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qPCR assay for detecting round goby invasive fish species *Neogobius melanostomus*

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ABSTRACT

Members of Ponto-Caspian gobies are predatory fish which have colonized freshwaters and brackish waters in Europe and North America causing a lot changes in the native ecosystems.

Several eDNA assays were developed to identify round goby fish. We are going to describe two successful assays.

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GUIDELINES

Handling high concentration of positive controls was performed in a post-PCR room which is physically separated from the pre-PCR room to avoid contamination.
Always add your samples first and seal them before adding the serial dilutions of positive control (standard) at the end.

MATERIALS TEXT

UltraPure™ DEPC-treated WaterThermo FisherCatalog #10813012
TaqMan™ Environmental Master Mix 2.0 https://www.thermofisher.com/order/catalog/product/4396838#/4396838
Interbal control http://www.primerdesign.co.uk/assets/files/internal_control_handbook_dna.pdf?timestamp=1504081027

SAFETY WARNINGS

Negative controls of DNase/RNase free water in each qPCR assay.

DISCLAIMER:

BEFORE STARTING

Laboratory work space and equipment were sterilized by UV-light and DNase solution and 70% ethanol. Filter pipet tips were used in all steps of the laboratory work.

DNA extraction 2h 30m

1 A tissue of round goby was extracted with Qiagen DNeasy blood and tissue extraction kit
https://www.qiagen.com/us/shop/pcr/dneasy-blood-and-tissue-kit/
The quality of DNA and 260/280 ratio were checked by NanoDrop instrument for nucleic acid measurements.

2 To check for inhibitors in DNA samples, internal control was used,
http://www.primerdesign.co.uk/assets/files/internal_control_handbook_dna.pdf?timestamp=1469446474

OR: https://www.thermofisher.com/order/catalog/product/4308321.

3 Primers

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A	B	C	D	E	F
Neogobius melanostomus	Primer	Reference	Temperature	Length	GC content
Neo_Mel_COI_F01	5'-CTTCTRGCCTCCTCTGGWGTTG-3'	Andersen et al., 2016	59.6 -62.8	22	54-59
Neo_Mel_COI_R01	5'-CCCWAGAATTGASGARATKCCGG-3'		58.9 -63.9	23	47-56
Neo_Mel_COI_P01	5'-FAM-CAGGCAACTTRGCACATGCAG-BHQ-3'		60.1 - 62.9	21	52-57
NeoMel_IK_F1	5'- TATGTGATGATCGGACAGC-3'	Adrian-Kalchhauser & Burkhardt-Holm, 2016.		19	53-56
NeoMel_IK_R1	5'- GTTCTCTAGTCAGCTCGCT-3'			19	45-51
NeoMel_IK_Probe	5'-FAM-CATCTTTCTCGGCTTATTCCCCA-BHQ-3'			23	

30m

4 **2 µl** Standard DNA dilution

To determine both limit of quantification and limit of detection, genomic DNA of round goby was serially diluted from $1e^{2}$-$1e^{-4}$.

1h 30m

5 The composition of PCR mixture

A	B	C	D
PCR reagent	Stock solution	Working solution	Final concetration (µL)
TaqMan Environmental Mastermix 2	2X	1X	10
Forward primer	10 µM	0.4 µM	1
Reverse primer	10 µM	0.4 µM	1
TaqMan probe	2.5 µM	0.1 µM	1
Internal control (IC) primer/probe mix			1
IC-DNA			0.5
DNase/RNase free water			8.5
Template			2
Total volume			25

RNase/DNase free water was used as a negative control.
Lower volume of PCR mixture was tested and efficiency was evaluated.

6 **- °C** Amplification conditions

A	B	C	D
	Step	**Time**	**Temp**
50 cycles	Enzyme activation	10 min	95
	Denaturation	15 s	95
	Data collection	1 min	60

Enzyme activation temperature depends on the type of qPCR master mixture.

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7 QPCR was performed in BioRad qPCR machine CFX96.

Analysis of qPCR results was performed through CFX maestro software
https://www.bio-rad.com/en-se/product/cfx-maestro-software-for-cfx-real-time-pcr-instruments?ID=OKZP7E15

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The analysis of PCR internal control
The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. Cq values of 26 or 27±3 are within the normal range.

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