Adaptation and Validation of the Purcell method of IHNv detection

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Protocol

Qiagen Quantitect Probe Rt-PCR kit
Primer and probe concentrations ⇝ Purcell et al (900nM of each primers, 200nM of probe)

Reagents	Initial Concentration	Final Concentration	Volume for 1 reaction	Volume for 9 reactions
QT Probe master mix	2x	1x	12,50	112,50
pPVP377	20	900	1,13	10,13
pPVP378	20	900	1,13	10,13
qPVP23	20	200	0,250	2,25
QT RT mix			0,250	2,25
H_2O			4,75	42,75
Volume total (µl)			20,00	180,00
Add	5	µl ARN to	20	µl MasterMix

Programme		
1.	30 min	50°
2.	15 min	95°
3.	15 sec	94°
4.	60 sec	60° *** coll. endpoint data FAM
5.	goto 3.	39x

Tests of ruggedness

- Slight variations for :
  - Primers and probe concentrations (+/- 20%)
  - PCR program
- Two-step versus one-step
- 5 ten-fold dilutions (supernatant of N61, internal French reference strain)

No significative CT difference with +/- 20% [primers] or [probe]

Efficiency

Between **91** and **96%** (slopes ranging from -3,4 to -3,5)

Repeatability

1 operator, 2 days

Nb of sessions	Operators	Nb of dilutions	Replicates/dilution
2	1	6	2

Concentrations (PFU /mL)	1.10^7	1.10^6	1.10^5	1.10^4	1.10^3	1.10^2
06/05/2015	15,76	18,36	22,31	25,72	28,82	31,7
12/05/2015	15,78	18,51	22,48	25,99	29,43	32,45
Mean value	15,77	18,44	22,4	25,86	29,12	32,08
Standard deviation	0,08	0,15	0,11	0,16	0,36	0,72
CV %	**0,51%**	**0,82%**	**0,51%**	**0,62%**	**1,22%**	**2,25%**

CV < 3%

Within operators

Nb of sessions	Operators	Nb of dilutions	Replicates/Dilution
3	3	6	2

3 operators

Concentrations (PFU/mL)	1.10^7	1.10^6	1.10^5	1.10^4	1.10^3	1.10^2
EG 07/05/2015	16,3	18,77	22,77	26,26	29,57	33,11
LL 06/05/2015	17,35	19,96	23,74	27,3	29,85	32,2
LM 06/05/2015	16,98	19,81	23,52	26,71	28,98	31,28
Mean value	16,88	19,52	23,34	26,76	29,47	32,20
Standard deviation	0,49	0,58	0,46	0,4899	0,40	0,93
CV%	**2,93%**	**2,96%**	**1,95%**	**1,83%**	**1,36%**	**2,89%**

CV < 6%

Analytical specificity

40 supernatants

		Sample status	
		Positive	Negative
Status delivered by the method	Positive	34 true positive	0 false positive
	Negative	0 False Negative	6 True Negative

100% positive supernatant detected

Analytical sensitivity (limit of detection)

	Nb of séances	Operators	Nb of dilutions	Replicates/dilution
LOD				
DNA plasmid	3	1	6	8
N61 RNA	3	1	6	2

- **25 copies / reaction** using plasmid pPVP22 (Ct=33,4)
- **10 PFU / reaction** using N61 IHNv strain **(2.10^3 PFU/mL)**; Ct=32,6

Diagnostic specificity : Dsp = 100%

		Sample status	
		Positive	Negative
Status delivered by the method	Positive	8 true positive	0 false positive
	Negative	0 False Negative	2 True Negative

Diagnostic sensitivity

2 methods :

- organ homogenate spiked with plasmidic DNA = **10 cp / reaction** (Ct=34,5)
- organs collected after experimental infections ; serial dilutions (Ct=35,2)

Quite difficult to find Dse (random signal with late Ct)

Perspectives

❶ **production of RNA Transcript** to determine precisely LD_{PCR} (copies of RNA/reaction)

❷ Add an **exogenous control** to validate the whole process (extraction + amplification)

Choice : bacteriophage T4 (DNA) and MS2 (RNA) to spike samples before extraction

Diagnostic sensibility : 10 fold serial dilutions with and without control

❸ **Reproductibility :** interested in participating a proficiency test for IHNv detection

❹ To be accredited for IHNv detection by RT-PCR in september 2017
↳ Transfer the method to the French accredited labs (7 labs)

Then deployment of the range of analysis (VHSv and KHV in 2018 ? IPNv in 2019 ?) while maintaining accreditation in cellular virology …