**Interlaboratory study on qPCR (with and without fluorescently labelled probes) on anglerfish, seabream and pike DNA**

**Content**

[**1.** **General information** 3](#_Toc148974764)

[**2.** **Contact** 3](#_Toc148974765)

[**3.** **Materials** 4](#_Toc148974766)

[**3.1.1.** **Materials provided to the participating laboratories** 4](#_Toc148974767)

[**3.1.2.** **Samples provided** 4](#_Toc148974768)

[**3.1.3.** **Reagents provided** 5](#_Toc148974769)

[**3.1.4.** **Materials and equipment to be provided by the participating laboratory** 6](#_Toc148974770)

[**4.** **Protocols** 6](#_Toc148974771)

[**4.1.** **EvaGreen Supermix** 6](#_Toc148974772)

[**4.2.** **Probe mix** 6](#_Toc148974773)

[**5.** **Short description** 7](#_Toc148974774)

[**6.** **General instructions** 7](#_Toc148974775)

[**7.** **Protocols** 7](#_Toc148974776)

[**7.1.** **Plate 1: Single anglerfish qPCR with EvaGreen® dsDye** 8](#_Toc148974777)

[**7.2.** **Plate 2: Triplex qPCR with EvaGreen® dsDye** 10](#_Toc148974778)

[**7.3.** **Plate 3: Single anglerfishqPCR with fluorescently labelled probe** 12](#_Toc148974779)

[**7.4.** **Plate 4: Multiplex qPCR with fluorescently labelled probes** 14](#_Toc148974780)

[**8.** **Data Processing** 16](#_Toc148974781)

[**9. Documentation of results** 16](#_Toc148974782)

# **General information**

This protocol describe conditions of qPCR protocols for detection and quantification of three fish kinds, namely *Esox lucius*, *Lophius piscatorius/Lophius budegassa* and *Spondyliosoma cantharus.* The method was developed and optimized using both EvaGreen and TaqManTM probes and is applicable to raw and processed materials. The designed primers and probe amplify sequence of parvalbumin gene:

* *Esox lucius* 223 bp fragment, probe is labelled by FAM
* *Lophius piscatorius/Lophius budegassa* 196 bp fragment, probe is labelled by Cy5 or FAM
* *Spondyliosoma cantharus* 77 bp fragment, probe is labelled by HEX/VIC

The purpose of this interlaboratory comparison is to test the performance of the method and not the laboratory. Therefore, each laboratory should perform the experiments exactly as described in the protocol. Any deviation from the described procedure should be reported.

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# **Materials**

# **Materials provided to the participating laboratories**

All materials should be stored at – 20°C. After first thawing, materials should be stored at + 4°C for a maximum of two weeks.

# **Samples provided**

23 samples (to be plated in 3 replicates, in one plate) labelled from 1 to 23. Each tube contains approximately 110 µL of DNA solution. This is 1.7 times the amount required in the analysis.

Two calibration curves will be used as part of the interlaboratory comparison, one prepared from the artificial dsDNA standard (St1\_RJ) and the other from the DNA of the selected fish (St2)

Sample for calibration curves:

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Sample | St1\_V  IDT\_RJ\_V | St1\_VI  IDT\_RJ\_VI | St1\_VII  IDT\_RJ\_VII | St1\_VIII  IDT\_RJ\_VIII | St1\_IX  IDT\_RJ\_IX | St1\_X  IDT\_RJ\_X | St1\_XI  IDT\_RJ\_XI |
| Copies in 1 ul | 1.531E+06 | 1.531E+05 | 1.531E+04 | 1.531E+03 | 1.531E+02 | 15 | 1.5 |
| Copies in reaction - single target | 6.124E+06 | 6.124E+05 | 6.124E+04 | 6.124E+03 | 6.124E+02 | 61 | 6.1 |
| Copies in reaction - three targets | 1.837E+07 | 1.837E+06 | 1.837E+05 | 1.837E+04 | 1.837E+03 | 184 | 18.4 |

* St1\_RJ standard, one tube label **St1**, volume 50 µL; dilution of calibration curve (KK) from St1 standards is 10x (1:9 volume); this dilution will be done in participant´s laboratory
* St2; dilution of calibration curve (KK) from genomic DNA is 4x (1:3 volume); this dilution will be done in participant´s laboratory

# **Reagents provided**

**Reaction Mix components**

* + Hot firepol® EvaGreen® qPCR Supermix
  + GoTaq Probe qPCR Master Mix
  + Nuclease free water: one tube
  + Primers and Probes

***Esox Label provided***

• forward primer (20 µM) E-F one tube with 200 µL

• reverse primer (20 µM) E-R one tube with 200 µL

• probe (20 µM) E-P one brown tube with 55 µL

NOTE: the pike probe is FAM labelled and BHQ quenched.

***Lophius Label provided***

• forward primer (20 µM) D-F one tube with 200 µL

• reverse primer (20 µM) D-R one tube with 200 µL

• probes (20 µM) D-P (D-P FAM or D-P Cy5) brown tubes with 85 µL

NOTE: the anglerfish probe is Cy5 and FAM labelled and BHQ quenched.

***Black seabream Label provided***

• forward primer (20 µM) P-F one tube with 200 µL

• reverse primer (20 µM) P-R one tube with 200 µL

• probe (20 µM) P-P one brown tube with 55 µL

NOTE: the seabream probe is HEX/VIC labelled and BHQ quenched.

# **Materials and equipment to be provided by the participating laboratory**

• 96-Well Reaction Plates

• Optical caps/adhesion covers

• Vortex

• Micropipettes

• Racks for reaction tubes, also cooled

• 0.5 mL and 2 mL DNase free reaction tubes

• Real-time PCR detection system and appropriate analysis software

• Standard bench top centrifuge with rotor or standard microfuge fit for reaction tubes, centrifuge for 96-Well reaction plates

# **Protocols**

# **EvaGreen® Supermix**

The procedure follows the basic principles of the real-time amplification method. PCR products are measured during each cycle (real-time) by fluorescence of the dsDNA intercalating dye. For the specific detection of fish species, a pair of specific primers is used to amplify a 223 bp fragment pike, 196 bp anglerfish and 77 bp for black seabream.

# **Probe mix**

The procedure follows the basic principles of the real-time amplification method. PCR products are measured during each cycle (real-time) by fluorescence of the labelled probes. For the specific detection of *esox lucius*, a pair of specific primers is used to amplify a 223 bp fragment. The esox specific probe uses FAM as a reporter dye at its 5’ end and BHQ. The 5’-nuclease activity of the Taq DNA polymerase is exploited, which results in the specific cleavage of the probe, leading to increased fluorescence that is then monitored.

For the specific detection of seabream, a pair of specific primers is used to amplify a 77 bp fragment. The seabream specific probe uses VIC/HEX as a reporter dye at its 5’ end and BHQ. The 5’-nuclease activity of the Taq DNA polymerase is exploited, which results in the specific cleavage of the probe, leading to increased fluorescence that is then monitored.

For the specific detection of anglerfish, a pair of specific primers is used to amplify a 196 bp fragment. The anglerfish specific probe uses Cy5 as a reporter dye at its 5’ end and BHQ3. The 5’-nuclease activity of the Taq DNA polymerase is exploited, which results in the specific cleavage of the probe, leading to increased fluorescence that is then monitored.

# **Short description**

The procedure follows the basic principles of the real-time amplification method. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probes or intercalation dye. For the specific detection the size of product/melting curve or fluorescence of probe is monitored.

For quantification of fish DNA will be done by standard calibration curves of know amount of targeted DNA.

# **General instructions**

* The procedure requires working under sterile conditions.
* Maintain separate working areas for DNA handling, reaction set-up and amplification.
* The use of filter-plugged pipette tips in order to avoid cross-contamination is recommended.
* Use powder-free gloves is recommended.
* Clean lab-benches and equipment periodically with 10% sodium hypochlorite solution or equivalent.
* All pipettes should be checked regularly for precision and, if necessary, should be calibrated.
* All reaction positions of the plate must be identified according to the attached reporting sheet.

# **Protocols**

For this interlaboratory study, four plates should be done. All four have the same layout of samples, but each record has a different mastermix.

* Plate A use mastermix for single qPCR with EvaGreen intercalating dye
* Plate B use mastermix for triplex qPCR with EvaGreen intercalating dye
* Plate C use mastermix for single qPCR with fluorescently labeled probe
* Plate D use mastermix for triplex qPCR with fluorescently labeled probes

IMPORTANT NOTE: although an excess of reagents and samples is provided, please perform only the experiments specifically required, i.e. one quantitative run. Please use the excess material to repeat a run only in the case of a major technical problem (i.e. erroneous preparation of reaction mix, power failure, general amplification failure etc.). Thank you.

# **Plate 1: Anglerfish single qPCR with EvaGreen® dsDye**

Final volume - Vf in one reaction is 20 µL, 4 µL od sample are in each reaction.

**Plate set-up and loading order**:



**Protocol**

1. Thaw, mix and centrifuge the components needed for the run. **Keep thawed reagents on ice**.

2. Dilute your standards (10x dilution of IDT\_RJ and 4x dilution of St2, see paragraph 3.1.2)

3. In one 2 mL tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mixes for the plate 1.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |
|  |  | stock concentration | concentration in reaction | 1 | 103 |
|  | NFW |  |  | 11.6 | **1195** |
|  | Hot firepol® evagreen® qPCR supermix/ MM-gre | 5x | 1x | 4 | **412** |
| **single** | primer D-F | 20 [µM] | 0.2 | 0.2 | **20.6** |
| primer D-R | 20 [µM] | 0.2 | 0.2 | **20.6** |
|  | Sample DNA [µl] |  |  | 4 | 4 |

Please note that additional volume is included in the total to cover pipetting variability due to the viscosity of the solution.

4. Mix well and centrifuge briefly.

5. Add into each reaction tube the amount of reaction mix needed = 16 µL.

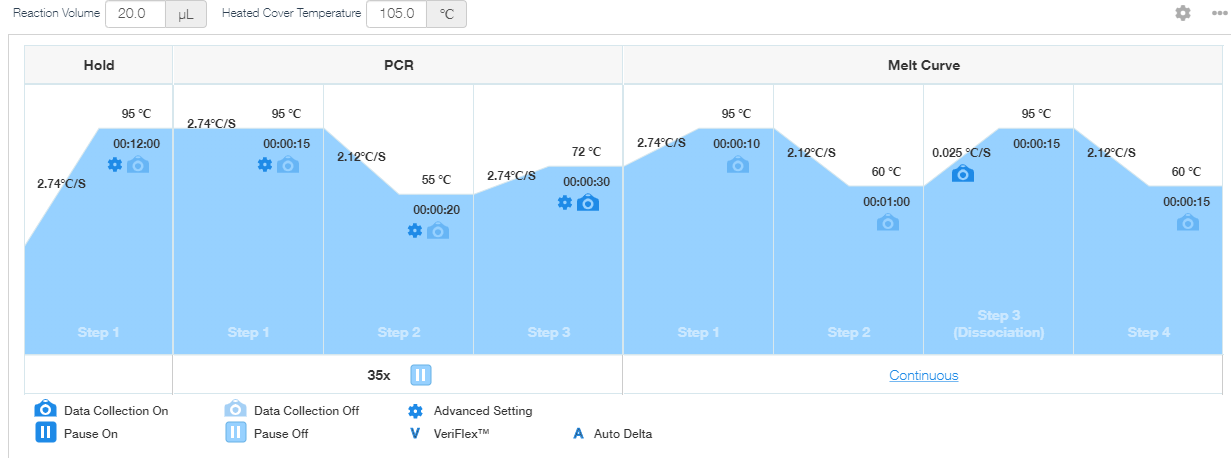
6. Add 4 µL of samples in each reaction.

7. Spin down plate in a micro-centrifuge.

8. Place an optical cover on the reaction plate and briefly centrifuge the plate.

9. Place the reaction plate in the Real-time PCR apparatus and start the run (possibly apply a compression pad, depending on the model).

10. Cycling



11. After finishing the runs, evaluate the run and generate excel with the results. Sent the provider excel and also raw data from qPCR cycler labelled **Plate\_A\_IDT\_labnumber**.

# **Plate 2: Triplex qPCR with EvaGreen® dsDye**

Final volume - Vf in one reaction is 20 µL, 4 µL od sample are in each reaction.

**Plate set-up and loading order**:



**Protocol**

1. Thaw, mix and centrifuge the components needed for the run. **Keep thawed reagents on ice**.

2. Use dilution of standards IDT\_RJ and St2 (see paragraph 3.1.2)

3. In one 2 mL tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mixes for the plate 2.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |
|  |  |  | stock concentration | concentration in reaction | 1 | 103 |
|  |  | NFW |  |  | 10.8 | **1112** |
|  |  | Hot firepol® evagreen® qPCR supermix/ MM-gre | 5x | 1x | 4 | **412** |
|  | **target 1** | primer D-F | 20 [µM] | 0.2 | 0.2 | **20.6** |
|  | primer D-R | 20 [µM] | 0.2 | 0.2 | **20.6** |
|  | **target 2** | primer E-F | 20 [µM] | 0.2 | 0.2 | **20.6** |
|  | primer E-R | 20 [µM] | 0.2 | 0.2 | **20.6** |
|  | **target 3** | primer P-F | 20 [µM] | 0.2 | 0.2 | **20.6** |
|  | primer P-R | 20 [µM] | 0.2 | 0.2 | **20.6** |
|  |  | sample DNA [µl] |  |  | 4 | 4 |

Please note that additional volume is included in the total to cover pipetting variability due to the viscosity of the solution.

4. Mix well and centrifuge briefly.

5. Add into each reaction tube the amount of reaction mix needed = 16 µL.

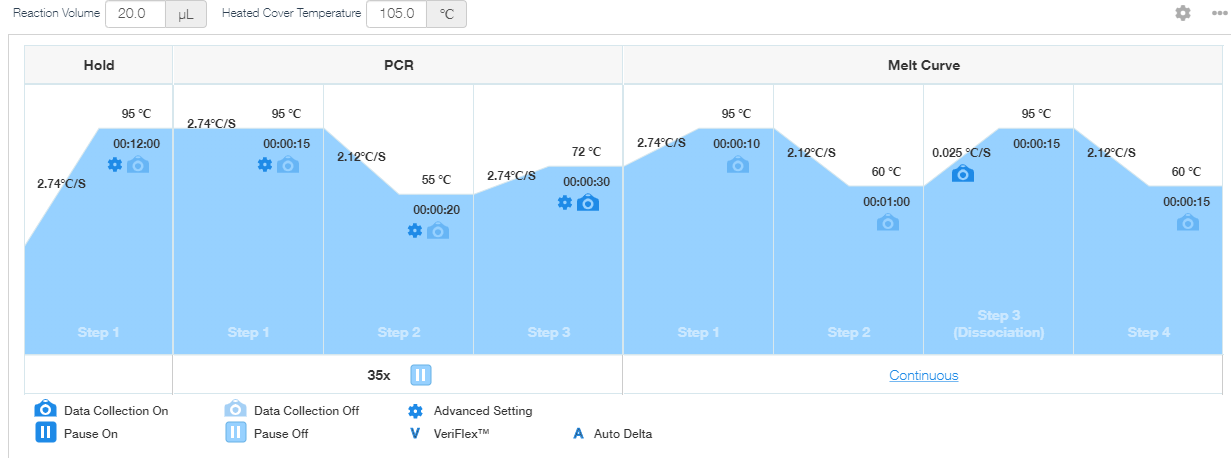
6. Add 4 µL of samples in each reaction.

7. Spin down plate in a micro-centrifuge.

8. Place an optical cover on the reaction plate and briefly centrifuge the plate.

9. Place the reaction plate in the Real-time PCR apparatus and start the run (possibly apply a compression pad, depending on the model).

10. Cycling



11. After finishing the runs, evaluate the run and generate excel with the results. Sent the provider excel and also raw data from qPCR cycler labelled **Plate\_B\_IDT\_labnumber**.

# **Plate 3: Anglerfishsingle qPCR with fluorescently labelled probe**

Final volume - Vf in one reaction is 20 µL, 4 µL od sample are in each reaction, Cy5 or FAM channel used for signal detection.

**Plate set-up and loading order**:



**Protocol**

1. Thaw, mix and centrifuge the components needed for the run. **Keep thawed reagents on ice**.

2. Use dilution of standards IDT\_RJ and St2 (see paragraph 3.1.2)

3. In one 2 mL tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mixes for the plate 3.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | stock concentra-tion | concentra-tion in reaction | 1 | 103 |
|  | NFW |  |  | 4.95 | **509.9** |
|  | GoTaq® Probe qPCR Master Mix | 2x | 1x | 10 | **1030** |
| **target 1** | primer D-F | 20 [µM] | 0.4 | 0.4 | **41.2** |
| primer D-R | 20 [µM] | 0.4 | 0.4 | **41.2** |
| probe D | 20 [µM] | 0.25 | 0.25 | **25.75** |
|  | sample DNA [µl] |  |  | 4 |  |
|  |  |  |  |  |  |

Please note that additional volume is included in the total to cover pipetting variability due to the viscosity of the solution.

4. Mix well and centrifuge briefly.

5. Add into each reaction tube the amount of reaction mix needed = 16 µL.

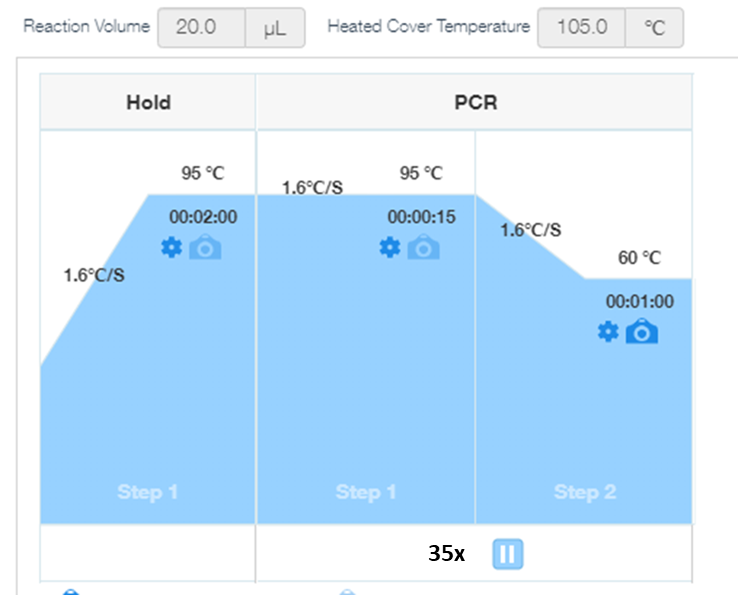
6. Add 4 µL of samples in each reaction.

7. Spin down plate in a micro-centrifuge.

8. Place an optical cover on the reaction plate and briefly centrifuge the plate.

9. Place the reaction plate in the Real-time PCR apparatus and start the run (possibly apply a compression pad, depending on the model).

10. Cycling



11. After finishing the runs, evaluate the run and generate excel with the results. Sent the provider excel and also raw data from qPCR cycler labelled **Plate\_C\_IDT\_labnumber**.

# **Plate 4: Multiplex qPCR with fluorescently labelled probes**

Final volume - Vf in one reaction is 20 µL, 4 µL od sample are in each reaction.

**Plate set-up and loading order**:



**Protocol**

1. Thaw, mix and centrifuge the components needed for the run. **Keep thawed reagents on ice**.

2. Use dilution of standards IDT\_RJ and St2 (see paragraph 3.1.2)

3. In one 2 mL tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mixes for the plate 1.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | stock concentra-tion | concentra-tion in reaction | 1 | 103 |
|  | NFW |  |  | 2.85 | **293.6** |
|  | GoTaq® Probe qPCR Master Mix | 2x | 1x | 10 | **1030** |
| **target 1** | primer D-F | 20 [µM] | 0.4 | 0.4 | **41.2** |
| primer D-R | 20 [µM] | 0.4 | 0.4 | **41.2** |
| probe D | 20 [µM] | 0.25 | 0.25 | **25.75** |
| **target 2** | primer E-F | 20 [µM] | 0.4 | 0.4 | **41.2** |
| primer E-R | 20 [µM] | 0.4 | 0.4 | **41.2** |
| probe E | 20 [µM] | 0.25 | 0.25 | **25.75** |
| **target 3** | primer P-F | 20 [µM] | 0.4 | 0.4 | **41.2** |
| primer P-R | 20 [µM] | 0.4 | 0.4 | **41.2** |
| probe P | 20 [µM] | 0.25 | 0.25 | **25.75** |
|  | sample DNA [µl] |  |  | 4 |  |

Please note that additional volume is included in the total to cover pipetting variability due to the viscosity of the solution.

4. Mix well and centrifuge briefly.

5. Add into each reaction tube the amount of reaction mix needed = 16 µL.

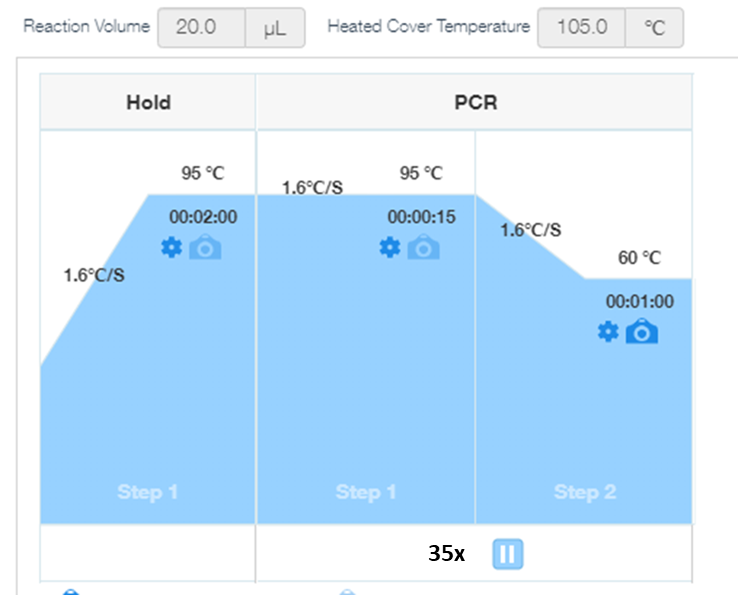
6. Add 4 µL of samples in each reaction.

7. Spin down plate in a micro-centrifuge.

8. Place an optical cover on the reaction plate and briefly centrifuge the plate.

9. Place the reaction plate in the Real-time PCR apparatus and start the run (possibly apply a compression pad, depending on the model).

10. Cycling



11. After finishing the runs, evaluate the run and generate excel with the results. Sent the provider excel and also raw data from qPCR cycler labelled **Plate\_D\_IDT\_labnumber**.

# **Data Processing**

***Important preliminary notes:***

* Use the sample labelling as described in points above.
* Eliminate only obvious outliers from the calculation of the calibration curves and blind samples.

# **9. Documentation of results**

Please send the following data of **all** analytical runs without changes. Thank you for participation.