RT-qPCR: genome detection of the listed fish diseases, IHN and VHS

Heike Schütze (FLI, Germany)

FRIEDRICH-LOEFFLER-INSTITUT
seit 1910
FLI
Bundesforschungsinstitut für Tiergesundheit
Federal Research Institute for Animal Health

RT-qPCR (EC 2015/1554)

EU-RL Diagnostic manual

	IHNV				VHSV			
assay	reference	gene	product	remark	reference	gene	product	remark
RT-PCR	Emmenegger et al.2000 *	G	693 bp	1-step, without nPCR	Snow et al. 2004	N	512 bp (505 bp)	1-step; BF-2 Detection
					Kim et al.2018	N	320 bp	1-step
RT-qPCR	Purcell et al.2013	N	80 bp	2-step	Jonstrup et al. 2013 *	N	77 bp	1-step
	Cuenca et al. 2020			1-step				

* OIE recommendation

Other assays have not been sufficiantly tested and validated for isolates of different genotypes (OIE, 2017, 2020)

Improvement of IHNV RT-qPCR

IHNV RT-qPCR (Purcell et al. 2013):
- high risk of contamination (2-step)
- 1 µg RNA as template
- no stable clear detection of some IHNV field samples

⇒ **Goal:** the establishment of an efficient and rapid diagnostic method

1) Robust One-step RT-qPCR
2) Detection of IHNV-genome with good sensitivity and specifity
3) good price-performance ratio (probe: Taq-Man vs. FAM; kits)
4) endogenous control system
5) Optimisation of processes in daily routine diagnostics
6) meet MIQE guidelines and OIE criteria

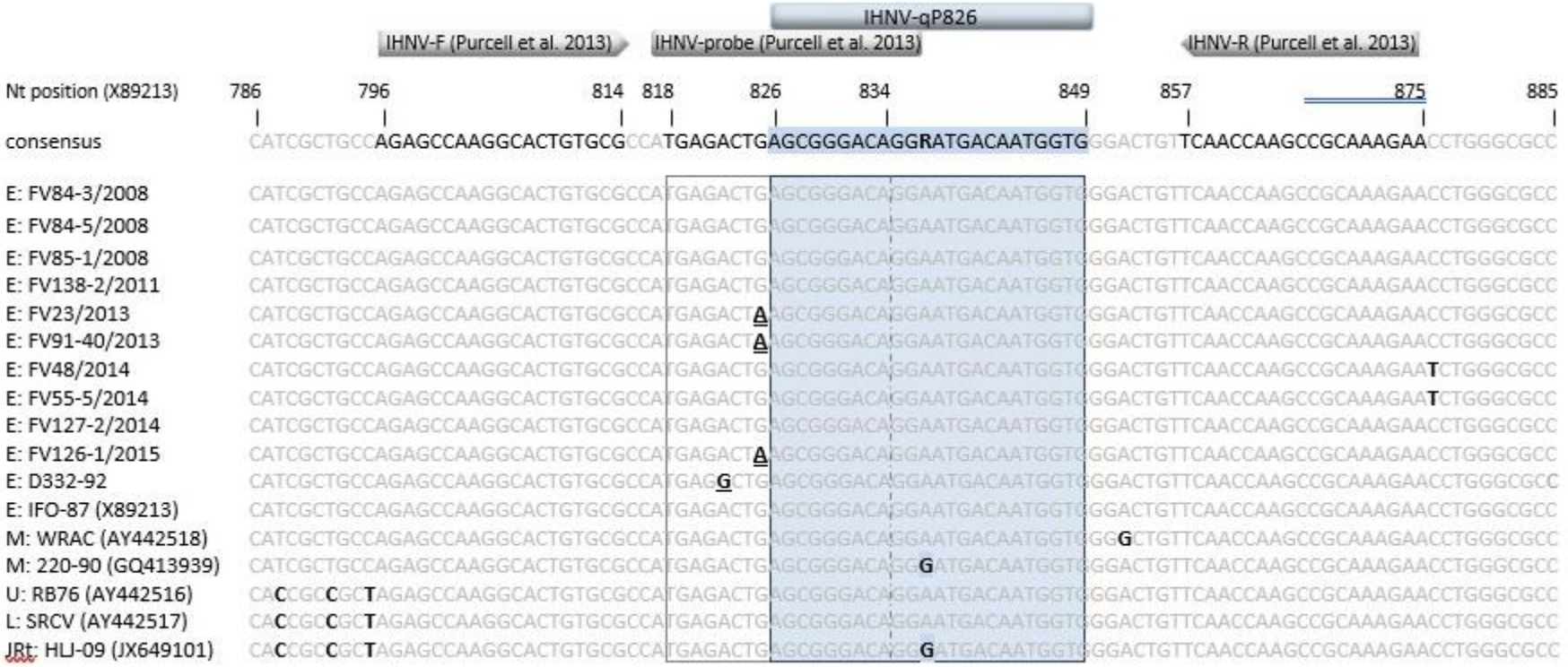
CVUA Stuttgart and NRL IHN, VHS (FLI, Germany): independent validation

Purcell et al.2013: 2-step RT-qPCR

- archieved diagnostic field samples
- Positive control: D332-92, WRAC, RB76, SRCV

$\Rightarrow$ Doubtful results (FV23, FV91-40 and D332-92)

(Discrepancy to virus isolation in cell culture with IFAT)



⇒ SNP within the binding site of IHNV-probe (Purcell et al. 2013)

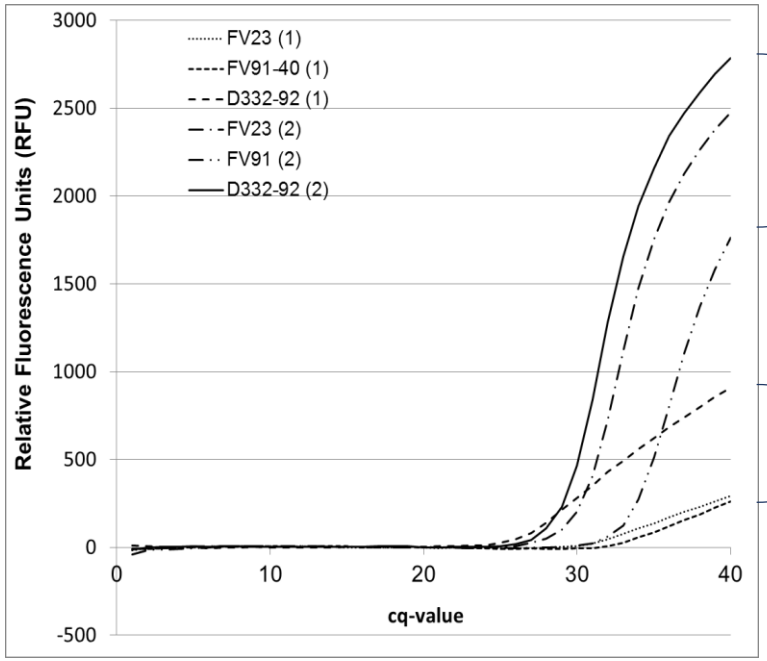
- in silico analyses with available IHNV sequence data

1) optimized FAM-labeled probe
2) modified thermal profile
3) internal control: ß Actin

IHNV RT-qPCR: modified one-step versus two step method

Sample	Two-step RT-qPCR (Purcell et al., 2013)		One-step RT-qPCR (new)	
	cq MV	SD	cq MV	SD
FV84-3	32.61	0.03	28.76	0.05
FV84-5	35.79	0.99	36.77	0.64
FV85-1	29.60	0.25	31.04	0.05
FV138-2	26.07	0.00	24.95	0.00
FV23[a]	33.31	1.47	28.68	0.03
FV91-40[a]	38.71	0.57	32.01	0.07
FV48	15.29	0.03	16.30	0.08
FV55-5	19.72	0.03	20.20	0.02
FV126-1	19.91	0.05	20.61	0.02
FV127-2	30.19	0.21	29.33	0.07
D332-92[a]	25.60	0.06	27.51	0.01
IHNV WRAC	12.81	0.04	13.40	0.08
IHNV RB76	13.14	0.05	13.62	0.04
IHNV SRCV	12.77	0.03	13.21	0.14

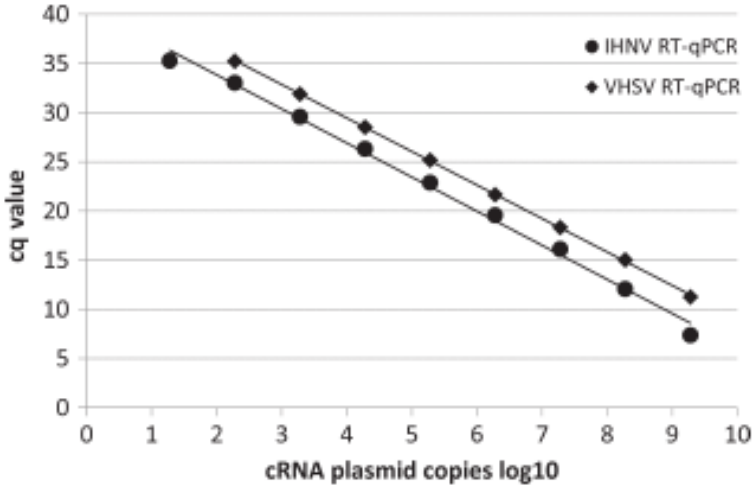
Notes. Mean cq value (MV) and standard deviation (*SD*) are given.
[a]Isolates with doubtful results in the two-step RT-qPCR.



⇒ Taq-Man Probe = critical for samples with SNP and low virus load
⇒ Good perfomance of the new method

Technical sensitivity

Standard curves for serial diluted IHNV and VHSV transcripts (cRNA of IHNV-N- and VHSV-N-plasmid)

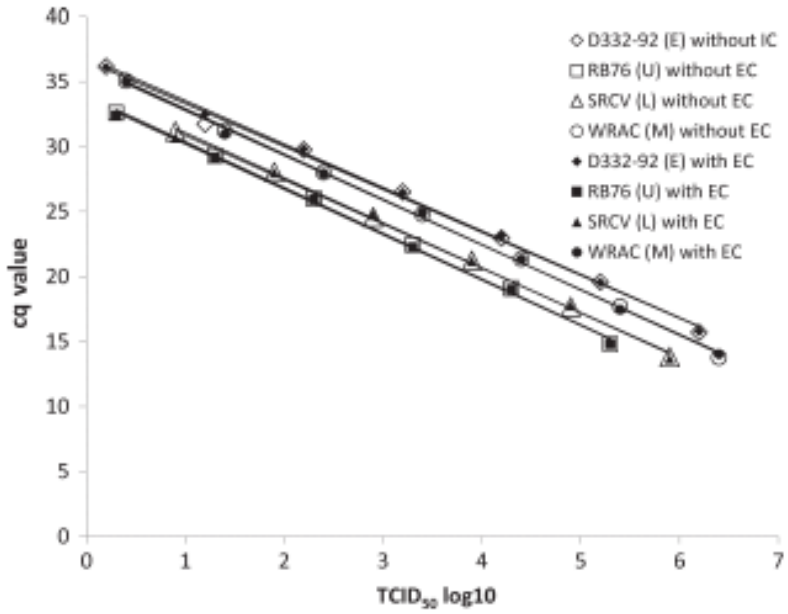


cRNA (plasmid copies)	IHNV RT-qPCR		VHSV RT-qPCR	
	cq MV	SD	cq MV	SD
1.90E+09	7.38	0.56	11.28	0.77
1.90E+08	12.09	1.22	15.04	0.11
1.90E+07	16.12	0.06	18.35	0.10
1.90E+06	19.58	0.20	21.68	0.12
1.90E+05	22.88	0.15	25.20	0.11
1.90E+04	26.33	0.21	28.53	0.07
1.90E+03	29.58	0.05	31.91	0.10
1.90E+02	33.02	0.25	35.23	0.42
1.90E+01	35.27	0.74	no cq	no cq

	IHNV RT-qPCR	VHSV RT-qPCR
Slope	−3.4673	−3.4054
Efficiency ($E = 10^{-1/slope}$)	1.9427	1.9663
% Efficiency	94.27	96.63
Y-intercept	40.78	43.08
R^2	0.9949	0.9998
Limit of detection (cq)	19 (35.27)	190 (35.23)

Analytical sensitivity

standard curves for serial diluted viruses of IHNV D332-92, RB76, SRCV, WRAC
with vs. without EC



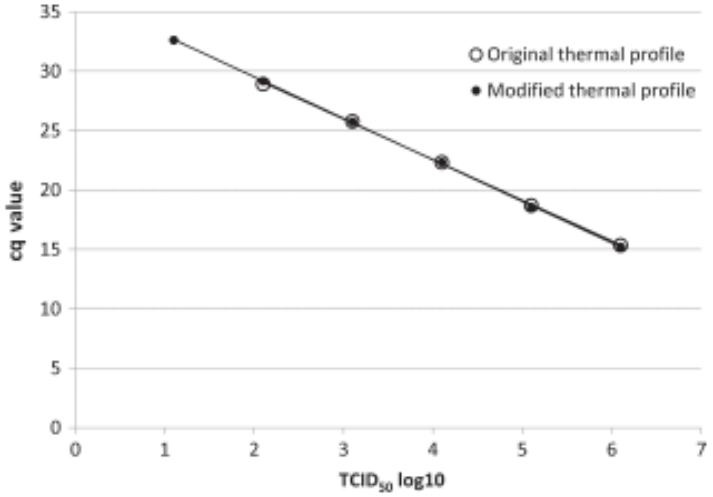
	Without EC				With EC			
	D332-92	RB76	SRCV	WRAC	D332-92	RB76	SRCV	WRAC
Slope	−3.3039	−3.5191	−3.4649	−3.4850	−3.3421	−3.4906	−3.4046	−3.4464
Efficiency ($E = 10^{-1/slope}$)	2.0076	1.9238	1.9436	1.9362	1.9916	1.9341	1.9666	1.9505
% Efficiency	100.76	92.38	94.36	93.62	99.16	93.41	96.66	95.05
Y-intercept	36.64	33.89	34.52	36.40	36.88	33.71	34.25	36.2353
R^2	0.9951	0.9980	0.9991	0.9989	0.9983	0.9981	0.9964	0.9985
Limit of detection (cq)	1.6 (36.17)	2.0 (32.59)	7.9 (31.18)	2.5 (35.06)	1.6 (36.12)	2.0 (32.40)	7.9 (30.68)	2.5 (35.01)

Virus isolate ($TCID_{50}$/ml)	Virus re-isolation		Without EC		With EC	
	$TCID_{50}$/ml	CPE	cq MV	SD	cq MV	SD
IHNV D332-92	1.58+06	+	15.71	0.03	15.79	0.06
	1.58+05	+	19.59	0.15	19.54	0.26
	1.58+04	+	22.96	0.19	23.12	0.04
	1.58+03	+	26.52	0.29	26.47	0.14
	1.58+02	+	29.77	0.05	29.81	0.11
	1.58+01	+	31.75	2.42	32.49	1.31
	1.58+00	+	36.17	0.71	36.12	0.67
IHNV RB76	2.00E+05	+	14.80	0.15	14.81	0.35
	2.00E+04	+	19.08	0.10	19.01	0.20
	2.00E+03	+	22.44	0.24	22.24	0.35
	2.00E+02	+	26.00	0.08	25.95	0.40
	2.00E+01	+	29.30	0.06	29.18	0.16
	2.00E+00	+	32.59	0.03	32.40	0.43
IHNV SRCV	7.94E+05	+	13.82	0.17	13.69	0.03
	7.94E+04	+	17.66	0.16	17.76	0.22
	7.94E+03	+	21.21	0.07	21.19	0.20
	7.94E+02	+	24.57	0.10	24.74	0.10
	7.94E+01	+	28.03	0.44	27.98	0.08
	7.94E+00	+	31.18	0.13	30.68	0.32
IHNV WRAC	2.51E+06	+	13.76	0.11	14.04	0.06
	2.51E+05	+	17.70	0.02	17.46	0.26
	2.51E+04	+	21.35	0.10	21.34	0.04
	2.51E+03	+	24.79	0.05	24.89	0.25
	2.51E+02	+	28.01	0.01	27.93	0.03
	2.51E+01	+	31.21	0.11	30.96	0.12
	2.51E+00	+	35.06	0.16	35.01	0.16

EC = ß-Actin

Analytical sensitivity

standard curves for serial diluted viruses of **VHSV Fi13**
Jonstrup et al. 2013 vs. **modified thermoprofile**

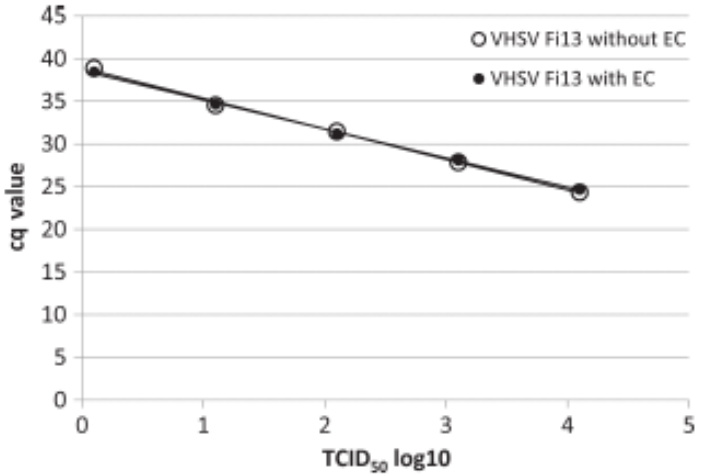


Virus re-isolation		Original thermal profile		Modified thermal profile	
$TCID_{50}$/ml	CPE	cq MV	*SD*	cq MV	*SD*
1,26E+06	+	15.35	0.05	15.22	0.25
1,26E+05	+	18.71	0.23	18.57	0.36
1,26E+04	+	22.32	0.21	22.33	0.14
1,26E+03	+	25.79	0.18	25.74	0.2
1,26E+02	+	28.91	0.17	29.17	0.26
1,26E+01	–	no cq	/	32.62	0.36

	Original thermal profile	Modified thermal profile
Slope	−3.4200	−3.4917
Efficiency ($E = 10^{-1/slope}$)	1.9606	1.9337
% Efficiency	96.06	93.37
Y-intercept	36.24	36.51
R^2	0.9995	0.9982
Limit of detection (cq)	126 (28.91)	13 (32.62)

Analytical sensitivity

standard curves for serial diluted viruses of **VHSV Fi13**
modified thermoprofile: **with vs. without EC**



Virus re-isolation		Without EC		With EC	
$TCID_{50}$/ml	CPE	cq MV	*SD*	cq MV	*SD*
1,26E+04	+	24.34	0.10	24.68	0.70
1,26E+05	+	27.86	0.11	28.08	0.04
1,26E+04	+	31.46	0.18	31.23	0.12
1,26E+03	+	34.52	0.42	34.65	0.29
1,26E+02	–	38.88	0.30	38.45	0.40

	Without EC	With EC
Slope	−3.5740	−3.4120
Efficiency ($E = 10^{-1/slope}$)	1.9046	1.9637
% Efficiency	90.46	96.37
Y-intercept	38.92	38.58
R^2	0.9974	0.9989
Limit of detection (cq)	1.3 (38.88)	1.3 (38.45)

Specifity

105 Archived IHNV field isolates collected between 1998-2015 in Europe

- detection by RT-qPCR with good signals

154 Field samples from clinically healthy fish within IHN prevention and monitoring programme

- 28 fish = IHNV positive in cell culture and RT-qPCR
- 14 fish = IHNV positive only in RT-qPCR

165 Archived VHSV field isolates collected between 1998-2015 in Europe

- detection by RT-qPCR with good signals

127 Field samples from clinically healthy fish within VHS prevention and monitoring programme

- 2 fish = VHSV positive in cell culture and RT-qPCR
- 1 fish = VHSV positive only in RT-qPCR

⇒ **Higher sensitivity of RT-qPCR compared to virus isolation**

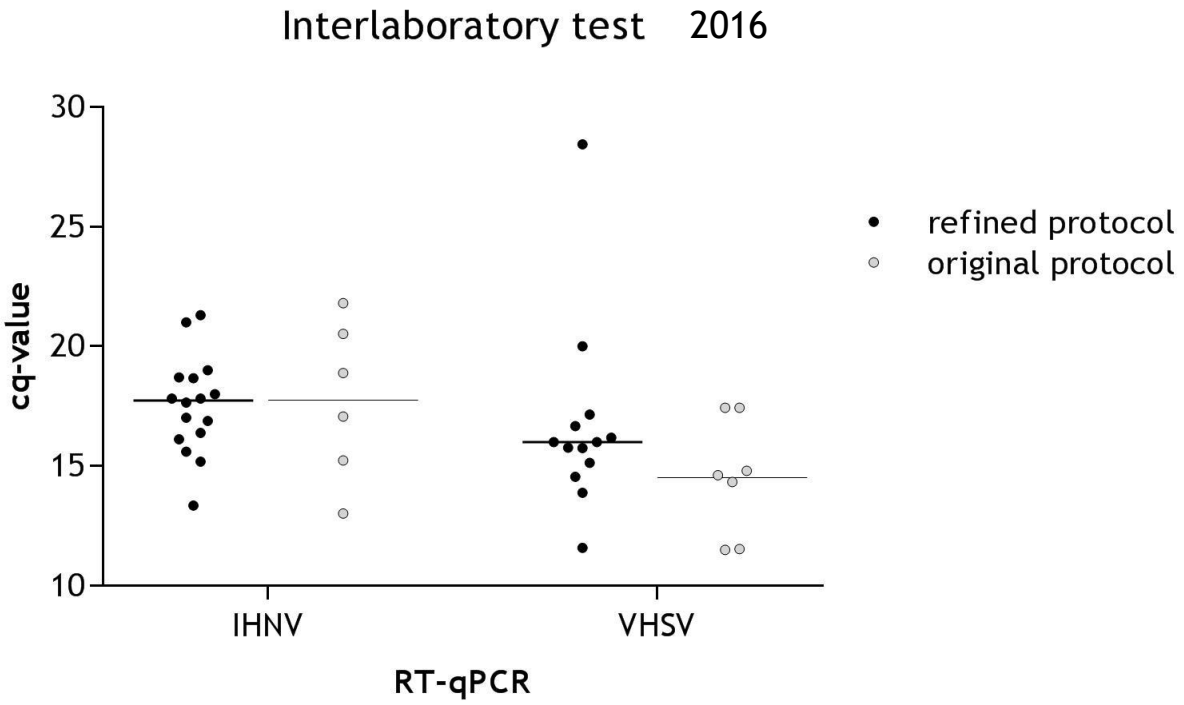
Specifity

The following samples were also included in this study :

- SVCV 56
- IPNV VR299, Ab, Sp, fiedl samples
- ISAV Glesvaer 2/90
- Ranaviruses ECV-24, EHNV
- Renibacterium salmoninarum
- Yersinia ruckeri
- Aeromonas hydrophila
- Aeromonas salmonicida
- Aeromonas veronii
- Tertracapsuloides bryosalmonae

Robustness

- validation in two independent laboratories (CVUAS, FLI)
- Annual proficiancy test in Germany for modified RT-qPCR



Summary

	IHNV	VHSV	IPNV
reaction	1-tube, 1-step		
primers	Purcell et al. 2013	Jonstrup et al. 2013	Hoferer et al. 2017
probe	Hoferer et al. 2019	Jonstrup et al. 2013	Hoferer et al. 2017
thermoprofile	Hoferer et al. 2019, 2017		

- High technical and analytical sensitivity
- High specifity
- High robustness
- Meets criteria of OIE and MIQE-guidlines
- Internal control ß-actin
- Same run of IHNV-, VHSV- and IPNV- RT-qPCR
- Comparable to gold standard (cell culture)
- RNA-extraction: automatic, manuel
- RNA-reagents: independent
- RT-PCR reagents: independent

Received: 24 August 2018 | Revised: 19 December 2018 | Accepted: 20 December 2018

DOI: 10.1111/jfd.12968

ORIGINAL ARTICLE

WILEY Journal of Fish Diseases

Improvement of a diagnostic procedure in surveillance of the listed fish diseases IHN and VHS

Marc Hoferer[1] | Valerij Akimkin[1] | Julia Skrypski[1] | Heike Schütze[2] | Reinhard Sting[1]