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Research paper

Evaluation of relative quantification of alternatively spliced transcripts using droplet digital PCR

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ABSTRACT

Introduction: For the relative quantification of isoform expression, RT-qPCR has been the gold standard for over a decade. More recently, digital PCR is becoming widely implemented, as it is promised to be more accurate, sensitive and less affected by inhibitors, without the need for standard curves. In this study we evaluated RT-qPCR versus RT-droplet digital PCR (ddPCR) for the relative quantification of isoforms in controls and carriers of the splice site mutation *BRCA1* c.212 + 3A > G, associated with increased expression of several isoforms.
Materials and methods: RNA was extracted from EBV cell lines of controls and heterozygous *BRCA1* c.212 + 3A > G carriers. Transcript-specific plasmids were available to determine the efficiency, precision, reproducibility and accuracy of each method.
Results: Both ddPCR and RT-qPCR were able to accurately quantify all targets and showed the same LOB, LOD and LOQ; also precision and reproducibility were similar. Both techniques have the same dynamic range and linearity at biologically relevant template concentrations. However, a significantly higher cost and workload was required for ddPCR experiments.
Conclusions: Our study recognizes the potential and validity of digital PCR but shows the value of a highly optimized qPCR for the relative quantification of isoforms. Cost efficiency and simplicity turned out to be better for RT-qPCR.

1. Introduction

In modern day genetics the concept of alternative splicing is frequently investigated. Alternative splicing is a naturally occurring mechanism that increases the protein coding complexity of the genome. With the formation of several transcripts from one locus the number of proteins that can be formed out of the 20,000 genes, which make out the human protein coding genome, increases tremendously [1]. Besides naturally occurring alternative splicing, alternative (aberrant) transcripts can also arise from mutations in the genome leading to the formation of new splice sites or the removal of existing ones [2,3]. However, the interpretation of variants modifying alternative transcript ratios in combination with the induction of novel transcripts is less straightforward. Therefore, a first step towards understanding the pathogenicity of a variant, suspected to alter splicing, is accurate quantification of the naturally occurring transcripts together with the discovery and quantification of aberrant transcripts induced by the variant. In case of sufficient expression of normal, functional transcripts, the phenotypic effect of the deleterious variant might be minimal, because the remaining level of the normal transcript can maintain protein functionality [3].

For transcript-specific quantification of RNA RT-qPCR has been the gold standard for several years [4,5]. Here, quantification with intercalating dyes is the simplest and most cost efficient solution, although this method is prone to detection of non-specific amplification, because the dye can intercalate non-specifically (in amplicons not of interest). Non-specific amplification is expected to be less of a problem when using isoform-specific hydrolysis probes. Here fluorescence is only measured when the probe can anneal to a specific target and subsequently gets cleaved during elongation. Accurate quantification of all

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targets can however be impaired if one or more transcripts are significantly overrepresented in the sample [5] and the design of the probes in a transcript-specific sequence may not always be feasible for each isoform in combination with the design of transcript-specific primers. This and the added cost of fluorescently labelled probes, are the major limitations for qPCR with probes. Theoretically the number of target amplicons doubles every cycle during a PCR reaction, making it possible to perform relative quantifications (by calculating relative differences in amplification of target between two samples or between targets in a single sample) or absolute quantifications (by calculating the absolute amount of target based on a standard curve of known quantities). To be able to compare quantities between samples, typically several stable reference genes are measured in parallel with the expression of the target for normalization [6,7].

Recently, droplet digital PCR (ddPCR) is becoming a widely used alternative to qPCR for the quantification of nucleic acids in specific applications such as copy number quantification [8]. In ddPCR thousands of nanoliter scale droplets are generated, each containing none, one or a handful of target molecules. During PCR, each of these droplets acts as a separate reaction volume, amplifying the target. After amplification, each droplet is read out individually and droplets with a higher fluorescence than the threshold are deemed positive. Using Poisson distribution statistics, it is possible to estimate the starting concentration of target in each sample, allowing for both relative and absolute quantification at the same time without the need for a standard curve [9]. Therefore, at first sight, the calculation of target concentration is simpler with ddPCR than with qPCR and accuracy of quantification is not dependent on the accuracy of a standard curve. However, determining the threshold position for ambiguous droplets is a matter of debate and has an impact on the accurate quantification of the target. New ways to tackle this problem have been proposed [10,11]. Most recently, a data-driven method was developed, that allows for threshold calculation using extreme value theory. According to Trypsteen et al. [12] this method is more accurate than its predecessors because it imposes no assumptions on the distribution of the data and it corrects for the baseline shift between no-template-controls (NTCs) and samples. Using an appropriate method for data analysis, ddPCR is potentially more precise than qPCR [10,13].

The introduction of digital PCR raises the question if qPCR should remain the gold standard for quantifying alternative transcripts. Here, we make a comparison between qPCR and ddPCR for the relative quantification of such transcripts. Hereto, we use a deleterious *BRCA1* mutation (c.212 + 3A > G) as a model for the evaluation of qPCR and ddPCR-based quantification. *BRCA1* c.212 + 3A > G is a Belgian founder mutation associated with an increased risk for breast and ovarian cancer [14,15]. The variant was shown to induce a shift in the ratio of naturally occurring isoforms. Three naturally occurring transcripts were identified at this locus. An isoform containing the full-length exon 5 (BRCA1-ex5FL; r.135_212), a transcript with a total skip of exon 5 (BRCA1-Δex5; r.135_212del) and a transcript where the last 22 nucleotides of exon 5 are deleted (BRCA1-Δ22ntex5; r.190_212del) [16]. Several other publications confirmed the expression of these isoforms in relation to this variant [17,18]. ddPCR was evaluated in comparison to qPCR in terms of accuracy, linearity, dynamic range, precision and reproducibility for the quantification of transcript isoforms.

2. Methods and materials

2.1. Samples

For this study Epstein-Barr Virus (EBV) immortalized B cell lines derived from individuals with a germline *BRCA1* c.212 + 3A > G mutation were used as carrier samples. For the control samples EBV cell lines were used from individuals not carrying a germline *BRCA1/2* mutation as determined by screening the entire coding region of both genes. In total 6 carrier and 4 control samples were used. EBV cell lines were made according to Hui-Yuen et al. [19]. Approval for generation and usage of the EBV cell lines for fundamental research purposes was granted by the Gent University Hospital Ethical Committee and by the individuals whom these cell lines were derived from in the form of an informed consent. All experiments were done in accordance with the recommendations and restrictions set by the Gent University Hospital Ethical Committee in compliance with the WMA declaration of Helsinki regarding medical research on human subjects.

No nonsense mediated decay inhibitors were added to the cell cultures only stable transcripts were of interest in this study. For RNA extraction 1.5×10^6 cells were pelleted and resuspended in 3 mL culture medium (RPMI medium 1640, foetal bovine serum 10%, sodium pyruvate 1.11%, β-mercaptoethanol 0.11%, interleukin 2 0.11%, glutamine 1% and penicillin-streptomycin 0.5%; Thermo Fisher Scientific, Waltham, USA). Cells were counted using a Bürker counting chamber. From 1 mL RNA was extracted right away (0 h samples). 1 mL was left in culture for another 4 h (4 h samples) and another 1 mL for 24 h (24 h samples) before starting RNA extraction. RNA extraction was done using RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's protocol (without the optional DNase treatment), after which RNA was measured on DropSense96 (Trinean, Gentbrugge, Belgium) and stored at −80 °C. Removal of contaminating gDNA was done prior to RT with Heat & Run DNA removal (ArticZymes, Tromsø, Norway) following the manufacturer's instructions. For RT, the iScript cDNA Synthesis Kit (Bio-Rad, Temse, Belgium) was used in compliance with the manufacturer's recommendations. DNase treatment and RT were done as consecutive steps on the same batch of 1 μg of total RNA.

A number of dilution series were constructed. Dilutions were made from either EBV derived samples (controls or *BRCA1* c.212 + 3A > G carriers) or transcript-specific plasmids. In total 11 dilution points were made and a no template control. 5 ng/μL yeast tRNA carrier used in all dilutions. Plasmid series were diluted 1/10, EBV series 1 in 2. Template concentration in all dilution points was calculated from DropSense96 measurements of the undiluted sample. A 424 bp amplicon (spanning exons 2–7 of *BRCA1*) was amplified using cDNA from a patient heterozygous for *BRCA1* c.212 + 3A > G as described before [16]. This amplicon was cloned in a pCR2.1-TOPO vector (Invitrogen, Carlsbad, USA). Individual clones were investigated through Sanger sequencing; three clones were retained, each containing one of the three transcripts under investigation. In all experiments these plasmids were used as circular molecules without enzymatic linearization.

2.2. Quality control

For both qPCR and ddPCR the same primer sets were used. Specificity of primers for all three assays was verified with qPCR on plasmid constructs, each containing a specific isoform (Supplementary Data A). Furthermore, specificity was also investigated for each individual qPCR reaction with melting curve analysis. All reactions contained only one detectable amplification product.

Quality control (QC) on cDNA and original RNA samples was done via qPCR using an artificial SPUD assay [20], an exon-spanning assay from exon 2–4 in MKNK2 (reference sequence NM_199054), which doesn't yield a product on gDNA as the involved introns have a combined length of > 4 kb, (160 bp, F: 5′-CCAGCCGAACTTCAGGGTTT-3′, R: 5′-CGTCCGGGATGTCAATGGG-3′; forward primer sequence in exon 2, reverse primer sequence in exon 4) and an intronic assay located between exon 30 and 31 in ABCA4 (bp, F: 5′-CCAAGCCTACCTACATGGTGT-3′, R: 5′-AGGGATCCCAAAAGAAGGAC-3′; both primers are entirely located within the same intron; reference sequence NM_000350). Amplification as described in the qPCR section.