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Reliable and robust droplet digital PCR (ddPCR) and RT-ddPCR protocols for mouse studies

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Abstract

Droplet digital PCR (ddPCR) is a recent method developed for the quantification of nucleic acids sequences. It is an evolution of PCR methodology incorporating two principal differences: a PCR reaction is performed in thousands of water-oil emulsion droplets and fluorescence is measured at the end of PCR amplification. It leads to the precise and reproducible quantification of DNA and RNA sequences. Here, we present quantitative methods for DNA and RNA analysis using Bio-Rad QX100 or QX200 systems, respectively. The aim of these methods is to provide useful molecular tools for validating genetically altered animal models such as those subject to CRISPR/Cas9 genome editing, as well for expression or CNV studies. A standard procedure for simultaneous DNA and RNA extraction adapted for mouse organs is also described. These methods were initially designed for mouse studies but also work for samples from other species like rat or human. In our lab, thousands of samples and hundreds of target genes from genetically altered lines were examined using these methods. This large dataset was analyzed to evaluate technical optimizations and limitations. Finally, we propose additional recommendations to be included in dMIQE (Minimum information for publication of quantitative digital PCR experiments) guidelines when using ddPCR instruments.

Keywords

Genetically modified animals, droplet digital PCR (ddPCR), RT-ddPCR, Research reproducibility, Quality control, dMIQE

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Abbreviations: cDNA: complementary DNA, CNV: copy number variation, ddPCR: droplet digital polymerase chain reaction, DNA: deoxyribonucleic acid, GMO: genetically modified organism, dMIQE: Minimum information for publication of quantitative digital PCR experiments; MIQE: Minimum information for publication of quantitative Real-Time PCR experiments; RNA: ribonucleic acid, RIN: RNA integrity number, RQS: RNA quality score, RT: reverse transcription

Highlights

- Droplet digital PCR for DNA and mRNA target quantification are described.
- We present molecular tools for validating animal models genetically altered by CRISPR/Cas9 genome editing, for example, and for expression and CNV studies.
- The protocol is validated for mouse studies using a large dataset and its technical optimizations and limitations are evaluated.
- We present a new procedure for simultaneous DNA and RNA extraction adapted for mouse organs.
- Additional recommendations are given for dMIQE (Minimum information for publication of quantitative digital PCR experiments) guidelines when specifically using ddPCR QX100 or QX200 instruments.

1. Introduction

Quantitative PCR (qPCR) and reverse transcriptase-qPCR (RT-qPCR) are basic methods in molecular biology for quantifying DNA and RNA sequence. Droplet digital polymerase chain reaction (ddPCR) is a recent evolution of PCR methods used to quantify nucleic acids proposing two principal modifications in comparison with qPCR: the ddPCR reaction is performed in thousands of water-oil emulsion droplets and the fluorescence is measured at the end of the PCR amplification. Compared to qPCR, linear and highly efficient PCR amplification is less critical, generally resulting in more accurate and reproducible results. [1]. It also allows quantification without the requirement of standard curves, making this technology much easier to use.

ddPCR and RT-ddPCR are suitable replacements for qPCR and RT-qPCR in a broad range of applications such as in human medicine or for regulatory purposes (e.g. pathogen or GMO quantification in food). ddPCR and RT-ddPCR are also being increasingly applied to research such as the detection of rare sequence variants [2], precise CNV quantification [3], cell karyotyping [4] and gene expression analyses [5]. They are undoubtedly valuable tools for characterizing genetically modified lines.

The use of quantitative methods for DNA/RNA analysis is more challenging than what some researchers might imagine. Without the following of strict practices [6], these methods do not lead to accurate quantification and therefore directly impact the reproducibility of published data [7,8]. To improve data comparability and reproducibility, it is crucial to provide a thorough description of the experimental results for qPCR or ddPCR, as highlighted by the MIQE and digital MIQE guidelines [9,10]. A careful assessment of

these quantification protocols using a large number of samples and assays is also required to evaluate technical optimizations and limitations.

In this paper, we describe three protocols that have been validated on large datasets. The first provides a method to quantify DNA copy numbers by ddPCR and the second a method for RNA expression quantification by RT-ddPCR. The final protocol is an extraction optimization that allows the simultaneous purification of high-quality DNA and RNA. These protocols are designed to be easy-to-use, reliable and robust and we have used them successfully on a large panel of organs extracted from genetically engineered mouse models (and to a much lesser extent rat and human samples). This large dataset is analyzed in this paper to provide recommendations. Though initially designed for mouse studies these protocols can be employed for DNA and RNA sequence quantification in other species.

2. Materials and methods

2.1. Preparation of DNA quantification by ddPCR

2.1.1. Primer and probe design

Primers and probes are designed using the PrimerQuest online web interface from IDT (<https://www.idtdna.com/Primerquest/Home/Index>), using the default criteria of qPCR parameters (qPCR 2 Primers + Probe button). Special attention was paid to avoid amplicons containing repeat sequences as these sequences may impact the PCR amplification step. Assays are designed to amplify 60 to 300 bps within the target region (Sup table 1). For target genes with paralogue(s) or pseudo gene homologues, we select one primer in an exonic sequence and the other one in the neighbor intron to avoid unspecific design.

ZENTM double-quenched probes are used as 5' nuclease probes. They include FAM or HEX fluorescent dye and Iowa black hole double quencher dyes. Compared with TaqMan, minor groove binder TaqMan or Universal Probe Library (UPL) probes, ZENTM probes increase discrimination between positive and negative droplets. They also reduce the negative droplet background fluorescence level (data not shown and [11,12]). FAM fluorescent probes show better discrimination between positive and negative droplets than HEX fluorescent probes (data not shown). It is therefore recommended to use a FAM ZENTM probe (named 6-FAM/ZEN/IBFQ in PrimerQuest) for the target gene and a HEX ZENTM probe (named HEX/ZEN/IBFQ in PrimerQuest) for normalization assays. Primers and probes are ordered as a mix with

a primer to probe ratio of 3. The mix is suspended in water for a final concentration of 7.5 nM primers and 2.5 nM probes.

2.1.2. Genomic DNA extraction

Organs are harvested and placed in a 2 ml Safe-Lock tube following the recommended organ volume for DNA given in Table 1. The tissues are immediately frozen in liquid nitrogen to avoid any degradation.

Genomic DNA is then extracted using the phenol/chloroform method:

1. Add one volume of Proteinase K solution (62 mM Tris, 6.2mM EDTA, 200mM NaCl, 1% SDS and 20mg/ml of proteinase K (Sigma)) to the tube.
2. Incubate at 55°C overnight with agitation.
3. Check the complete digestion of your organ and add 1 volume of Tris saturated Phenol/Chloroform, pH 8 (Biosolve ref 169123).
4. After agitation and centrifugation for 15 min, take the aqueous upper phase and put into a new 1.5ml tube. Check that the aqueous phase is transparent and does not contain an organic phase.
5. Add 0.8 volume of Isopropanol
6. After shaking for 20s, centrifuge at 11 000 rcf speed for 20 min.
7. Remove the supernatant, wash with 2 volumes of Ethanol 70% and centrifuge for 1 min at 11 000 rcf
8. After drying (about 10 min), add pure water as described in Table 1.

DNA quality and concentration are assessed using Nanodrop (Thermo Scientific). The A260/A280 ratio is used to measure protein contamination and should be higher than 1.8. DNA concentration is adjusted with water to 0.3 µg/µl and the samples are stored at -20°C.

2.2. Preparation of RNA quantification by RT-ddPCR

2.2.1. Primer and probe design

Primers and probe are designed as described in 2.1.1. with special attention to avoid unspecific design. Before ordering, primers are blasted on the target gene map to verify that they span the exon/exon boundaries on the RNA. This method avoids the risk of amplifying contaminating DNA due to the size of the exon/intron/exon genomic structure, which should not be amplified following the conditions described here.

2.2.2. Total RNA extraction

Organs are harvested and placed in NucleoSpin® Bead Type D tubes (Macherey Nagel) following the recommended organ volume for RNA presented in Table 1. Tissues are immediately frozen in liquid nitrogen to avoid any degradation.

Total RNA is then extracted, by following the method below:

1. Add 1 ml of TRIzol™ reagent (Invitrogen).
2. Lyse and homogenize the tissue in Precellys 24 (Bertin technologies) according to the parameters in Table 1.
3. Add 200 µl of dichloromethane, shake for 20 s and centrifuge for 20 min at 12 000 rcf at room temperature.
4. Prepare a 1.5 ml tube with 500 µl ethanol 70% per sample.
5. Transfer 500 µl of aqueous phase from TRIzol™ tube in the 1.5 ml ethanol 70% tube and mix.
6. Transfer the entire volume into NucleoSpin® RNA column (Machery-Nagel).
7. Perform steps 5 to 8 of the NucleoSpin® RNA protocol following the manufacturer's instructions.
8. Elute the RNA with an appropriate RNase free water volume as described in Table 1.

RNA quality and concentration are assessed using Nanodrop (Thermo Scientific). A A260/A280 ratio is used to measure protein contamination and should be higher than 1.8. RNA concentration is adjusted with RNase free water to 0.33 µg/µl and the samples are found to be stable at -20°C for more than 3 years (data not shown). Small RNA molecules below 50 bps are eliminated with this protocol. This method, the combination and adaptation of TRIzol and NucleoSpin® RNA (Machery-Nagel) extractions, allows using a single protocol for efficient and high quality RNA extraction from a variety of organs though it is not adapted for feces and blood extractions.

2.2.3. RNA reverse transcription (RT)

Three microliters of total RNA are used as a template for RT. cDNA synthesis is performed using the QuantiTect Reverse Transcription Kit (Qiagen). The final reaction is diluted five times and stored at -20°C.

2.3. *Optimized condition for simultaneous DNA and RNA extraction*

The quantification of both DNA and RNA targets on the same sample requires a protocol that preserves the integrity of both DNA and RNA molecules while efficiently removing inhibitors. For simultaneous DNA and RNA extraction, organs are harvested and placed in NucleoSpin® Bead Type D tubes (Macherey Nagel) following the recommendations given in Table 1. 700 µl of lysis buffer from the NucleoSpin® RNA kit (Macherey Nagel) is added and the organ is lysed and homogenized also as described in Table 1 (Homogenization RPM-duration column). The lysate is then split in half, with one half being extracted according to section 2.1.2. (starting at step 4.) and the other following section 2.2.2 (skipping step 2).

As this optimization avoids the need to separate the sample into two parts, the protocol is particularly useful for heterogeneous samples that must be compared more precisely for RNA and DNA sequence quantification. For example, samples from cancer biopsy, from tissue-specific Cre or CreER^{T2}-driver mouse lines or from vectors (viral or non-viral) *in vivo* delivery can be easily analyzed.

2.4. *Optimization of assay conditions*

Droplet digital PCR duplex reaction is prepared by adding 2 µl of template (25 ng genomic DNA or 2 µl diluted cDNA), 10 µl of 2X ddPCR Supermix for probes (no dUTP) from Bio-Rad, 1 µl of target probe (ZENTM FAM) / primers mix (final concentration of 750 nM of each primer and 250 nM of probe) and 1 µl of reference probe (ZENTM HEX) / primer mix (final concentration of 750 nM of each primer and 250 nM of probe) in a total volume of 20 µl. Droplets are generated using the QX100 or QX200 droplet generator as per Mazaika and Homsy [13]. Forty µl of droplets containing reaction is transferred in a semi-skirted plate (Bio-Rad) for thermal cycling. Amplification is subjected to the following thermal conditions: 95°C for 10 min (Hot Start polymerase activation) followed by 40 cycles of 95°C for 20 s, a gradient of annealing between 53°C to 63°C for 1 min and a final deactivation step of 98°C for 10 min. All the steps are performed with a ramp rate of 2°C/s. The ddPCR amplification outcome is analyzed using a QX100 or QX200 Droplet Reader (Bio-Rad), as described in Mazaika and Homsy [13].

The PCR gradient is used to determine the optimum annealing temperature giving the greatest positive-negative droplet separation. The mean positive droplet amplitude / mean negative droplet amplitude ratio should be over 2 for both the target (FAM dye) and the reference (HEX dye) assays.

The setting up ddPCR is performed using wild type material with appropriate controls (water controls, no reverse transcriptase control for RT-ddPCR experiments). For RT-ddPCR, wild-type organs are selected for

good expression of the target gene using BioGPS (<http://biogps.org>) [14]. mRNA expression correlation between BioGPS and RT-ddPCR data reveals that BioGPS is a useful tool for selecting organs expressing the target gene (Sup Fig. 1).

This method is highly robust with 96.4 % of setups validated on more than 250 designs (data not shown). For the rare cases of poor results, a new assay design is performed. Assay optimizations are described in chapter 3.5.

2.5. *Data analysis and interpretation*

Analysis of QX100 / QX200 droplet reader data is performed with Quantasoft™ Analysis Pro (version 1.0.596) [13]. All samples are inspected manually. Samples with fewer than 8,000 droplets are repeated. A low number of droplets can usually be explained by over-robust pipetting after the droplet generation step.

In ddPCR, DNA fragments or cDNA are distributed randomly into the droplets during the partitioning process. Each droplet may thus contain only the target sequence, the reference sequence, both or neither (Fig 1a).

A one-dimensional (1D) display is provided by the Quantasoft™ software which represents for each of the thousands of droplets its fluorescent amplitude for FAM dye emission or for HEX dye emission. Two droplet populations should be observed. The droplets with high fluorescence values are the positive droplets while those droplets with low fluorescence values are negative for the assay (Fig 1). If additional populations or droplets with intermediate fluorescence are observed, they must be characterized further (see 3.5.). The QuantaSoft™ software automatic setting will apply a threshold directly over the negative droplets to differentiate positive and negative ones. In some cases, the automatic setting does not detect the threshold correctly. Therefore, for each sample, the threshold must be verified and reset if necessary using this 1D visualization display.

The QuantaSoft™ Analysis Pro Software also provides a FACS-like 2 dimensional (2D) plot visualization that allows visualizing for each single droplet the fluorescent amplitude for both FAM dye emission (channel 1) and for HEX dye emission (channel 2). The inspection of the 2D visualization is performed for each sample. Up to four populations should be observed: negative, FAM positive, HEX positive and FAM/HEX positive droplets (Fig 1). If additional populations are present, they must be characterized further (see section 3.5). The Poisson distribution 95% confidence interval is provided by the software for each sample. A large error

bar indicates that the target/reference concentration in the sample is too low or too high. In this case, the experiment is repeated on a concentrated (if possible) or a diluted template, respectively.

3. Optimizations, limitations and troubleshooting

3.1. Evaluation of RNA extraction

The RNA Quality Score (RQS, Perkinelmer LabChip) and the RNA Integrity Number (RIN, Agilent) are equivalent metrics used to assess the integrity of extracted RNA [15]. Samples with an RIN/RQS value ≥ 7 are usually considered to be of good quality [16]. The RNA extraction method described here allows the isolation of good quality RNA, with the exception of pancreas and ventral skin (Fig 2a).

For pancreatic tissue containing a large amount of RNase, quick dissection followed by overnight submersion at 4°C in 5 volumes of RNeasy lysis solution improves mean RQS values by 3.2 (3.9 to 7.1) (Fig 2a). Good lysis and homogenization of ventral skin is difficult to achieve with our protocol (Table 1) and may explain the low RNA quality. The adaptation of the existing protocol to mouse skin samples must be done to ensure accurate quantification [17].

3.2. Efficiency of the RT step in RT-ddPCR

The efficiency of reverse transcription between samples or even between replicates can be very variable. RT efficiency is impacted by a large number of parameters [18], especially the target to be amplified [19]. With our protocol, between the three enzymes tested (Fig 2b&c), the QuantiTect Reverse Transcription Kit (Qiagen) reveals the best results and the lowest coefficient of variation (as defined by the standard deviation divided by the mean calculated in percent (%CV)) and it was therefore selected for the RT step (Fig 2c). Four different targets were tested with the three enzymes in 8 replicates in up to two organs.

3.3. Tolerance of ddPCR & RT-ddPCR to inhibitory substances

Purification of DNA and RNA are undertaken before ddPCR or RT-ddPCR to remove contaminants from the samples to be analyzed. PCR amplification and reverse transcription are sensitive to some contaminants impacting the accuracy of the quantification [20]. Some of these inhibitors may not be eliminated during the purification step. As fluorescence quantification is performed after the last PCR amplification cycle in ddPCR,

the variability of PCR amplification efficiency has a lower impact on quantification accuracy. Consequently, ddPCR is generally more tolerant to inhibitors than qPCR but without being fully tolerant [5,21–25].

3.4. 2D visualization for evaluating complex results and multiple droplet populations

1D visualization (Fig 1) is frequently used by researchers to validate ddPCR results. It is necessary to provide qualitative information but is not sufficient to verify either the specificity of the assays or the presence of possible unexpected PCR events. For example, in 1D visualization, droplets with intermediate fluorescence falling between positive and negative (called rain) are frequently observed. They can be true positive or false positive depending on their possible origins (see Witte *et al.* [11] for possible sources). With the protocol described here, this rain effect is mostly negligible. We analyzed the impact of rain on 20 randomly selected designs corresponding to a total of 773 samples. We found that these intermediate droplets count for less than 0.90 % of the positive droplets, indicating that they can be counted as either positive or negative droplets (Fig 1c). The threshold can thus be set up indifferently either directly above negative droplets or directly below positive droplets (Fig 1a).

In more rare cases, droplet rain can be very abundant and impacts on accurate quantification (e.g. Witte *et al.* [11]). Mazaika and Homsy suggested that insufficient homogenization of the reaction mix could be an explanation [13]. According to our tests, mix vortexing did not affect the presence of rain (data not shown). We found that suboptimal PCR amplification due to sequence variation or completion in the duplex reaction can explain certain rain effects (Fig 1b). The two-dimensional (2D) display allows a clearer distinction between droplet populations than in 1D visualization and should be used systematically to discriminate between possible causes of rain. For example, 1D visualization may be misleading if a wild-type probe cross-reacts with a mutant amplicon, as observed when quantifying a slight variation of nucleotides on DNA or mRNA (Fig 1). In Fig 3, all the assays are competing duplex reactions with both mutant (MT) and wild-type (WT) probes located at the exact same position on the target mRNA or DNA (Fig 3a). In the first example (Fig 3b), WT and MT alleles differ from three bps. The 1D display detects two positive droplet populations corresponding to WT and MT mRNA. In 2D visualization, each population is correctly separated and quantified. In the second example (Fig 3c), WT and MT alleles differ from two bps (GGA>AGC). In 1D visualization, rain is observed for both channels in MT heterozygote animals (GCA/AGC) but not in MT homozygote mice (AGC/AGC) nor WT animals (GCA/GCA). This indicates competition between WT and MT

amplification, as only double positive droplets derived from heterozygote mouse present reduced and variable fluorescence levels.

Finally, 2D display can detect additional PCR products. In the example in Fig 3d, CRISPR/Cas9 genome editing with an ssDNA donor was used to introduce a point mutation by homologous directed repair (HDR) in a defined locus. In founders, a large panel of mutations can be observed following double strand break repair: non homologous end-joining (NHEJ) will lead to small (or larger) deletions or indels at the target whereas HDR using the ssDNA as donor will lead to the replacement of the wild-type sequence by the MT sequence [26]. Founder animals were genotyped using ddPCR with two probes, one located on the WT locus sequence (channel 2 – HEX) and the other corresponding to the point mutation (channel 1- FAM). In founder #3, five major droplet populations (not including double positive droplets) were detected. PCR amplification followed by sequencing show that in addition to wild-type and point mutation allele detection, the two additional populations (labelled 4 and 5) correspond to different NHEJ events.

These examples indicate that careful analyses of droplet populations using 2D visualization is a key step to guarantee the quality of data interpretation. If rain impacts the quantification, our recommendation is to first try to optimize ddPCR amplification (see next chapter) and, if unsuccessful, in a second step to design a new assay. If unexpected droplet populations are observed, sequencing of the PCR product corresponding to the ddPCR assay will also be required.

3.5. *Optimization of ddPCR amplification step*

For the vast majority of ddPCR designs, no optimization of PCR amplification is required. However, in rarer cases, poor separation of positive and negative droplets or droplet rain can be observed. With our protocol, on 250 designs done in our lab, only 3.6% showed poor or no ddPCR amplification. These issues can be explained by multiple causes like poor quality of the reference genome sequence in the target region (primers and/or probe do not match the target as anticipated), partial PCR inhibition [11], biased amplification efficiency [11], nonspecific amplification [11], DNA structure (loops, repeats, high G/C content, etc.) [27] or even the performance of the thermal cycler (Sup Fig 2 [28]). In PCR or qPCR, a large panel of chemical (addition of Taq, probe concentration, MgCl₂ etc.) and physical parameters (elongation time, ramp rate, etc.) can be adapted when no specific DNA amplification is observed. In ddPCR, the variation of chemical parameters does not seem to really increase the quality of the results whereas adjustment of physical parameters successfully improved poor ddPCR amplification (Table 2).

3.6. RT-ddPCR normalization using a reference gene

Gene expression quantification is prone to errors due to pipetting errors and variations in yield and efficiency during RNA isolation, reverse transcription and, to a much lesser extent, due to ddPCR amplification [5]. To accommodate this intrinsic variability, as in RT-qPCR, the normalization of a target gene expression to one or more reference genes is usually performed. Duplexing the reference (HEX probe) and the target (FAM probes) in the same sample is the first optimization that can be done to reduce data variability [29]. Whatever the reference gene used, its expression level and stability can vary considerably between different tissues, cell types, analysis timing and/or experimental conditions [30]. In our design, the *Hprt* gene is commonly used as a reference gene. The selection of a suitable reference gene for mRNA expression studies in mice is problematic because no reference gene remains very stable when a large panel of organs is analyzed. In RT-qPCR, *Hprt* can be a good normalization gene for some organs and conditions (e.g. in mouse or human [31–34]) and other normalization genes can perform much better in other set ups (e.g. [31,35,36]). Here, the variability of *Hprt* was analyzed on 684 WT samples (10 different organs and 21 different projects). The impact of its variability is analyzed using 3 methods (Fig 4b):

- Global variation per organ: [%cDNA sample] / mean global [WT]. *Hprt* mRNA expression is analyzed by RT-ddPCR. 100% represents the mean value of *Hprt* expression from all samples per organ.
- Variation per batch and per organ: [%cDNA sample] / mean batch [WT]. 100% represents the mean value of *Hprt* expression per batch and per organ.
- Ratio: result of target variation using *Hprt* in each sample. The experimental target is normalized using the *Hprt* gene. 100% is set per experimental target and per batch.

Our data reveal that technical factors (mRNA quantification, RT efficiency) and batch effects are important factors in gene quantification errors. With our protocol, *Hprt* showed low variability in the 4 organs tested (Fig 4b) and is sufficient for good mRNA quantification by RT-ddPCR. However, when very precise measurements are required, it is strongly advised to evaluate and select specific reference genes for each experimental design and to normalize by using several reference genes [37,38].

3.7. Multiplexing

The quantification of more than two targets in a single reaction can be achieved easily by using ddPCR. Amplitude-based multiplexing uses probes conjugated with a single dye at different concentrations [39] or is based on ddPCR designs that present different final fluorescence amplitudes [4]. Ratio-based multiplexing assays use a combination of two dyes at different concentrations [39]. From our experience, ratio-based multiplexing (Fig 5a) is easier to set up than amplitude-based multiplexing (Fig 5b) because the latter requires assays that show clearly different fluorescence amplitudes. From our experience (~100 different multiplexing assays), up to 4 targets can be easily and precisely quantified routinely, using ratio-based multiplexing (Fig 5a) whereas up to 3 targets can be used easily with amplitude-based multiplexing.

Analyses of ddPCR multiplex data using 2D views are easy to perform with QuantaSoft™ Analysis Pro software, as described in the user manual. In Fig 5c, we also present a competing duplex + amplitude base multiplexing technique that we developed for the quantification of small variations. It allows the calculation of a CNV value for DNA quantification for both wild-type and mutant alleles instead of a ratio with only a competing duplex assay. This is especially useful when the target region is not present in 2 copies (e.g. trisomic animals, cancer models, etc.). At RNA level, it permits the inclusion of a reference assay like *Hprt* and thus improves the accuracy of quantification. Additional details on multiplexing with digital PCR are described in Whale *et al.* [39].

3.8. Concluding remarks

Droplet digital PCR and RT-ddPCR are powerful methods for the quantification of DNA and RNA targets. They can be used to complement or replace qPCR. Compared with qPCR, they are less sensitive to technical bias, but good protocols and a broad understanding of the technical limitations remain essential to ensure the quality of published results and to avoid experimental nonsense data. As highlighted by Huggett *et al.*, extensive description of ddPCR experiments following dMIQE is mandatory to ensure comparability and reproducibility [10]. In table 3, we propose minor additions to Huggett *et al.*'s dMIQE checklist when using ddPCR instruments.

Author's contributions

LL, PC designed the different protocols and performed the experiments. LL, SJ, MC, YH, GP designed the study. LL, SJ, MC, YH, GP contributed to the writing of the manuscript. All the authors have read and approved the final manuscript.

Competing interest

The authors declare no competing interest

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Tables

Table 1. Details of DNA and RNA extraction conditions used in this protocol for a wide range of mouse organs.

Organ	Quantity of organ to harvest ¹	DNA extraction		RNA extraction	
		Elution volume (µl)	Homogenization (RPM- cycle × duration) ²	Elution volume (µl)	
aorta	all	20	5000- 1 × 20s	20	
bone marrow	100 mg	20	5000- 1 × 20s	20	
brain - whole	1 hemisphere	150	5000- 1 × 20s	100	
brain region - brainstem (midbrain+pons+medulla)	all	50	5000- 1 × 15s	50	
brain region - cerebellum	all	70	5000- 1 × 15s	70	
brain region - cortical and subcortical region	1 hemisphere	70	5000- 1 × 15s	70	
brain region - hippocampus	all	20	5000- 1 × 15s	20	
brain region - hippocampus + thalamus + septal area	all	30	5000- 1 × 15s	30	
brain region - hypothalamus	all	20	5000- 1 × 15s	20	
brain region - thalamus + septal area	all	30	5000- 1 × 15s	40	
brown adipose tissue	100 mg	50	6000- 1 × 20s	30	
cecum	100 mg	50	5000- 1 × 20s	50	
colon	100 mg	30	6000- 1 × 20s	70	
duodenum	100 mg	70	5000- 1 × 20s	150	
esophagus	all	20	5000- 1 × 20s	30	
eye	both	20	5000- 1 × 20s	20	
femur	half	20	6500- 2 × 20s	20	
gastrocnemius	100 mg	40	5000- 1 × 20s	40	
heart	half	30	5000- 1 × 20s	30	
ileum	100 mg	70	5000- 1 × 20s	150	
jejunum	100 mg	70	5000- 1 × 20s	150	
kidney	100 mg	150	5000- 1 × 20s	100	
liver	100 mg	150	5000- 1 × 20s	100	
lung	100 mg	30	5000- 1 × 20s	30	
lymph node	100 mg	30	6000- 1 × 20s	30	
mammary gland	100 mg	50	6000- 1 × 20s	30	
olfactive bulb	all	30	5000- 1 × 15s	30	
ovary	both	20	5000- 1 × 20s	30	
pancreas	50 mg	100	5000- 1 × 20s	150	
prostate	100 mg	50	5000- 1 × 20s	50	
quadriceps	100 mg	50	5000- 1 × 20s	40	
rectum	100 mg	30	5000- 1 × 20s	50	
salivary gland	100 mg	50	6000- 1 × 20s	50	
soleus	100 mg	40	5000- 1 × 20s	40	

spinal cord	50 mg	20	5000- 1 × 15s	30
spleen	50 mg	50	5000- 1 × 20s	70
stomach	100 mg	50	6000- 1 × 20s	30
testis	100 mg	70	5000- 1 × 20s	70
thymus	100 mg	50	5000- 1 × 20s	70
thyroid	all	30	5000- 1 × 20s	20
tibialis	100 mg	40	5000- 1 × 20s	40
tongue	100 mg	50	5000- 1 × 20s	30
urinary bladder	all	40	5000- 1 × 20s	20
uterus	100 mg	30	5000- 1 × 20s	50
vagina	100 mg	50	5000- 1 × 20s	50
ventral skin ³	100 mg	20	6000- 3× 15s	30
white adipose tissue	100 mg	50	6000- 1 × 20s	30

¹ maximum recommended quantity of mouse organ to harvest to avoid poor DNA or RNA extraction.

² program the Precellys with RPM, number of shredding cycles and duration of shredding cycle. A 10s break between each cycle is programmed to avoid overheating of the sample.

³ before homogenization, cut the skin with scissors into small pieces to improve shredding.

Table 2. Parameters influencing ddPCR amplification

MODIFIED PARAMETER	IMPROVEMENT OF DDPCR AMPLIFICATION	REFERENCES
Variation of chemical parameters		
Supplementary addition of Taq Polymerase	No	[11]
Supplementary addition of MgCl ₂	No	[11]
Supplementary addition of dNTPs	No	[11]
Probe concentration	No	[11,40,41]
Probe type	No	[11]
Primer concentration	No	[11,42]
Variation of physical parameters		
Temperature gradient	Yes	[11,40,42–44]
Ramp rate	Yes	[11,43,44]
Elongation & denaturation time	Yes	[11,43,44]
Number of cycles	Yes	[11,41,43,44]
Thermal cycler	Yes	Sup Fig 2 in this paper

Table 3. Huggett *et al.* dMIQE checklist updated with additional recommendations when using ddPCR instruments

ITEM TO CHECK	IMPORTANCE	ITEM TO CHECK 2	IMPORTANCE
Experimental design		dPCR oligonucleotides	
Definition of experimental and control groups.	E	Primer sequences and/or amplicon context sequence.	E
Number within each group.	E	RTPrimerDB (real-time PCR primer and probe database) identification number.	D
Assay carried out by core lab or investigator's lab?	D	Probe sequences.	D
Power analysis.	D	Types of probes and types of fluorophores¹	REF or E
Sample		Location and identity of any modifications.	E
Description.	E	<i>Manufacturer of oligonucleotides.</i>	<i>REF or D</i>
<i>Volume or mass of sample processed.</i>	<i>REF or E</i>	<i>Purification method.</i>	<i>REF or D</i>
Microdissection or macrodissection.	E	dPCR protocol	
<i>Processing procedure.</i>	<i>REF or E</i>	<i>Complete reaction conditions.</i>	<i>REF or E</i>
<i>If frozen—how and how quickly?</i>	<i>REF or E</i>	<i>Reaction volume and amount of RNA/cDNA/DNA.</i>	<i>REF or E</i>
<i>If fixed—with what, how quickly?</i>	E	<i>Primer, (probe), Mg⁺⁺ and dNTP concentrations.</i>	<i>REF or E</i>
Sample storage conditions and duration (especially for formalin-fixed, paraffin-embedded samples).	E	<i>Polymerase identity and concentration.</i>	<i>REF or E</i>
Nucleic acid extraction		<i>Buffer/kit catalogue no. and manufacturer.</i>	<i>REF or E</i>
<i>Quantification—instrument/method.</i>	<i>REF or E</i>	Exact chemical constitution of the buffer.	D
<i>Storage conditions: temperature, concentration, duration, buffer.</i>	<i>REF or E</i>	<i>Additives (SYBR green I, DMSO, etc.).</i>	<i>REF or E</i>
DNA or RNA quantification	E	Plates/tubes Catalogue No and manufacturer.	D
Quality/integrity, instrument/method, e.g. RNA integrity/R quality index and trace or 3':5'.	E	<i>Complete thermocycling parameters.</i>	<i>REF or E</i>
Template structural information.	E	<i>Reaction setup.</i>	<i>REF or D</i>
Template modification (digestion, sonication, preamplification, etc.).	E	Gravimetric or volumetric dilutions (manual/robotic).	D
<i>Template treatment (initial heating or chemical denaturation).</i>	<i>REF or E</i>	<i>Total PCR reaction volume prepared.</i>	<i>REF or D</i>
Inhibition dilution or spike.	E	<i>Partition number.</i>	<i>REF or E</i>
DNA contamination assessment of RNA sample.	E	Individual partition volume.	E
<i>Details of DNase treatment where performed.</i>	<i>REF or E</i>	Total volume of the partitions measured (effective reaction size).	E
Manufacturer of reagents used and catalogue number	D	Partition volume variance/SD.	D
<i>Storage of nucleic acid: temperature, concentration, duration, buffer.</i>	<i>REF or E</i>	Comprehensive details and appropriate use of controls.	E
RT (If necessary)		<i>Manufacturer of dPCR instrument.</i>	<i>REF or E</i>
<i>cDNA priming method + concentration.</i>	<i>REF or E</i>	Calibration of dPCR instrument and thermal cyclers²	D
<i>One- or 2-step protocol.</i>	<i>REF or E</i>	dPCR validation	
<i>Amount of RNA used per reaction.</i>	<i>REF or E</i>	Optimization data for the assay.	D
<i>Detailed reaction components and conditions.</i>	<i>REF or E</i>	Specificity (when measuring rare mutations, pathogen sequences etc.).	E
RT efficiency.	D	Limit of detection of calibration control.	D
Estimated copies measured with and without addition of RT. ^b	D	If multiplexing, comparison with singleplex assays.	E
Manufacturer of reagents used and catalogue number.	<i>REF or D</i>	If multiplexing, detail of multiplexing strategy (ratio-based, amplitude-based, competing assay)	E
<i>Reaction volume (for 2-step RT reaction).</i>	<i>REF or D</i>	Data analysis	

<i>Storage of cDNA: temperature, concentration, duration, buffer.</i>	<i>REF or D</i>	Mean copies per partition (λ or equivalent).	E
dPCR target information		<i>dPCR analysis program (source, version).</i>	<i>REF or E</i>
Sequence accession number.	E	Outlier identification and disposition.	E
Amplicon location.	D	Results of no-template controls.	E
Amplicon length.	E	Examples of positive(s) and negative experimental results as supplemental data.	E
<i>In silico specificity screen (BLAST, etc.).</i>	<i>REF or D</i>	Examples of significant results analyzed using 2D visualization as supplemental data.³	E
Pseudogenes, retropseudogenes or other homologs?	D	<i>Where appropriate, justification of number and choice of reference genes.</i>	<i>REF or E</i>
Sequence alignment.	D	<i>Where appropriate, description of normalization method.</i>	<i>REF or E</i>
Secondary structure analysis of amplicon and GC content.	D	Number and concordance of biological replicates.	D
Location of each primer by exon or intron (if applicable).	E	Number and stage (RT or dPCR) of technical replicates.	E
Where appropriate, which splice variants are targeted?	E	Repeatability (intraassay variation).	E
		Reproducibility (interassay/user/lab etc. variation).	D
		Experimental variance or CI. ^d	E
		Statistical methods used for analysis.	E
		Data submission using RDML (Real-time PCR Data Markup Language).	D

This table updates the information recommended by Huggett *et al.* to be included in any paper containing ddPCR data. All essential information (E) must be submitted with the manuscript. Desirable information (D) should also be submitted if possible. For authors who apply this protocol strictly, REF indicates that the citation of this paper is sufficient to be consistent with the corresponding dMIQE information. Our additional recommendations (not included in Huggett *et al.*'s dMIQE paper) are indicated in red.

¹ Types of probes and types of fluorophores impact ddPCR results (data not shown and [11,12]) and should be indicated.

² As described by Kim *et al.* [28] or illustrated in Supplementary figure 2, poorly calibrated instruments are a source of non-reproducible results.

³ As described in chapter 3.4 and Figure 3, analyses of ddPCR data using 2D visualization are requested for proper data interpretation.

Sup table 1 Parameters and rules for primer and probe

Primer criteria

PARAMETER	RANGE	IDEAL
Tm	59°C- 65°C	62°C
Tm differences	1°C - 2°C	0°C
Length (in bp)	17-30	22
%GC	35-65	50

Probe criteria

PARAMETER	RANGE	IDEAL
Tm	64°C - 72°C	68°C
Tm above primers	>4°C to 6°C	>5°C
Length (in bp)	20-30	24
%GC	40-60	50

Amplicon criteria

PARAMETER	RANGE	IDEAL
Size (in bp)	60-300	150

Figures

Figure 1. **1D & 2D visualization and evaluation of rain impact on ddPCR quantification.**

(A) 1D and 2D data view and evaluation of rain effect. Because in ddPCR, DNA fragments (or cDNA) are randomly distributed in droplets, after amplification each droplet can be HEX positive (3), FAM positive (2), HEX+FAM positive (2∩3) or negative (1). In 1D visualization, simple and double positive droplets cannot be distinguished. 2D analysis is therefore always required.

The rain effect was analyzed on 20 randomly selected designs corresponding to a total of 773 samples. For each sample, the threshold is placed at two levels (α and β). The mean number of these rain droplets is calculated and presented as a histogram (B). The change in concentration (C) is calculated as a percentage of variation between both threshold levels $\left(1 - \left(\frac{[\beta]}{[\alpha]}\right)\right) \times 100$ and presented as a histogram. The impact of rain effect is very moderate on ddPCR quantification by changing the final quantification from less than 1%. Error bars correspond to a 95% confidence interval.

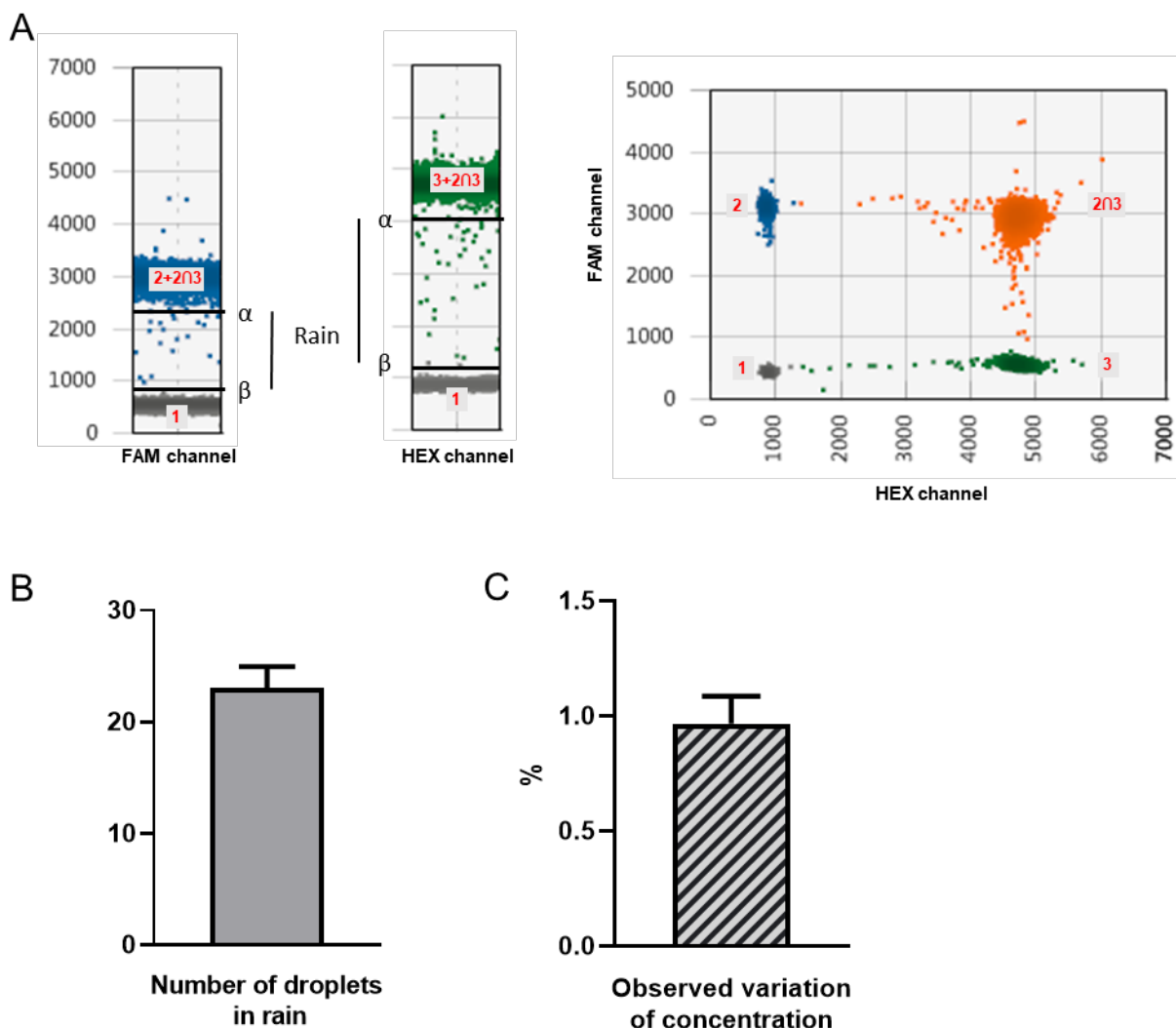


Figure 2. Quality assessment of ddPCR and RT-ddPCR protocol

(A) The quality of mRNA preparation is evaluated using the RQS score [15]. Boxplots represent median RQS.

The upper and lower limits of the boxplot are the 75th and 25th percentiles, respectively. Whiskers represent

a 5–95 % coincidence interval. Samples with an RIN value ≥ 7 are usually considered as good quality [16].

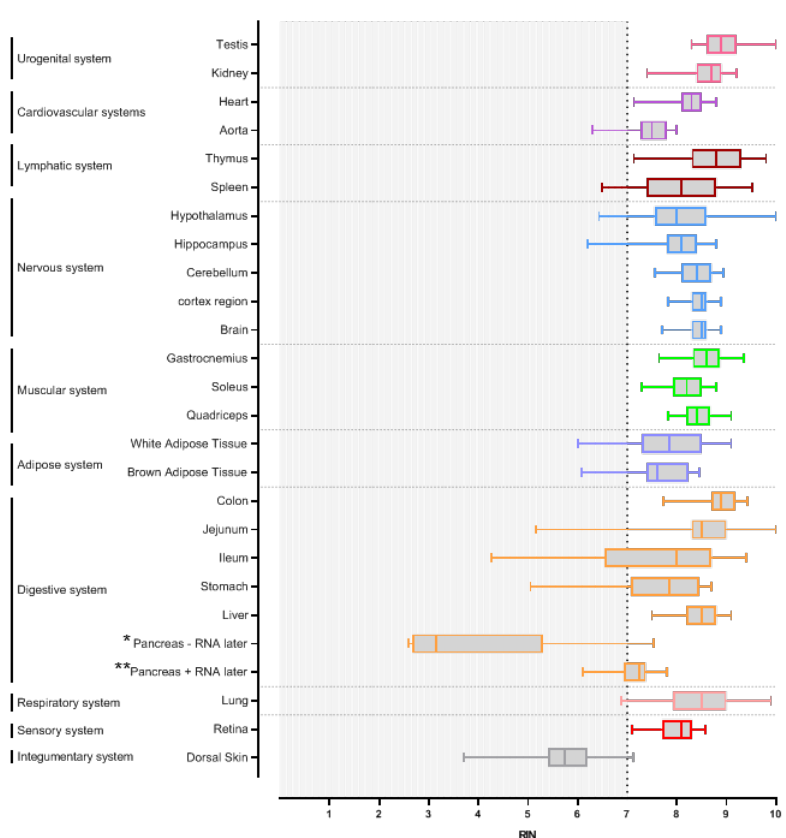
For pancreas tissue that contains a high amount of RNase, quick dissection followed by overnight submersion at 4°C in 5 volumes of RNAlater™ solution (labelled **) greatly improves mean RQS values compared to quick-frozen isolation (labeled *).

(B) Comparison of RT enzymes. The protocol described in this paper was tested using three different reverse transcriptases for 4 targets. Eight replicates were performed for each target transcript. **(C)** Relative standard

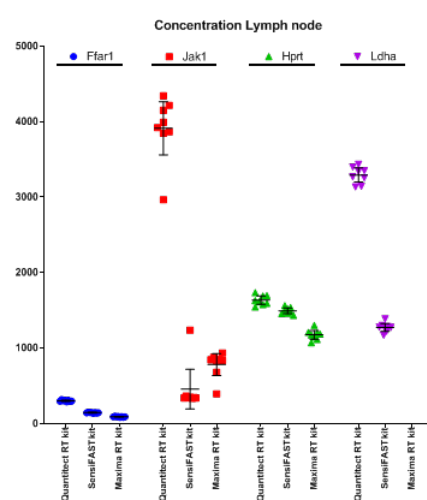
deviation expressed in %CV (percent of coefficient of variation as defined by standard deviation divided by the mean) is used to evaluate the dispersion of the data and asses the precision and repeatability of each

test. QuantiTect RT and SensiFAST™ RT enzymes reveal similar %CV values but the QuantiTect enzyme produces a higher cDNA yield and was therefore preferentially selected for the RT-ddPCR experiments.

A



B



C

Reverse Transcriptase	Provider	<i>Ffar1</i>			<i>Jak1</i>			<i>Hprt</i>			<i>Ldha</i>		
		Mean	%CV	Replicates	Mean	%CV	Replicates	Mean	%CV	Replicates	Mean	%CV	Replicates
QuantiTect RT kit	QIAGEN	301.9	3.3	8	3911.3	10.8	8	1634.9	4.1	8	3291.9	3.4	8
SensiFAST™ kit	Bioline	144.7	3.5	8	456.4	69.1	8	1493.8	2.9	8	1273.1	4.9	8
Maxima™ RT kit	Thermo Scientific	90.1	5.2	8	781.9	22.1	8	1175.8	5.9	8	ND	ND	ND

Figure 3. **2D visualization is essential for accurate ddPCR and RT-ddPCR data analysis**

(A) Schematic representation presenting primer and probe hybridization arrangements in a competing duplex reaction used for mutant (MT) mRNA quantification versus a wild type (WT) sequence.

(B) Comparison of 1D and 2D views for mRNA expression analyses of an AAG wild type (WT) > GCC mutation (MT). Each droplet population is labeled on the 1D and 2D views as follows. 1: negative droplets, 2: droplets that are only positive for the MT (GCC) sequence, 3: droplets that are only positive for the WT (AAG) sequence, 2∩3: double positive droplets: MT (GCC) and WT (AAG) sequences are detected.

In 1D visualization, an additional droplet population is observed. This additional population can be explained by cross reaction between the WT and the MT assay. In the FAM channel, not only the MT droplets are detected, i.e. 2 + 2∩3, but also the WT droplets, i.e. population 3. Likewise, in the HEX channel, the MT droplets are detected too, i.e. population 2. In 2D visualization, each population is correctly separated, cross reaction can be seen by the fact that populations 1 and 2 are not aligned vertically and populations 1 and 3 are not aligned horizontally.

(C) Comparison of 1D and 2D views for expression analyses of AGC wild type (WT) and GCA mutation (MT) mRNA. In 1D visualization, rain is observed for both channels in heterozygote MT/WT mice but not in homozygote mutant or wild type animals, indicating probe competition between WT and MT amplification. 2D analysis allows identifying the different droplet populations and leading to a conclusion of the genotype of the animal. 1: negative droplets, 2: droplets only positive for MT (GCA), 3: WT (AGC) droplets, 2∩3: double positive droplets MT (GCA) and WT (AGC)

(D) 2D view of CRISPR/Cas9 edited founder animals. Presence of the mutation was analyzed by PCR + sequencing and ddPCR on ear DNA and is presented for 3 representative founders. Founder 1# is revealed as WT by PCR + Sanger sequencing; Founder 2# presents both WT and MT alleles by PCR + Sanger sequencing. Founder 3# presents WT, MT alleles and 2 additional NHEJ events by PCR + Sanger sequencing. ddPCR allowed detecting these different populations. 1: negative droplets, 2: MT droplets, 3: WT droplets, 4: NHEJ#1 droplets, 5: NHEJ#2 droplets.

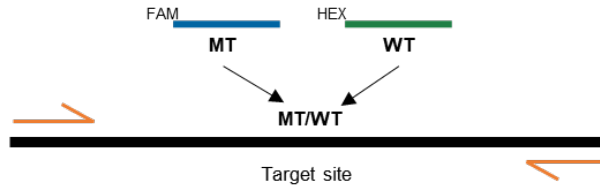
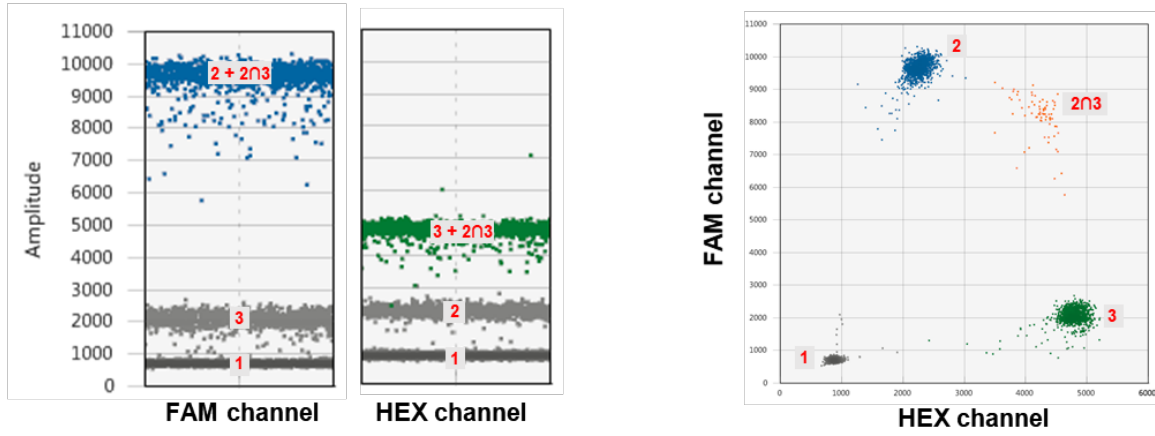
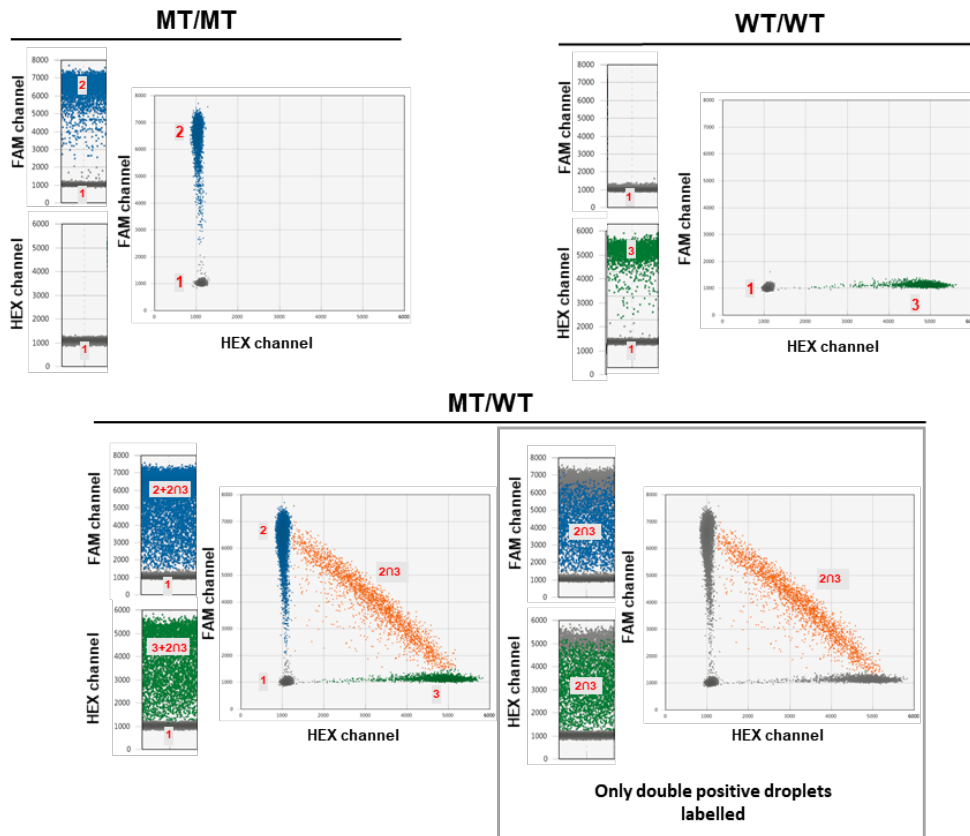
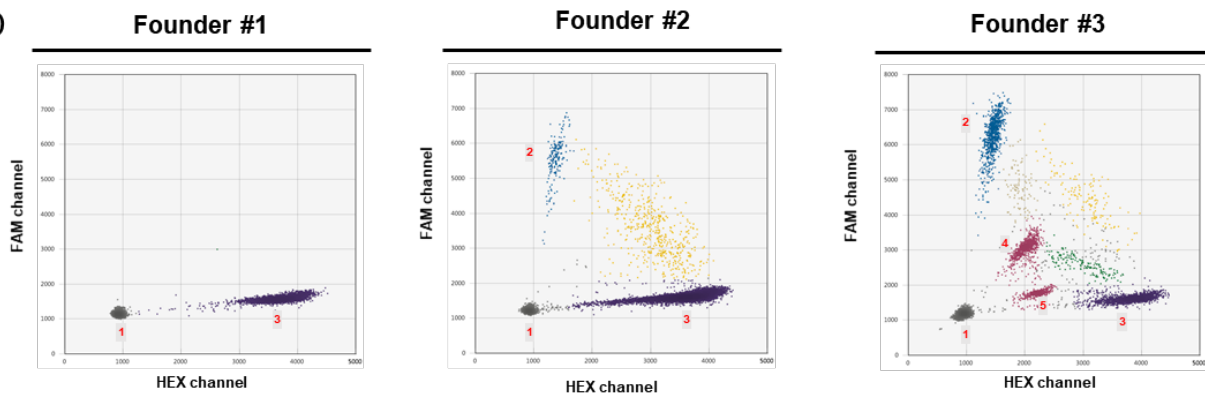
A**B****C****D**

Figure 4. **Evaluation of *Hprt* as reference gene for mRNA expression normalization.**

(A) Expression of *Hprt* in 10 different organs. The bar in boxplot represents the median concentration detected by ddPCR. The upper and lower limit of the boxplot represent the 75th and 25th percentiles, respectively. Whiskers represent a 5–95 % confidence interval. *Hprt* expression was normalized using cDNA concentration measured by NanoDrop. In the table, %CV as defined in Figure 1 was used to evaluate the dispersion of the data.

(B) Evaluation of *Hprt* variability in 4 organs using three different methods. The global ratio displays mean *Hprt* expression in each organ without any normalization to another gene; variation per batch evaluates the batch effect presenting *Hprt* expression normalized by batch (but not using another gene as reference). The ratio displays the target gene normalized by *Hprt* which was used as reference.

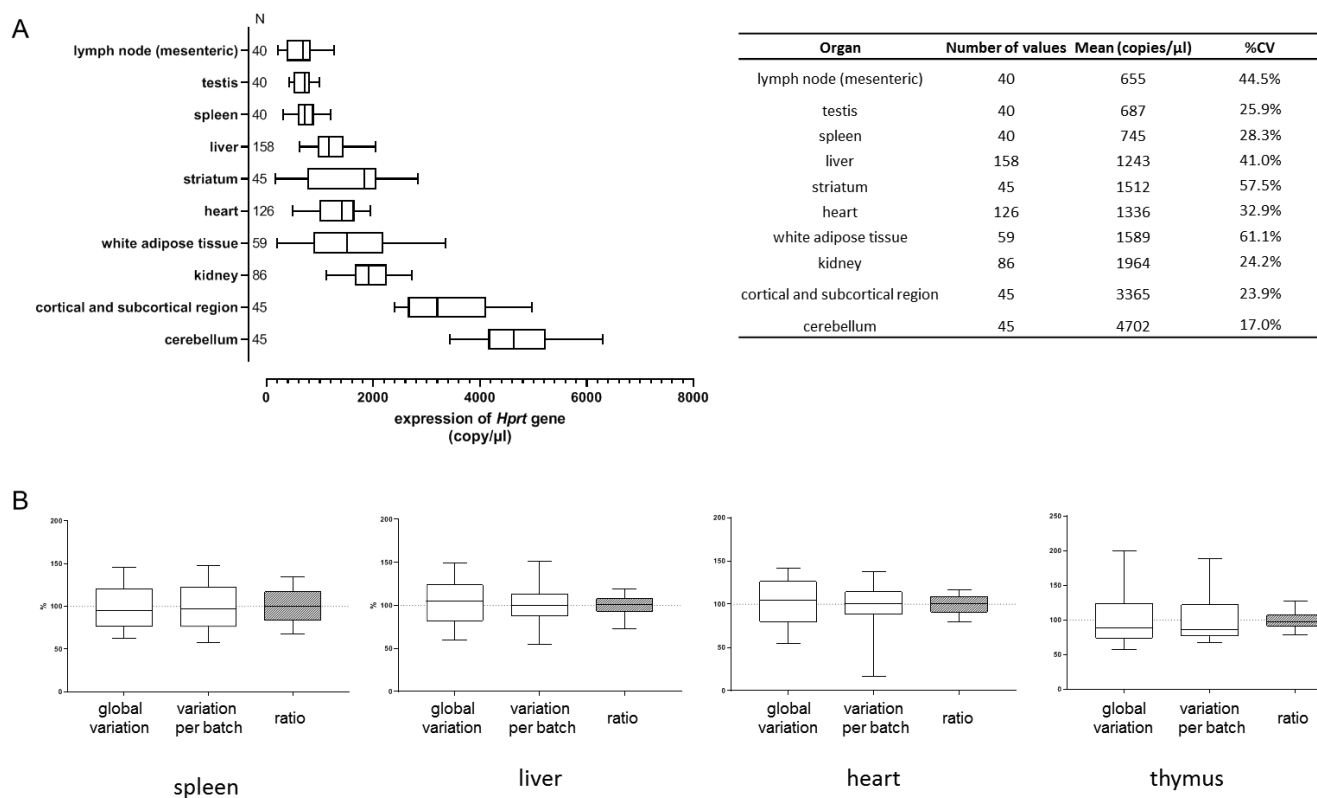
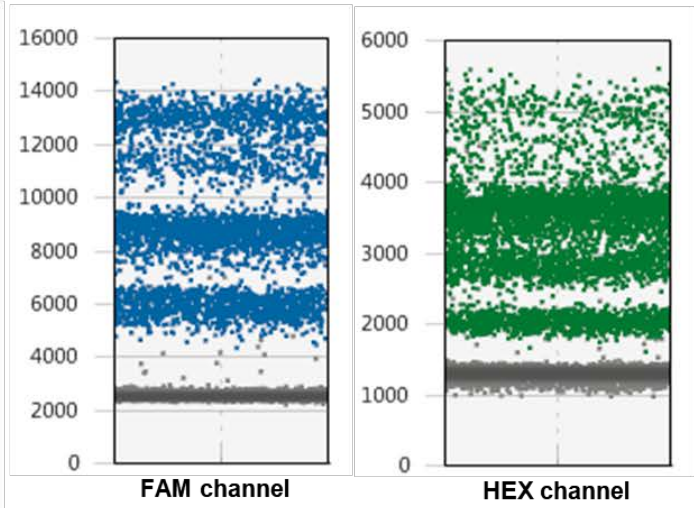
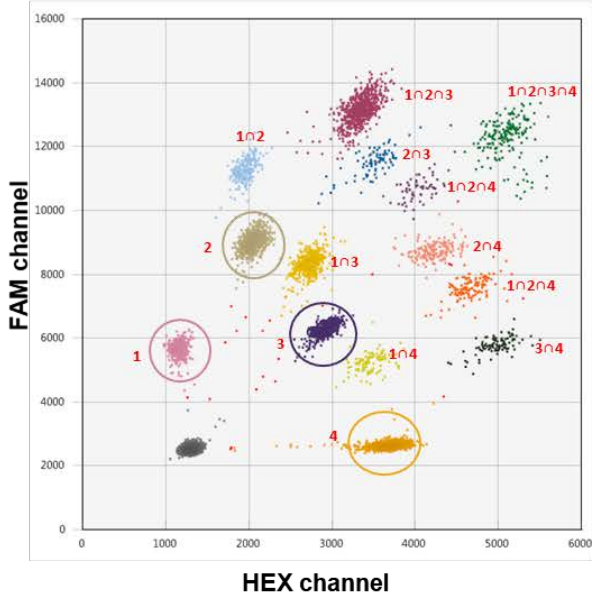
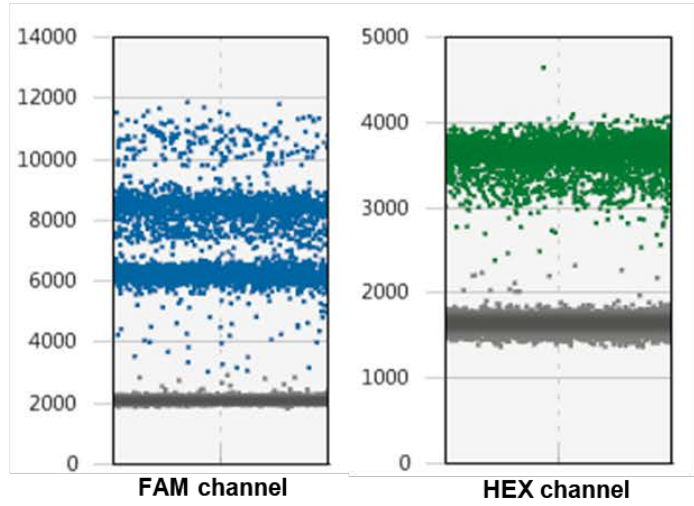
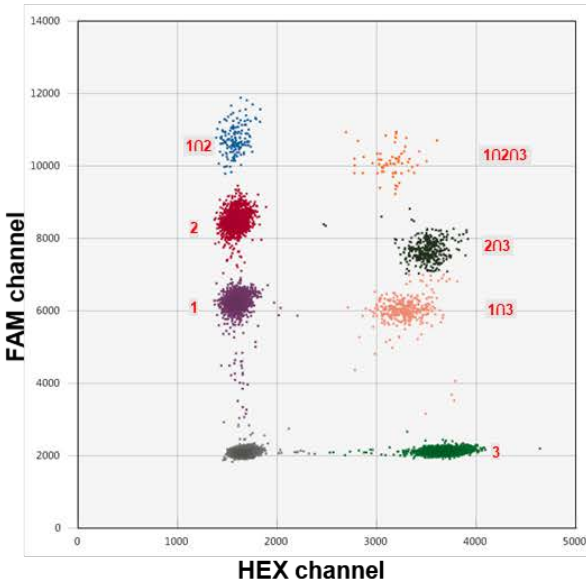


Figure 5. **Comparison of multiplexing strategies.** 1D and 2D displays are presented. For each multiplexing strategy a 2D plot is given with an FAM and HEX channel. In the 2D plot, each droplet population is numbered (1, 2, 3 or 4) and double, triple or quadruple positive droplets are represented by the population digit separated by an intersection sign (for example, triple positive droplets for 1, 2 and 3 are represented as $1 \cap 2 \cap 3$). (A) ratio-based multiplexing assay including four different probes. Each single positive droplet population is surrounded by a colored circle. 1: the pink circle represents a 100% FAM probe, 2: the brown circle represents the mix of the same probe with 75% labeled with FAM dye and 25% labeled with HEX dye, 3: deep purple represents the mix of the same probe with 25% labeled with FAM dye and 75% labeled with HEX dye 4: the gold circle represents a 100% HEX probe. (B) Amplitude-based multiplexing assay including three probes. 1: FAM probe presenting a lower amplification efficiency, 2: FAM probe presenting a higher amplification efficiency, 3: HEX probe (C) Competing duplex reactions combined with amplitude-based multiplexing using three probes. 1 and 2 assays are a competing duplex reaction with the addition of a reference gene HEX probe (3).

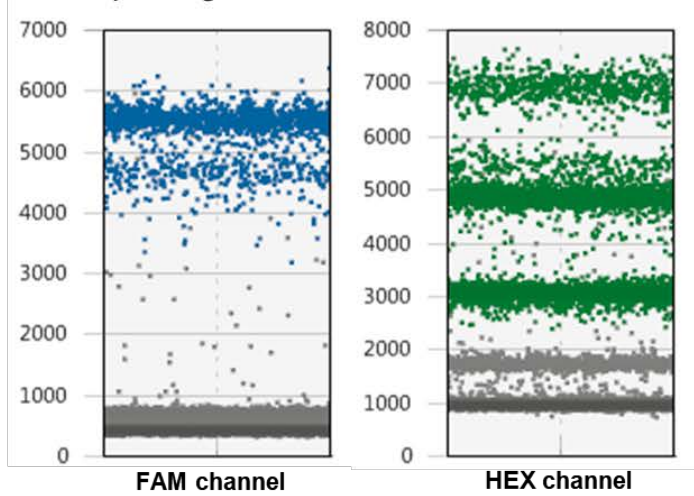
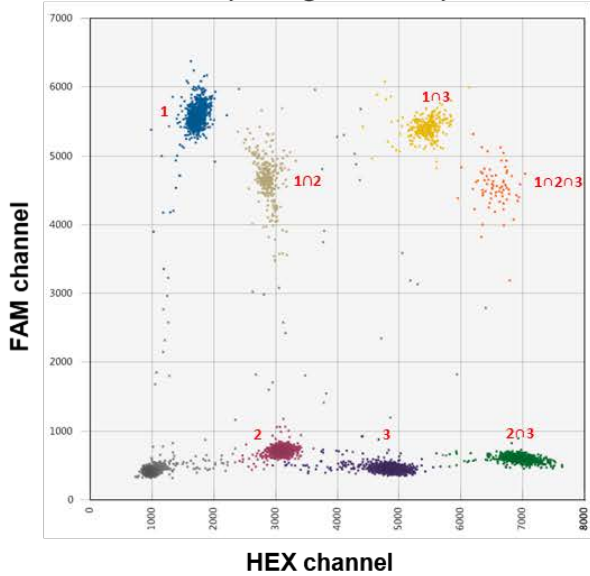
A Ratio based multiplexing



B Amplitude based multiplexing

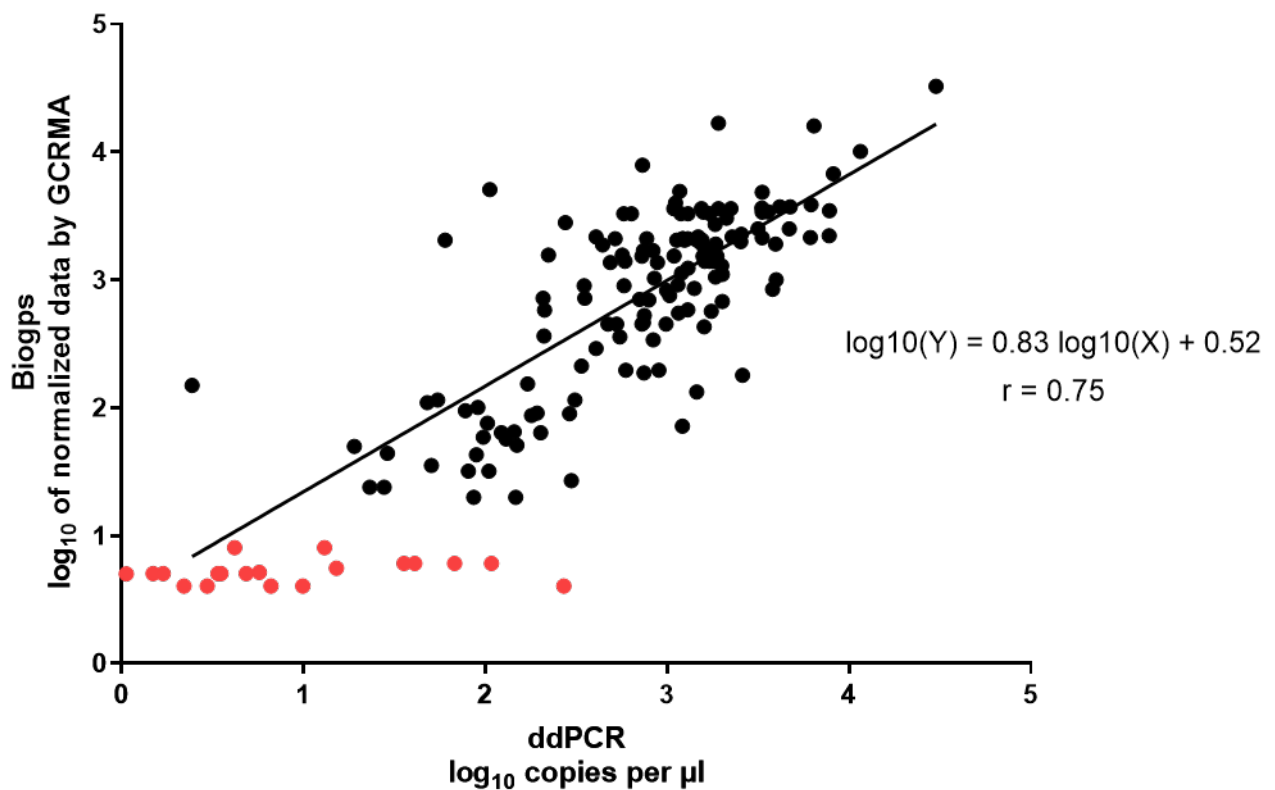


C Probe-competing with amplitude based multiplexing



Sup figure 1. **Comparison of RT-ddPCR and BioGPS mRNA expression data.**

RT-ddPCR values are indicated on the x-axis as log₁₀ of copies per microliter. BioGPS values are indicated in the y-axis as log₁₀ of GC Robust Multi-array Average (GC-RMA). 145 assays were analyzed from 47 different targets in several organs. Affymetrix GeneAtlas[®] microarrays were used to generate BioGPS data in mouse. Red dots are values for which the Affymetrix GC-RMA value corresponds to the background level (<1). These red dots were excluded from the correlation analysis as they were not successfully quantified by Affymetrix GeneAtlas[®] microarray.



Sup figure 2. **Impact of thermal cycler on droplet separation as an example of causes of poor ddPCR quantification.**

Representative 2D plots of the same experiments done with two different thermal cyclers are presented in two different sites. For each experiment, the DNA probe & primer batches are identical. The exact same thermal conditions, including following the same ramping on each thermal cycler, were also applied. The same scale is used to represent fluorescence amplitude. This result was repeated a least 3 times in each site. As previously described by Kim et al. [28] for multiplex PCR, the performance of the thermal cycler can strongly impact the reliability of ddPCR.

1: negative droplets; 2: wild-type positive droplets; 3: mutant positive droplets; 2∩3 double positive droplets

