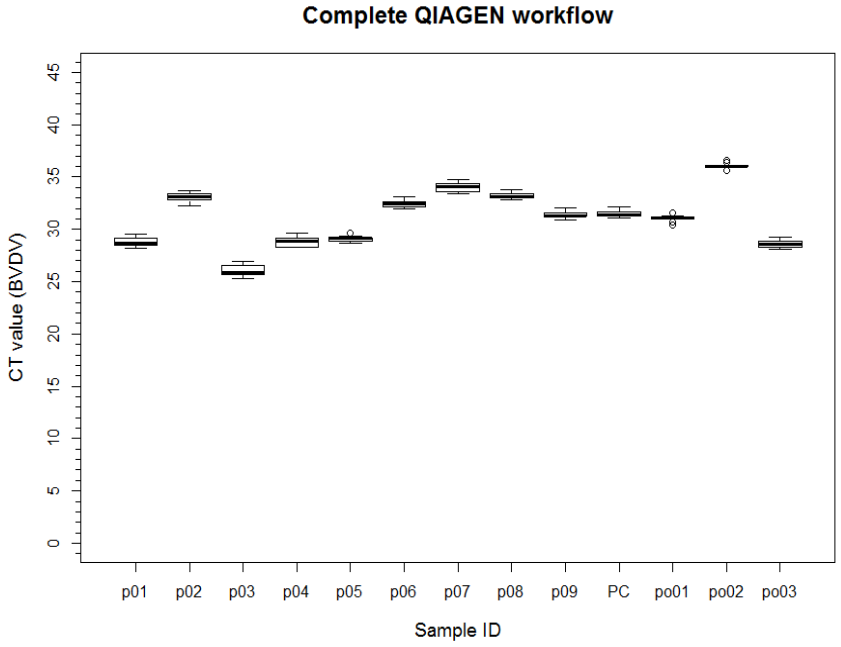
Average standard deviation but one false-negative samples

BVDV Detection:
QIAGEN Extraction and in-lab RT-qPCR (Q-L)
Complete Qiagen Workflow (Q-Q)



SD: 0.48 (0.10 – 2.11)

Low-medium standard deviation with in-lab RT-qPCR



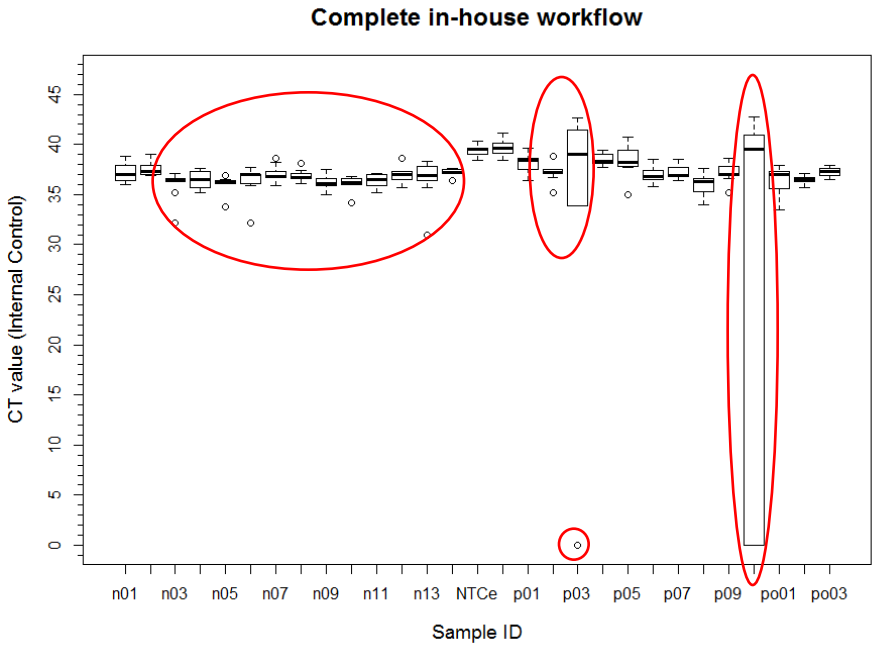
SD: 0.38 (0.27 – 0.52)

Lowest standard deviation and high sensitivity with QIAGEN workflow (MagAttract extraction and *virotype* BVDV Reagents)

Results:
Internal Control Amplification

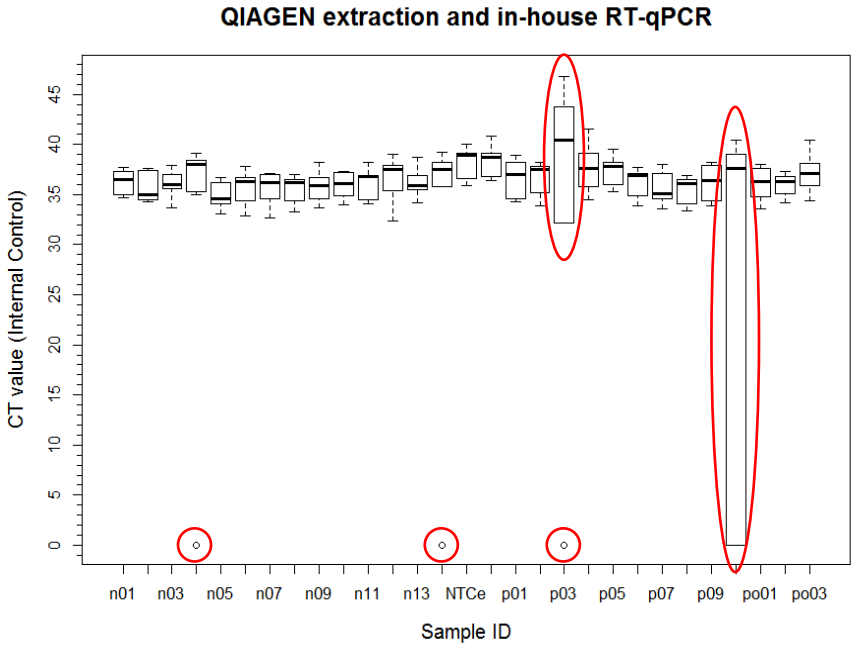
Internal Controls										
Sample	Q-Q Mean	Q-L Mean	L-Q Mean (all)	L-Q Mean (-Rep1)	L-L Mean	Q-Q SD	Q-L SD	L-Q SD (all)	L-Q SD (-Rep1)	L-L SD
Positive 1	**24.95**	36.80	26.41	24.63	38.16	**0.56**	1.73	2.55	0.56	0.90
Positive 2	**25.07**	36.63	26.45	24.79	37.15	**0.51**	1.62	2.44	0.69	0.89
Positive 3	**25.30**	41.12	26.87	25.15	39.15	**0.61**	17.59	2.53	0.87	16.49
Positive 4	**25.22**	37.59	26.73	25.00	38.50	**0.55**	2.07	2.53	0.74	0.56
Positive 5	**25.23**	37.31	26.98	25.41	38.51	**0.44**	1.40	2.33	0.87	1.64
Positive 6	**25.30**	36.20	26.94	25.24	37.00	**0.43**	1.30	2.56	1.05	0.85
Positive 7	**25.59**	35.67	27.21	25.65	37.23	**0.36**	1.55	2.40	1.17	0.64
Positive 8	**25.24**	35.52	26.69	25.08	36.14	**0.51**	1.32	2.38	0.86	1.09
Positive 9	**24.99**	36.23	27.08	25.64	37.22	**0.66**	1.72	2.44	1.62	0.95
Negative 1	**25.15**	36.18	27.15	25.70	37.14	**0.43**	1.15	2.36	1.42	0.90
Negative 2	**25.23**	35.85	27.41	26.12	37.49	**0.60**	1.47	2.20	1.50	0.65
Negative 3	**25.35**	35.99	27.78	26.40	35.92	**0.39**	1.40	2.33	1.53	1.43
Negative 4	**25.57**	37.57	27.81	26.52	36.45	**0.47**	11.89	2.24	1.57	0.89
Negative 5	**25.10**	34.98	27.43	26.13	36.07	**0.57**	1.22	2.22	1.52	0.85
Negative 6	**25.34**	35.81	27.36	26.01	36.31	**0.44**	1.52	2.24	1.43	1.57
Negative 7	**25.22**	35.49	26.85	25.26	37.05	**0.41**	1.52	2.38	0.95	0.83
Negative 8	**25.16**	35.46	27.53	26.24	36.87	**0.54**	1.24	2.36	1.81	0.57
Negative 9	**25.30**	35.86	27.39	26.02	36.25	**0.58**	1.50	2.36	1.68	0.66
Negative 10	**25.18**	36.03	27.50	26.26	36.12	**0.61**	1.16	2.18	1.59	0.76
Negative 11	**25.49**	36.05	27.37	26.05	36.37	**0.47**	1.43	2.28	1.61	0.68
Negative 12	**25.43**	36.63	27.98	26.49	36.93	**0.53**	1.92	2.58	1.68	0.81
Negative 13	**25.23**	36.25	27.11	25.56	36.40	**0.59**	1.46	2.49	1.43	2.08
Negative 14	**26.15**	37.69	26.93	25.09	37.20	**0.29**	15.70	2.67	0.72	0.35
Pool 1	**25.15**	35.86	26.82	25.11	36.51	**0.45**	1.61	2.57	1.02	1.34
Pool 2	**25.19**	36.00	26.52	24.73	36.44	**0.46**	1.03	2.61	0.76	0.44
Pool 3	**25.37**	37.14	26.86	25.22	37.25	**0.46**	1.82	2.49	1.11	0.46
NTCe	**25.12**	38.26	26.64	24.94	39.37	**0.55**	1.44	2.51	0.80	0.56
PC	**25.55**	38.53	27.14	25.35	39.87	**0.46**	18.20	2.64	0.89	18.92
NTPq	**25.48**	38.48	26.77	25.16	39.59	**0.39**	1.54	2.41	0.90	0.76
avg	**25.30**	36.66	27.09	25.55	37.26	**0.49**	3.47	2.42	1.18	2.05
min	**24.95**	34.98	26.41	24.63	35.92	**0.29**	1.03	2.18	0.56	0.35
max	**26.15**	41.12	27.98	26.52	39.87	**0.66**	18.20	2.67	1.81	18.92
sd	**0.23**	1.26	0.40	0.56	1.11					

Internal Control Detection:
Complete in-Lab Work Flow (L-L)
QIAGEN Extraction and in-lab RT-qPCR (Q-L)



SD: 2.05 (0.35 - 18.92)

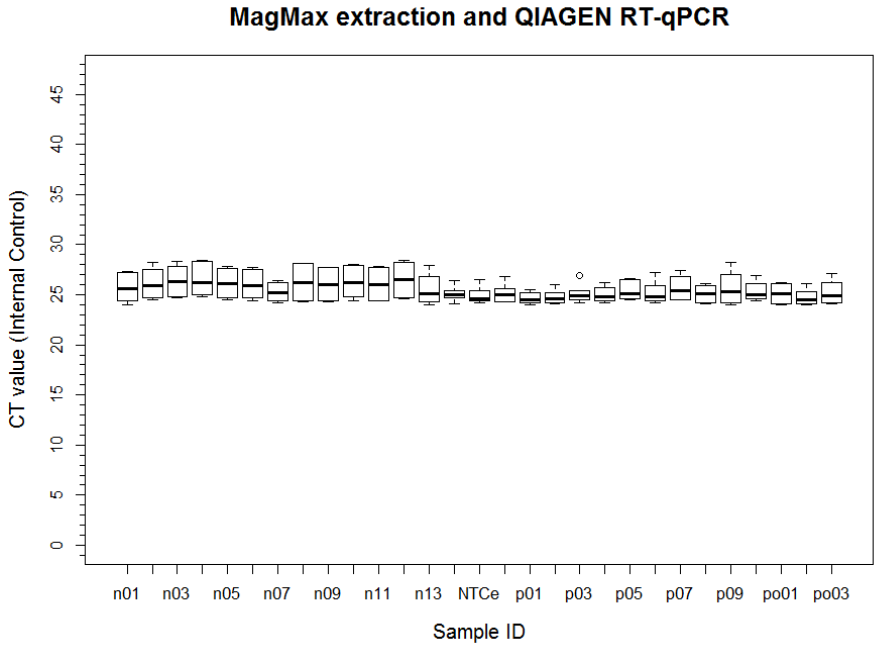
Average standard deviation between triplicates and two samples without internal control (sub-optimal internal control)



SD: 3.47 (1.18 - 18.20)

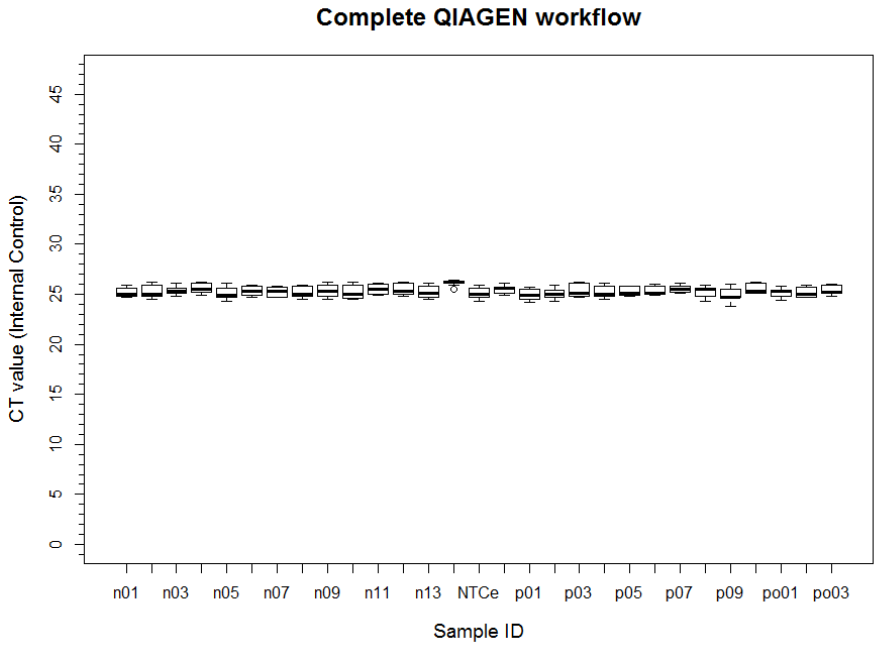
Average standard deviation between triplicates and four samples without internal control (sub-optimal internal control)

Internal Control Detection:
MagMax Extraction and Qiagen virotype RT-qPCR (L-Q)
Complete Qiagen Workflow (Q-Q)



SD: 1.18 (0.56 - 1.81)

Average standard deviation and good internal control signal (MagMax extraction and *virotype* BVDV Reagents)



SD: 0.49 (0.29 - 0.66)

Lowest standard deviation and good internal control signal with QIAGEN workflow (MagAttract extraction and *virotype* BVDV Reagents)

Conclusions

- The sensitivity of the QIAGEN workflow was higher than the in-house duplex assay, evidenced by a signal threshold that was on average 3 CT values lower than the in-lab assay
- The IC of the *virotype* BVDV RT-PCR Reagent performed better than the IC of the in-lab assay
- The standard deviation of the CT value for each positive sample (extracted and tested in triplicate) was lower when using the QIAGEN workflow
- The QIAGEN workflow was more cost-efficient

(QIAGEN MagAttract 96 cador Pathogen Kit and *virotype* BVDV Reagents)