Startdate: 01 Sep 2023

Author: Fabian Knofl

**GN004773-010**

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ddPCR

# Devices

|  |  |
| --- | --- |
| Cooling device +4°C | KG6568 / KG5908 / KG5032 |
| Cooling device -20°C | KG5961 / KG6032 / CEKG6237 / KG6030 |
| Cooling device -60°C | KG5962 / KG6459 |
| Incubator | VWR / Inv.Nr  118861  118860 |
| Centrifuge | Eppendorf Inv.Nr  119028 / Thermo Fisher Inv.Nr.  087972 |
| Droplet Generator | AutoDG Biorad Inv.Nr. 122920 |
| PX1 PCR Plate Sealer | Biorad / Inv.Nr. 123000 |
| QX200 Droplet Reader | Biorad / Inv.Nr. 115002 |
| Laminar Flow Hood | Heraeus /  LF5181  LF5182  IBS /  LF5206  LF5220  LF5217  LF5224 |
| Pipettes (calibrated) | Thermo Fisher / Eppendorf / Sartorius |
| Thermocycler | Bio-Rad C1000 Touch Inv.Nr. 123001 /  Bio-Rad C1000 Touch Inv.Nr. 121363 |
| Hamilton Vantage | Inv.Nr. 121089 |
| QX ONE | Serial nr.: 766BR2023 |

# Controls

| **Project** | **Control name** | **Lot number** | **Supplier** |
| --- | --- | --- | --- |
| ID tag/ITR | Negative control  (ddH2O) | P/N: AM9932 | Thermo Fisher |
| Plasmid control  (pXL029\_SacI) | Lot# 190820/01/AL  DFM10120 | In house |
| Reference control  (PP073\_1933\_FDP) | Lot# 221230/01/FB  GN004308-009 | In house |

# Primers and Probes

| **Project** | **Primer or Probe Name and Primer or Probe sequence** | **Lot number** | **Supplier** |
| --- | --- | --- | --- |
| ID tag | Primer: 1179\_ID Tag F-1 (HPLC)  (CCC CGT GTG AAC GAT TGG T) | 230404/03/KK  GN004512-006 | Microsynth |
| Primer: 1180\_ID Tag R-1 (HPLC)  (CGT ATT TCC CGT TTA GGC TTT CG) | 230404/04/KK  GN004512-006 | Microsynth |
| Probe: 1147\_Shire ID tag P-1  (FAM-AAC CCG GTG TCC TGT GAG-MGBNFQ) | 221228/05/PE  GN003826-034 | Microsynth |
| ITR | Primer: 1177 fwd ITR (HPLC)  (GGA ACC CCT AGT GAT GGA GTT) | 230404/01/KK  GN004512-006 | Microsynth |
| Primer: 1178 rev ITR (HPLC)  (CGG CCT CAG TGA GCG A) | 230404/02/KK  GN004512-006 | Microsynth |
| Probe: ITR probe (20µM)  (VIC-CAC TCC CTC TCT GCG CGC TCG-MGBNFQ) | 210811/02/RM | Applied Biosystems |

New ITR Primer:

|  |  |  |
| --- | --- | --- |
| Primer: 1228/30\_fwd ITR  (GGA ACC CCT AGT GAT GGA GTT) | 230728/01/FB GN004308-075 | Microsynth |
| Primer: 1229/31\_rev ITR  (CGG CCT CAG TGA GCG A) | 230728/02/FB GN004308-075 | Microsynth |

# Reagents and Consumables

| **Bezeichnung** | | **Lot number** | **Producer** |
| --- | --- | --- | --- |
| Ambion nuclease free H2O | | P/N: AM9932  Lot.: 2208525 | Thermo Fisher |
| DNase I (RNase-free) | | Cat. No. M0303L  Lot.: 10123433 | NEB |
| DNase I Buffer | | Cat. No. B0303S  Lot.: 10121416 | NEB |
| 0,5M EDTA | | Cat. No. E177-100mL  Lot.: 19/1856218 | VWR |
| 10x GeneAmp PCR Buffer | | Cat. No. 4379876  Lot.: 61161047 | Thermo Fisher |
| 10% Pluronic F-68 | | Cat. No. 24040032  Lot.: 2390101 | Thermo Fisher |
| SalmonSpermDNA [10 µg/mL] | | Lot.: GN000195-024 | In house |
| 2x ddPCR Supermix (no dUTP) | | Cat. No. 1863025  Lot.: 64537648 | Bio-Rad |
| QX ONE | QX ONE Droplet Generation Oil | Cat. No. 12006058  Lot.: 64509220 | Bio-Rad |
| QX ONE Droplet Reader Oil | Cat. No. 12006057  Lot.: 64515138 | Bio-Rad |
| QX ONE GCR96 Cartridges | Cat. No. 12006859  Lot.: 2205G | Bio-Rad |
| QX ONE ddPCR System Waste Bottle | Cat. No. 12006060 | Bio-Rad |
| Hamilton | Deepwell Plate 96/1000µl, white Border (Protein low DW Platte) | Cat. No. 0030 504.208  Lot.: | Eppendorf |
| PCR FramePlate 96 Well Skirted PCR Plate (DNase Platte) | Cat. No. 814302  Lot.: | Hamilton |
| PCR ComfortLid | Cat. No. 814300  Lot.: | Hamilton |
| Reagent Container 120ml | Cat. No. 194052  Lot.: | Hamilton |
| ddPCR Plates 96-Well, Semi-Skirted | | Cat. No. 12001925  Lot.: 64528141 | Bio-Rad |
| Pierceable Foil Heat Seal | | Cat. No. 1814040  Lot.: 101820 | Bio-Rad |
| GCR96 Foil Heat Seal | | Cat. No. 12006843  Lot.: 101889 | Bio-Rad |
| ddPCR Droplet Reader Oil | | Cat. No. 1863004  Lot.: | Bio-Rad |
| 1,5 mL Protein LoBind Tubes | | Cat. No. 525-0133 | Eppendorf |

Expiry dates were checked and have not been surpassed:

# Sample Table

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| vial nr. | target | sample name | Predilution  factor | Pluronic dilution  factor | final dilution factor | vial nr. |
| 1 | IDT/ITR | Negative control nuclease free dH20 | n.z. | **1E+02** | *1E+02* | **1** |
| 2 | IDT/ITR | Plasmid control (pXL029\_SacI) | n.z. | **1E+04** | *2.86E+04* | **2** |
| 3 | IDT/ITR | Reference control (PP073\_1933\_FDP) | 5E+02 | **1E+02** | *5E+04* | **3** |
| 4 | IDT/ITR | 1.1A | 5E+02 | **1E+03** | *5E+05* | **4** |
| 5 | IDT/ITR | 2.12 | 5E+02 | **1E+02** | *5E+04* | **5** |
| 6 | IDT/ITR | 2.19 | 5E+02 | **1E+03** | *5E+05* | **6** |
| 7 | IDT/ITR | 5.35A | 5E+02 | **1E+03** | *5E+05* | **7** |
| 8 | IDT/ITR | 5.36A | 5E+02 | **1E+03** | *5E+05* | **8** |
| 9 | IDT/ITR | 5.37A | 5E+02 | **1E+03** | *5E+05* | **9** |
| 10 | IDT/ITR | 5.38A | 5E+02 | **1E+03** | *5E+05* | **10** |
| 11 | IDT/ITR | 5.39A | 5E+02 | **1E+03** | *5E+05* | **11** |
| 12 | IDT/ITR | 5.40 | 5E+02 | **1E+03** | *5E+05* | **12** |
| 13 | IDT/ITR | 5.41 | 5E+02 | **1E+03** | *5E+05* | **13** |
| 14 | IDT/ITR | 5.42 | 5E+02 | **1E+03** | *5E+05* | **14** |
| 15 | IDT/ITR | 5.43 | 5E+02 | **1E+03** | *5E+05* | **15** |
| 16 | IDT/ITR | FF\_028\_A003\_c04\_BDS 6m, +4°C | 5E+02 | **1E+04** | *5E+06* | **16** |
| 17 | IDT/ITR | FF\_028\_A003\_c04\_FDP 6m, -60°C | 5E+02 | **1E+04** | *5E+06* | **17** |
| 18 | IDT/ITR | Plasmid control (pXL029\_SacI) -DNase | n.z. | **1E+04** | *2.86E+04* | **18** |
| 19 | IDT/ITR | Negative control nuclease free dH20 | n.z. | **1E+02** | *1E+02* | **19** |
| 20 | IDT/ITR | Plasmid control (pXL029\_SacI) | n.z. | **1E+04** | *2.86E+04* | **20** |
| 21 | IDT/ITR | Reference control (PP073\_1933\_FDP) | 5E+02 | **1E+02** | *5E+04* | **21** |
| 22 | IDT/ITR | Reference control (PP073\_1933\_FDP) | 5E+02 | **1E+02** | *5E+04* | **22** |

**TA19-22: Bridging of the new ITR primer**

# Experiment Set-Up

## Preparation of the Pluronic buffer

**ddPCR dilution buffer (Pluronic buffer)**

composition: 0.1% Pluronic, 2ng/mL salmon sperm DNA, 1x GeneAmp PCR Buffer

already manufactured in protocol.: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Reagenzien** | **Endvolumen** | **1 mL** | **10 mL** | **50 mL** | **100 mL** | **200 mL** |
| 10x GeneAmp PCR Buffer | | 0,1 mL | 1 mL | 5 mL | 10 mL | 20 mL |
| 10% Pluronic F-68 | | 10 µL | 100 µL | 500 µL | 1000 µL | 2000 µL |
| 10µg/mL Salmon Sperm DNA | | 0,2 µL | 2 µL | 10 µL | 20 µL | 40 µL |
| Nuclease free H2O | | 0,9 mL | 8,9 mL | 44,5 mL | 89 mL | 178 mL |

Preparation of samples (point 6.2 to 6.5):  manually  Hamilton Vantage

Production of the DNase mix (in 6.3):  manually  Hamilton Vantage

## Sample Predilution

Samples first undergo a serial dilution (final dilution factor 1:500)

**Dilution 1**: 90µl Pluronic buffer + 10µl sample

**Dilution 2**: 490µl Pluronic buffer +10µl from dilution 1.

-> Specific predilution for each sample (see column “predilution” in sample table)

## DNaseI Digestion

Overview of the DNase digestion

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sample type** | **DNase I Mix   µL** | **Nuclease free water** **µL** | **Prediluted sample µL** | **Plasmid control preparation µL** | **Total volume µL** |
| Negative control | 10 | 10 | / | / | 20 |
| Plasmid control | / | 16.5 | / | 3.5 |
| Reference control | 10 | / | 10 | / |
| Samples | 10 | / | 10 | / |

\*DNase Mix (40x) Preparation:

80µl NEB-DNaseI (4U) + 80µl 10xDNaseI buffer + 240µl Ambion nuclease free H2O.

2x



* Vortex gently and centrifuge the tubes in a microfuge briefly
* Incubate for NLT 60 min and NMT 90 min at 37°C
* After incubation, briefly spin tubes and add 6 µL 0.5 M EDTA to stop DNase I enzyme activity.
* Vortex and spin tubes briefly

If sample storage is necessary, at this stage controls and samples can be stored at NMT -20°C. Thaw at room temperature.

## final sample dilution

* Add 74 µL of the ddPCR Pluronic buffer to the sample preparation, 6.3. Resulting in a total volume of 100 µL, corresponding to a 1:10 dilution. Total dilution at this step is **1:5000 (5.00E+03)**.
* Prepare sample dilution according the next Table for each sample.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Total sample dilution** | **Required additional dilution** | **Reagent** | **Dilution**  **step 1**  **µL** | **Dilution**  **step 2**  **µL** | **Dilution**  **step 3 µL** |
| **5.00E+04** | **1:10** | Pluronic Buffer  Sample | 90  10 | N/A | N/A |
| **5.00E+05** | **1:100** | Pluronic Buffer  Sample | 990  10 | N/A | N/A |
| **5.00E+06** | **1:1 000** | Pluronic Buffer  Sample | 990  10 | 90  10 | N/A |

**Expected vector genome titer and optimal total dilution**

|  |  |
| --- | --- |
| **Expected sample titer vg/mL** | **Optimal total sample dilution** |
| NMT 1.00E+11 | 5.00E+04 |
| 1.00E+11 | 5.00E+05 |
| 1.00E+12 | 5.00E+06 |

## ddPCR Mastermix Preparation & PCR Set-Up

**Mastermix for DUPLEX ddPCR: IDT/ITR**



**Calculation verified by/on:\_\_\_\_\_\_\_\_\_**

## Hamilton vantage: automated sample preparation

The Hamilton vantage is a pipetting robot. Hamilton performs the sample preparation comparable to the manual sample preparation procedure described in chapters 6.2 to 6.5.  
After sample preparation (enzymatic digest and subsequent sample dilutions), droplet generation and subsequent reading can be performed either in the QX200 system or the QX One system. Each system requires different plasticware and a different handling procedure which will be described in the chapters below.   
If you choose QX One, the Hamilton Vantage pipettes the PCR Mix automatically in the corresponding GCR96 Cartridges, whereas when choosing the QX200 system Hamilton will stop after mixing the PCR reagents and the sample in the 96 well PCR plate.   
Refer to the respective experiment file for further information.

## Droplet generation and PCR

At this step the diluted samples and controls were put together with the prepared PCR master mix. The Auto DG QX200 or the QX One system is used to partition the ddPCR reaction mix into nanoliter-sized droplets. QX One system combines the function of droplet generator, thermal cycler and droplet reader system in one system. Using the QX200 system the droplet generation, the thermal cycling and the droplet reading are 3 steps that run in 3 respective instruments.

### *Automated droplet generation, thermal cycling and droplet reading using QX ONE*

The QX One system combines the function of droplet generator, thermal cycler and droplet reader system in one machine. Please noted that the respective plasticware differs from the consumables used in the Auto DG system.

* Add 19.8 µL of the PCR Master Mix (6.5) in each well of a ddPCR 96-well plate.
* Add 2.2 µL sample or control preparation (6.4). Each sample will be tested in duplicates.
* Seal the plate with a pierceable foil in the plate sealer for 5 sec at 180°C.
* Briefly vortex and centrifuge (e.g., 1150 rcf for 30 sec.) the plate.
* By piercing the foil of the PCR plate, add 20 µL of this PCR preparation in the appropriate wells of the QX ONE Cartridge (20 µL of column 1 of the 96-Well plate in column 1 in the QX ONE Cartridge).
* QX ONE Cartridge is heat sealed with a GCR foil: seal for 0.5 sec at 180°C in the heat sealer, turn the plate and seal again for 0.5 sec at 180°C. Centrifuge at exactly 1150 rcf for 30 sec..
* Put the Cartridge in the QX One system and start the plate template and thermocycler program.
* A full cycle from droplet generation to the finished analysis takes approximately 3 hours.

**There are further information in the Manual file**

name of the saved plate template: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

### *Semiautomated QX200 system (Droplet generation using Auto DG)*

The nanoliter-sized droplets were generated fully automatically with the Auto DG (QX200) machine. After this step, the PCR plate is placed in the thermal cycler and after cycling, the droplets were analyzed in the droplet reader.

* Add 19.8 µL of the PCR Master Mix (6.5) in each well of a ddPCR 96-well plate.
* Add 2.2 µL sample or control preparation (6.4). Each sample will be tested in duplicates.
* Seal the plate with a pierceable foil in the plate sealer for 5 sec at 180°C.
* Briefly vortex and centrifuge (e.g., 1150 rcf for 30 sec.) the plate.
* Put the ddPCR 96-well plate into the automated Droplet Generator and proceed according to the Instruction Manual.
* Droplets are stable for approximately 1 hour.

**There are further information in the Manual file**

#### PCR cycling Auto DG, QX200 only

* Run the PCR reaction (from 6.7.2) in an endpoint thermal cycler with cycling conditions shown in the Table below.
* A reduced ramp rate is a critical parameter and must be set to 2°C per second.
* Set the lid temperature of the thermal cycler at 105°C.

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Cycles** | **Temperature (°C)** | **Time** |
| 1 | 1 | 95 | 10 min. |
| 2 | 5 | 95 | 30 sec. |
| 65 | 60 sec. |
| 72 | 15 sec. |
| 3 | 42 | 95 | 30 sec. |
| 60 | 60 sec. |
| 72 | 15 sec. |
| 4 | 1 | 98 | 10 min. |
| 5 | 1 | 12 | ∞ |

After accomplished PCR reaction, the plate is placed into the droplet reader for sample analysis.

## Plate layout (full plate)

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| **A** | TA1 | TA1 | TA9 | TA9 | TA17 | TA17 | TA25 | TA25 | TA33 | TA33 | TA41 | TA41 |
| **B** | TA2 | TA2 | TA10 | TA10 | TA18 | TA18 | TA26 | TA26 | TA34 | TA34 | TA42 | TA42 |
| **C** | TA3 | TA3 | TA11 | TA11 | TA19 | TA19 | TA27 | TA27 | TA35 | TA35 | TA43 | TA43 |
| **D** | TA4 | TA4 | TA12 | TA12 | TA20 | TA20 | TA28 | TA28 | TA36 | TA36 | TA44 | TA44 |
| **E** | TA5 | TA5 | TA13 | TA13 | TA21 | TA21 | TA29 | TA29 | TA37 | TA37 | TA45 | TA45 |
| **F** | TA6 | TA6 | TA14 | TA14 | TA22 | TA22 | TA30 | TA30 | TA38 | TA38 | TA46 | TA46 |
| **G** | TA7 | TA7 | TA15 | TA15 | TA23 | TA23 | TA31 | TA31 | TA39 | TA39 | TA47 | TA47 |
| **H** | TA8 | TA8 | TA16 | TA16 | TA24 | TA24 | TA32 | TA32 | TA40 | TA40 | TA48 | TA48 |

**Important information**

|  |  |
| --- | --- |
| **IDT / ITR Duplex** | Predilution in Pluronic buffer 1:500   * all samples * reference control |
| Dilution factor   * negative control (final dilution factor 1E+02) * plasmid control (final dilution factor 2.86E+04) * reference control (final dilution factor 5E+04) |