# SOP for Fluidigm® Real-Time PCR TaqMan Assay

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## Chemical requirements

- STA: 2X TaqMan Preamp Master Mix (Applied Biosystems, PN 4361128)
- Exo I treatment: ExoSAP-IT (MJS Biolynx 78202- 4x 1ml, or PN# 78201 -1ml)
- Sample Pre-Mix for TaqMan: 2X TaqMan Gene Expression Master Mix (Applied Biosystems, PN 4369016)
- Sample Pre-Mix for TaqMan: 20X GE Sample Loading Reagent (Fluidigm PN 85000735)—store at 4ºC.
- Assay Mix for TaqMan: 2X Assay Loading Reagent (Fluidigm PN 85000736)—store at 4ºC.
- DNA Suspension Buffer, TE buffer: 10 mM Tris-HCl, 0.1 mM EDTA, ph 8.0 (Technova PN T0221)—store at room temperature.
- Deionized DNA-free, DNase-free, RNase-free water—store at room temperature.
- Primer/probes sets
- Samples of interest

**Fluidigm supports the following detection reagents with the BioMark System.**

Probe Types:

FAM-MGB

VIC-MGB

FAM-TAMRA

FAM-non fluorescent quencher

**Making cDNA :**

We use High Capacity RNA-to-cDNA Master Mix (PN4390799) from ABI, using 1 ug of starting RNA. These are incubated at 25$^{o}$ for 5 minutes, 42$^{o}$ for 60 minutes, then 85$^{o}$ for 5 minutes.

## Primers & Probes dilution

1. Resuspend or purchase primers or probes at 100 uM in 1X DNA suspension buffer.
2. Dilute and mix primer pairs (F & R) to 50 uM each.

   Mix an equal amount of F & R primers (100uM), eg. 20uL F + 20uL R
3. Dilute probes to 10 uM each.

Take 3 uL of 100 uM probe, add 27 uL 1X DNA Suspension Buffer.

## I. Fluidigm® Gene Expression Specific Target Amplification (STA)

Overview: The BioMark™ System uses a sample loading volume of 5 µL, and distributes this sample mixture across 48 or 96 reaction chambers in 9 or 6 nL aliquots, respectively. With these micro-volumes, detecting the specific targets requires a minimum of 500-1,000 copies in the original 5 µL loading volume. Because some genes exhibit low expression resulting in more dilute target concentrations, Fluidigm recommends using Specific Target Amplification (a pre-amplification) to increase target concentration.

### STA Primer Dilution

1.) Resuspend or purchase primers at 100 µM in 1X DNA Suspension Buffer.
2.) Create the Assay Mix Primer Pairs. Combine each forward and reverse primer pair to a final concentration of 50 uM each
3.) Make a 200 nM STA Primer Mix by combining equal volumes of each 50 µM primer pair and dilute using 1X DNA Suspension Buffer. Each primer is at a final concentration of 200 nM. This mix represents a 4X concentration of STA Primers.

Example preparation of 200nM pooled STA primer Mix

| 48 primer pairs (example) | Volume (uL) |
|---|---|
| 2uL each primer pair (50uM each) | 2 uL (x48 = 96 uL) |
| 1X DNA Suspension Buffer | 404 |
| Total | 500 |

### STA Thermal Cycling

1.) Combine the following:
STA Reaction solution

| Component | Volume for One Reaction (uL) | Volume for 60 Reactions |
|---|---|---|
| TaqMan PreAmp Master Mix (Applied Biosystems PN 4391128) | 2.5 | 150 |
| 200 nM pooled STA primer Mix | 1.25 | 75 |
| cDNA | 1.25 | |
| Total | 5 | 225 |

* Note: The final concentration of each primer pair in the STA reaction is 50 nM

2.) In a 96-well plate, combine 3.75 µL STA Pre-Mix with 1.25 µL each cDNA sample for a total 5 µL STA Reaction volume.
3.) Amplify for 14 cycles using the following thermal protocol as guide. (the same as TaqMan).

| Condition | Activate | 15 Cycles | | Hold |
|---|---|---|---|---|
| Temperature | 95°C | 95°C | 60°C | 4°C |
| Time | 10 min | 15 sec | 4 min | for ever |

## II. Exonuclease I (Exo I) Treatment Method

Cleanup step to remove unincorporated primers. Our lab uses ExoSAP-IT.

1.) Mix 5uL of post-PCR reaction product with 2ul of ExoSAP-IT for a combined 7ul reaction volume.

| Condition | Digest | Inactive | Hold |
|---|---|---|---|
| Temperature | 37°C | 80°C | 4°C |
| Time | 15 min | 15 min | for ever |

Dilute the final STA products 5-fold and store at -20oC or use immediately for on-chip PCR.

## Preparing the Sample Pre-Mix and Samples

Combine components in table below to make Sample Pre-Mix and final Sample Mixture (scale up appropriately for multiple runs).

| Component | Volume per inlet (uL) | Volume per inlet with Overage (uL) | Sample Pre-Mix for 48.48 (uL) | Sample Pre-Mix for 96.96 (uL) |
|---|---|---|---|---|
| 2X TaqMan Gene Expression Master Mix (ABI PN 4369016) | 2.5 | 3 | 180 | 360 |
| 20X GE Sample Loading Reagent (Fluidigm, PN 85000746) | 0.25 | 0.3 | 18 | 36 |
| STA and Exo I- treated sample | 2.25 | 2.7 | | |
| Total Volume | 5 | 6 | | |

*IMPORTANT: Do not use TaqMan Universal Master Mix or mixes containing 7-deaza-2'-deoxynucleotides, which may inhibit DNA binding dye interaction. Use TaqMan Gene Expression Master mix.

## Preparing the Assay Mix

### 1. TaqMan Assay:

Since we order primers and probes in separate tubes, prepare aliquots of 10X assays using volumes in table below:

We need to use different probe/primer concentrations for MGB (ABI) and Zen (IDT) probes:

| Component | Volume per inlet (uL) | Volume per inlet with Overage (uL) | Volume for 60 uL Stock (10 arrays) |
|---|---|---|---|
| 50 uM Primer pairs | 1 | 1.2 | 27 |
| 10 uM Probes | 1.5 | 1.8 | 3 |
| 2X Assay Loading Reagent (Fluidigm, PN 85000736) | 2.5 | 3 | 30 |
| Total Volume | 5 | 6 | 60 |

Final Concentration (at10X) Primers: 10 uM; Probe: 3 uM

## Priming the Chip and Loading Assay and Samples

1. Inject control line fluid into each accumulator on the chip (see Figure 1 for the 48.48 Dynamic Array IFC or Figure 2 for the 96.96 Dynamic Array IFC).
2. Remove and discard the blue protective film from the bottom of the chip.
3. Place the chip into the IFC Controller MX (for the 48.48 Dynamic Array IFC) or the IFC Controller HX (for the 96.96 Dynamic Array IFC), then run the **Prime (113x)** script (for the 48.48 Dynamic Array IFC, it takes about 11min.) or the **Prime (136x)** script (for the 96.96 Dynamic Array IFC, it takes about 20min.).
4. When the **Prime** script has finished, press **Eject** to remove the primed chip from the IFC Controller.

5. Pipette 5 µL of each assay and 5 µL of each sample into their respective inlets on the chip.
6. Return the chip to the IFC Controller.
7. Using the IFC Controller software, run the **Load Mix (113x)** script (for the 48.48 Dynamic Array IFC, it takes about 1hr.) or **Load Mix (136x)** script (for the 96.96 Dynamic Array IFC, it takes about 1.5hrs) to load the samples and assays into the chip.

* 20 minutes before the **Load Mix** script has finished, turn on the lamp.

8. When the **Load Mix** script has finished, remove the loaded chip from the IFC Controller.
9. Remove any dust particles or debris from the chip surface using scotch tape.

**Figure 1. 48.48 Dynamic Array IFC sample and assay inlets**

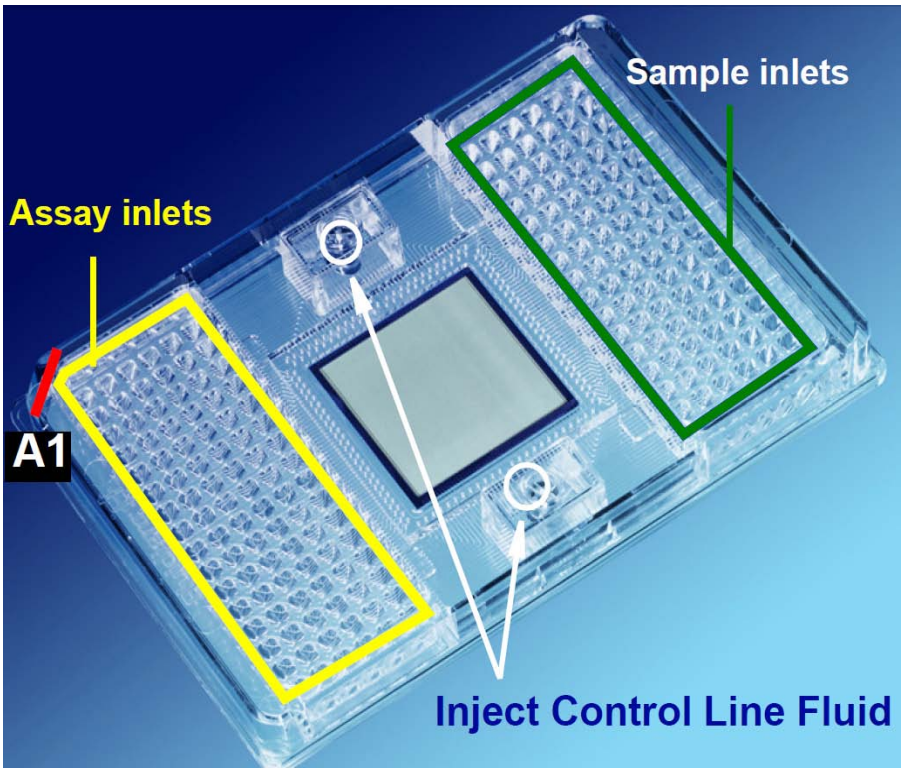


**Figure 2. 96.96 Dynamic Array IFC sample and assay inlets**

## Specific Details of ISAV TaqMan testing:

**We used a minimum of 2 replicate assays per individual and only scored as positive individuals showing a positive CT in both replicate assays.**

**We used a minimum of 2 Negative controls per Fluidigm run.**

**We did not have any positive controls for ISAV to run with these assays (these would not be provided to us). While this presents a difficulty in absolute quantification (we went solely by CT values) and carries the additional challenge in not knowing whether completely negative results were due to the absence of the virus or technical issues in running the assay, it does eliminate the possibility for contaminating the assays. Hence, because we have never conducted molecular analyses on ISAV and do not have samples in our lab from regions of the world known to have ISAV, if we are to obtain sequences with high identity to ISAV, they will reflect only what is observed in our tested samples.**

**We used the 96x96 array format, for which ISAV TaqMan assays were only a portion of the genetic markers being surveyed (we were also testing for other pathogens and host genes).**

**We ran all ISAV TaqMan assays simultaneously in different wells.**

**We included a series of "pool" samples, which contain the STA cDNA from all samples used in the study (separate pools were used for gill and liver). This is an important run to run control for host genes, but only picks up pathogens when they are highly prevalent.**

**We ran 160 sockeye salmon liver tissues and 414 sockeye salmon smolt gill tissues from individuals collected in the freshwater and marine environments from 2007-2010.**

**We repeated a small number of samples on the ABI 7900 to ensure that positives were detectable from both instruments. Most of these assays were performed using the STA (pre-amplified cDNA) samples, but we compared some against samples that were not pre-amplified. The CT values are always lower for STA samples (generally 5-10 cycles lower).**

**In order to obtain sequences, we used conventional PCR on STA samples that had tested TaqMan positive. These PCRs did not contain probe and were again run at an annealing temperature of $60^{o}$.**

> **We checked the size of the products obtained using the ABI Bioanalyzer. If they were of the correct size, they were TA cloned and sequenced on an ABI 3730. We sequenced multiple individuals per primer set.**