Guidelines for setting up Ultrasensitive qPCR assays for Microchimerism detection and quantification (for use on Thermofisher Scientific© QuantStudio 5 Real-Time PCR System, but adaptable to any qPCR system in principle)

Revised in July 2023.

S.B. Kanaan, PhD (Chimerocyte, Inc., Seattle, WA, USA & Fred Hutchinson Cancer Center, Seattle, WA, USA).

A- Primers and Probe preparation:

When ordering synthetic oligonucleotides, Forward and Reverse Primers are typically reconstituted or (if in lyophilized form) need to be reconstituted in a **100 u*M* (333.3x)** Low-TE (Quality Biological # 351-324-721) solutions. Probe is reconstituted in a **100 u*M* (1000x)** Low-TE solution as well.

Aliquot Primers/Probe in a **50x** solution using *extreme caution* and avoiding potential contamination at all costs.

1. Primer aliquot will be at **15 u*M*** (e.g. adding 30 uL from initial solution in 170 uL of Tris-Cl [Qiagen # 19086] per aliquot).
2. Probe aliquot will be at **5 u*M*** (e.g. adding 10 uL from initial solution in 190 uL of Tris-Cl per aliquot, in a dark tube to protect from light).
3. Keep aliquots frozen between −20 and −80°C until use. Thaw, use the same day, and refreeze.

   Note: to prepare aliquots, using Tris-Cl (10 m*M*, pH~8.5) solution is okay and provides good stability for the oligos. Limit using TE or other EDTA-containing buffers as EDTA can potentially interfere with downstream qPCR reactions.

   Note: avoid multiple cycles of thawing/freezing by keeping aliquots small. Frozen, oligos can be stable for relatively long periods.

B- Composition of a single reaction well in qPCR:

The following recommendations are valid for a **50 uL reaction** in a single well from a 96-well standard qPCR plate. For other reaction volumes, please scale accordingly. The composition of a single reaction well is:

- **25 uL** of **2x** Universal Master Mix (*All the assays have been validated with TaqMan™ Universal PCR Master Mix, ThermoFisher Scientific, but could also work on other mixes*)
- **1 uL** of **50x** Forward Primer
- **1 uL** of **50x** Reverse Primer
- **1 uL** of **50x** Taq-Man Probe
- **22 uL** of appropriate solution (e.g. DNA solution to be targeted)

  Note: thus, the working concentration (the "**1x**") is **0.3 u*M*** for each primer and **0.1 u*M*** for probe.

C- Inter and intra Microchimerism-specific qPCR assay homogeneity:

Microchimerism is often ultra-sensitively quantified by comparing amounts calculated in the targeted Microchimerism-specific reactions (Target-specific standard curve) versus amounts calculated in housekeeping-gene-specific reactions (Reference-specific standard curve) that are run in parallel. One of the mainstream housekeeping gene qPCR assays used in our lab targets the human β-globin locus on chromosome 11, and the primers and probe sequences are described in *Lo YMD et al., 1998, Am J Hum Genet*.

Note: in *Lo YMD et al.,* the β-globin probe is dually labeled with 6FAM and TAMRA, whereas we have successfully used in our work a variant of that probe with fluorescent dyes VIC and TAMRA.

The same housekeeping-gene reference-specific calibration standard curve should be used in all experiments to ensure inter- and intra-assay homogeneity. It is recommended that the DNA used in the standard curve derive from a commercially available human cell-line because it can be contamination-free and the same cell-line has the potential to be available forever.

> *Note*: we have worked successfully and reliably with standard female human genomic DNA from Promega (catalog# G152A) to produce accurate β-globin calibration curves.

A Microchimerism Target-specific calibration standard curve is run in parallel and compared to the housekeeping-gene calibration curve to make sure that DNA quantities are the same in both assays.

> *Note*: There is a cell bank that was established as part of an International Histocompatibility Working Group (IHWG) and includes DNA from cell-lines that are HLA-genotyped, extremely useful to generate Microchimerism-specific calibration curves when the assay is HLA-based. For more info, please visit: https://www.fredhutch.org/en/research/institutes-networks-ircs/international-histocompatibility-working-group.html

D- Designing a typical experiment:

It is *critical* that each experiment contains "***Non-Template Controls***" (NTC) for false positive assessment and "***Low-Concentration Positive Controls***" (LCPC) for false negative assessment (example in **Figure 1**).

It is convenient to convert genomic DNA concentrations to their equivalent in number of human cells. We estimate that the genome equivalent (gEq) of **1** human cell is **6.6 pg** of gDNA (*Saiki RK et al., 1988, Science*).

Tips for preparing the housekeeping-gene Reference-specific Calibration curve:

1. The titer of the human cell-line DNA should be well determined.
2. The Reference-specific curve should contain at least 5 dilution points.
3. The quantitative interval of the housekeeping-gene curve should be large. Because our Microchimerism-specific assays can amplify 1 gEq of Microchimerism is a background of up to 120,000 gEq of total DNA, the highest dilution point of the curve should be in that range. The lowest dilution point can go down to 10 gEq.
   > *Note*: it is not necessary to go below 10 gEq in the calibration curve. Indeed, the accuracy of pipetting the exact intended gEq becomes low under 10 gEq and the probabilistic randomness becomes more important.
4. Include at least 1 NTC reaction-well to assess potential false positives.

Tips for preparing the Microchimerism Target-specific Calibration curve:

1. The titer of the human cell-line DNA should be well determined.
2. The Target-specific curve should contain at least 5 dilution points.
3. Similar to the reference-specific curve, the quantitative interval of the Target-specific curve should be large.
4. Include more than 1 (typically 3) NTC reaction-well to assess potential false positives.
5. Include at least 4 LCPC reaction-wells to assess potential false negatives.
   > *Note*: an example of preparation of such positive controls can be found in Fig. S2 of *Kanaan SB et al., 2017, Oncoimmunol*. Among the four LCPC wells, two wells were set to contain 2 gEq each and two wells set to contain 0.5 gEq (i.e. 50% chance of containing only 1 gEq).
6. To mimic Microchimerism conditions, the calibration curve, the LCPC, and the NTC wells of the Microchimerism-specific assay are all diluted in a constant background of DNA that does not contain the Targeted chimeric polymorphism, of which the quantity typically ranges between 10,000 – 60,000 gEq.

Tips for preparing the test samples:

1. The test samples are run in both assays on same plate.
2. Typically, test samples are run in **duplicate** for the **reference** assay and in **six-replicates** for the **Target** assay (thus, eight wells in total).
3. For a total of 50 uL per well, it is advised not to load > 120,000 gEq, as assay sensitivity above these levels is not guaranteed (although we observed it can still work in most tested cases).

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	NTC	NTC	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1
B	All-purpose cell-line Dilution7	Assay-specific cell-line LCPC-2	Assay-specific cell-line LCPC-2	Assay-specific cell-line LCPC-2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2
C	All-purpose cell-line Dilution6	Assay-specific cell-line LCPC-1	Assay-specific cell-line LCPC-1	Assay-specific cell-line LCPC-1	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3
D	All-purpose cell-line Dilution5	Assay-specific cell-line Dilution5	Assay-specific cell-line Dilution5	Assay-specific cell-line Dilution5	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4
E	All-purpose cell-line Dilution4	Assay-specific cell-line Dilution4	Assay-specific cell-line Dilution4	Assay-specific cell-line Dilution4	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5
F	All-purpose cell-line Dilution3	Assay-specific cell-line Dilution3	Assay-specific cell-line Dilution3	Assay-specific cell-line Dilution3	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6
G	All-purpose cell-line Dilution2	Assay-specific cell-line Dilution2	Assay-specific cell-line Dilution2	Assay-specific cell-line Dilution2	Sample 7	Sample 7	Sample 7	Sample 7	Sample 7	Sample 7	Sample 7	Sample 7
H	All-purpose cell-line Dilution1	Assay-specific cell-line Dilution1	Assay-specific cell-line Dilution1	Assay-specific cell-line Dilution1	Sample 8	Sample 8	Sample 8	Sample 8	Sample 8	Sample 8	Sample 8	Sample 8

Figure 1: Example of a 96-well plate design. In **purple**, the wells corresponding to the Reference-specific assay. In **red**, the wells corresponding to the Target-specific assay. Column "1" contains the reference gene calibration DNA (the Reference standard curve). Columns "2", "3", and "4" contain the calibration DNA of the cell-line with the amplifiable target of the Microchimerism assay (the Target-specific standard curve) including the "low-concentration positive controls (LCPC). Columns "5" through "12" contain test samples DNA. NTC is non-template control.

E- Optimal conditions of a qPCR reaction:

They are as shown in **Figure 2**.

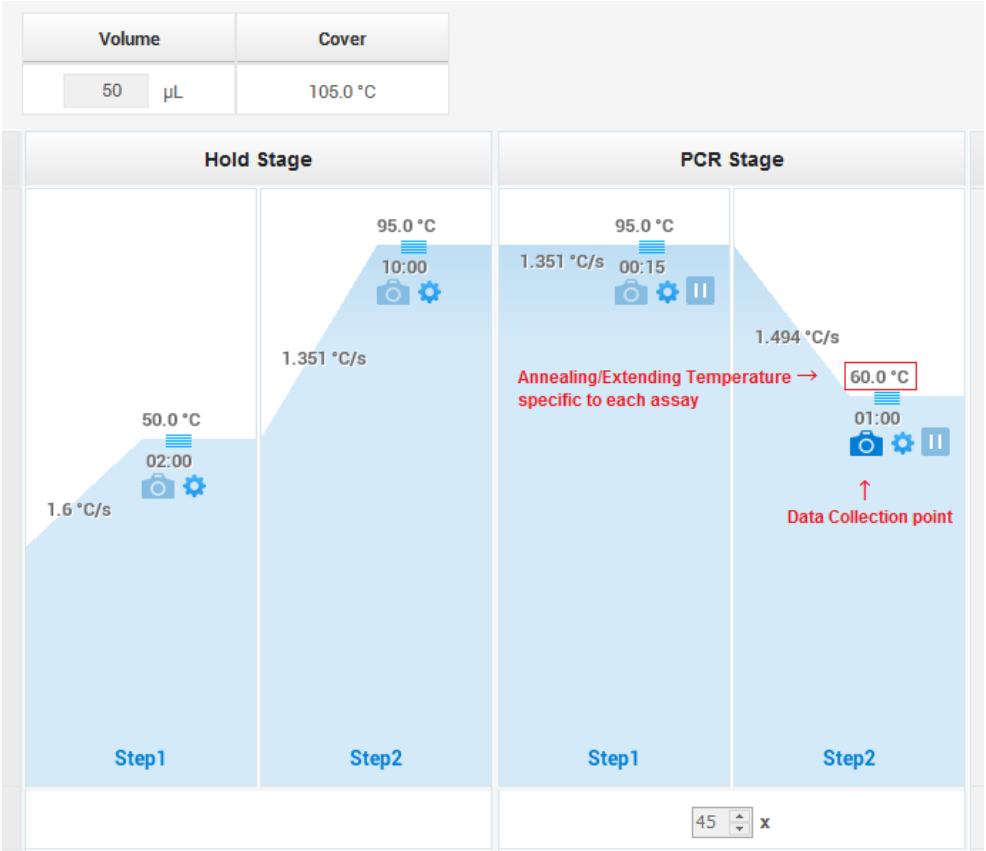

Figure 2: conditions of a Microchimerism-specific real-time quantitative PCR reaction.

F- Experimental procedure representation:

MC-SPECIFIC QPCR METHOD