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Optimisation of Uniplex and Duplex Reactions is not Required for Real-Time PCR Amplification of Target Genes in Endometrial Cancer [Version 1, 2 Approved with Reservations]

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Author contributions

The studies were conceptualized and designed by TA and AHT. TA performed the experiments in Leicester, under the supervision of AHT and JCK. All the authors contributed to analysing the data and TA wrote the first draft of the manuscript. All the authors contributed to the final draft of the manuscript. JCK, the chief investigator, is the guarantor of the research.

Ethics statement: All the volunteers gave a written informed consent to take part in the study, which was approved and conducted according to the guidelines of Leicestershire and Rutland Research Ethics Committee (reference number 06/Q2501/48).

Abstract

In quantitative real-time PCR experiments, reactions are commonly run separately (uniplex reactions), but when samples are precious or rare, the ability to combine reactions (duplex) expands study efficiency, although evidence that accurate quantification of both the gene of interest and the endogenous controls occurs is particularly important if the starting materials are different, i.e. normal tissue and tumour. In this study, the performance of uniplex and duplex reactions on cannabinoid receptor (CB1) expression in normal endometrial tissue and that of endometrial carcinoma were tested and normalised against three different endogenous controls, both as individual normalising references and when combined. The results demonstrated that both uniplex and duplex PCR yielded similar and reproducible results with all three housekeeping genes being used without any preceding optimisation, with CB1 expression decreased to 0.29 ± 0.07-fold (mean ± SD) in endometrial cancer using the uniplex method, and to 0.05 ± 0.03-fold using the duplex method, indicating a slight improvement with the duplex method. These data are similar to those generated for the CB1 repression in the endometrial cancer of a different patient cohort, suggesting that duplex quantitative real-time PCR experiments for differential gene expression in endometrial cancer studies is not only feasible, but desirable.

Keywords

Uniplex; Duplex; TaqMan; Validation; Cannabinoid Receptor

Introduction

The uniplex method of analysing transcript expression patterns in quantitative real-time PCR (qRT-PCR) has become an established technique [1]. In standard research settings, uniplex approaches are commonly used since optimisation or validation is simple because the gene-specific and control reactions are run separately. In contrast, duplexing or multiplexing in qRT-PCR, which is the simultaneous amplification and quantification of two or more genes in a single reaction, has become a popular method for the evaluation of mRNA levels, especially where the amount of sample is limited [2-4].

Primer and probes developed under the TaqMan® technology umbrella have become a popular research tool for the measurement of specific mRNAs *via* qRT-PCR. Although TaqMan® gene expression assays (Applied Biosystems) use a sophisticated design process to ensure that their use in uniplex reactions can be performed without optimisation and validation, and are claimed to provide reliable and reproducible results, rarely is this claim tested [5]. The use of a duplex method, which has many advantages over an uniplex method, such as minimising the impact of pipetting errors, maximising experimental efficiency by increasing sample throughput, preserving limited samples and saving reagent costs is even rarer, even when it is desirable. For example, when studying endometri-

al cancer, the tissues obtained from normal and cancerous biopsies could be very small [5], and so the use of a technique that utilises minimal tissues but can still give accurate results is essential. Thus, the duplex approach may be a more appropriate technique to evaluate mRNA levels in endometrial cancer tissues because of its economical use of limited amounts of tissues. The aim of this study was to determine if a duplex method with TaqMan® probes and primers provides a more efficient but accurate method for the measurement of important gene transcript levels.

Materials and Methods

Patients

Women undergoing a hysterectomy and bilateral salpingo-oophorectomy at the University Hospital of Leicester National Health Service Trust for endometrial carcinoma (study group) or a benign gynaecological condition, such as dysfunctional uterine bleeding of fibroids (control group) were recruited. All volunteers provided signed informed consent for the samples to be used. Fresh uteri samples from the surgery were transferred immediately on ice to the histopathology department where a senior gynaecology histopathologist divided the tissue into two; one piece for this study and the other for clinical diagnosis. The sample for this study was washed with phosphate buffered saline (PBS) to remove excess blood and stored at -80°C in RNA*later*®(Life Technologies, Paisley, UK) for further processing. Histopathological diagnosis was used to confirm the clinical diagnosis of cancer and the study group samples were divided into type and grade of cancer, according to the FIGO classification [6]. Endometria from the control group were divided into secretory, proliferative or atrophic according to the criteria described by Noyes [7]. There were nine samples in control group and confirmed as being secretory (n=3), proliferative (n=3) and atrophic (n=3). A total of 15 endometrial carcinoma samples were studied: type 1 grade 1 endometrioid adenocarcinoma (n=3), type 1 grade 2 endometrioid adenocarcinoma (n=3), type 1 grade 3 endometrioid adenocarcinoma (n=3), type 2 serous (n=3) and type 2 carcinosarcoma (n=3).

cDNA Preparation

The frozen endometrial tissues (100 mg) in RNA*later*® were allowed to defrost at room temperature for 20 minutes and then transferred into lysis buffer from the *mir*Vana™ miRNA Isolation Kit (Life Technologies, Paisley, UK) and disrupted/homogenised using the TissueRuptor system (Qiagen, Crawley, UK) and total RNA isolated according to the manufacturer's protocol. The RNA was quantified and its purity determined using a NanoDrop 2000c spectrophotometer (ThermoScientific, Loughborough, Leicestershire, UK), as the 260/280nm ratio. At this point, the amount of RNA was standardised to 10 µg of total cellular RNA and exogenous DNA removed by treating with TURBO-DNAse free kit (Life Technologies, Paisley, UK) at 37°C for 30 minutes according to the manufacturer's instructions. The DNase was inactivated by adding 10 µl of inactivation buffer and centrifugation for 1.5 minutes at 10000 x *g*. At this point, the purity and concentration of the soluble RNA was re-evaluated in triplicate on the Nanodrop spectrophotometer. The supernatants were subjected to first strand synthesis using the high capacity cDNA MultiScribe™ Reverse Transcriptase Kit (Life Technologies, Paisley, UK) according to the manufacturer's protocol; incubation at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and then cooled to 4°C. The cDNA was stored at -20°C.

Quantitative Real-Time PCR

Quantitative real-time PCR experiments were performed using TaqMan® human mitochondrial ribosomal protein L19 (MRPL19; NM_014763), peptidylpropyl isomerase A (cyclophilin A) (PPIA; NM_021330) and importin 8 (IPO8; NM_006390) primers and VIC labelled probes purchased from Applied Biosystems by Life Technologies, (Paisley, UK) as a set of normalising genes specific for the examination of gene expression in human tissues. The primers and probes for the gene of interest, the cannabinoid receptor 1 (CB1; Hs00275634_m1) were also purchased from Applied Biosystems by Life Technologies, (Paisley, UK), as 6-carboxyfluorescein /minor groove binder (FAM/MGB) labelled dyes. Minus reverse transcriptase (RT-) and no template (NTC) controls containing DNAse free water instead of template mRNA/cDNA were included in each run. No product was synthesised in the NTC and RT-minus reactions confirming the absence of contamination with exogenous DNA (data not shown). Uniplex and duplex reactions were performed in a final volume reaction of 20 µl. Uniplex reactions consisted of cDNA (2 µl), DNAse-free water (7 µl), TaqMan® universal PCR Master Mix (10 µl) and 1 µl of primers/probe for the endogenous controls (MRPL19, IPO8 and PPIA) or CB1. Duplex reactions consisted of cDNA (2 µl), DNAse-free water (6 µl), TaqMan® universal PCR Master Mix (10 µl) and 1 µl of primers/probe for the endogenous controls and 1 µl of primers/probe for CB1. The reaction plates were run on a StepOne Plus instrument (Applied Biosystems by Life Technologies, Paisley, UK) and the thermal cycler profile was as follows: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. All the reactions for the reference genes were performed in triplicate.

Analyses

The mRNA gene expression stability analyses were obtained using the mean qRT-PCR threshold cycle (Ct) value. The Ct values obtained were converted into quantitative relative expression values using the $2^{-\Delta\Delta Ct}$ method [8]. Statistical analyses of CB1 expression in normal and cancerous endometrium with the three endogenous controls were expressed as the mean ± standard error of the mean (SEM) or standard deviation (SD). One-way ANOVA with Dunnett's multiple comparison test and unpaired Student's t-tests were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla

California USA, www.graphpad.com) and a p-value of less than 0.05 was considered significant.

Results

The ages and body mass indices (BMI) of the volunteers are shown in Table 1. Even though the women with atrophic uteri appeared to be older and heavier than the premenopausal women, there was no statistically significant difference in age (years) or BMI (Kg/m^2) between these groups or the women and also between those with malignancy (Table 1). To increase study power, the samples taken from normal (proliferative, secretory and atrophic uteri; n=9) were pooled together. As a test group, all of the malignant samples (n=15) were combined. When the combined group ages and BMI were re-examined, the average ages and BMI significantly different ($p<0.01$ and $p<0.0001$; respectively) between the control and study groups; this is because the pre-menopausal women (by definition) were younger and thinner than the women with malignancy, who predominantly present at an older age and are more likely to be more obese women [9,10].

Table 1: Patient Characteristics.

Groups	Age	BMI
Control		
Atrophic	62.33 ± 4.61	26.67 ± 6.42
Secretory	46.00 ± 4.35	26.00 ± 1.00
Proliferative	47.33 ± 0.57	26.00 ± 1.73
Endometrial Carcinoma Type 1		
Grade 1	78.00 ± 13.23	31.33 ± 6.65
Grade 2	67.67 ± 11.06	30.67 ± 1.52
Grade 3	72.67 ± 12.06	35.33 ± 6.11
Endometrial Carcinoma Type 2		
Serous	59.00 ± 3.46	37.67 ± 2.51
Carcinosarcoma	50.00 ± 5.00	36.67 ± 6.42
Control	51.89 ± 8.48	26.22 ± 3.38
Endometrial carcinoma	65.47 ± 13.21*	34.33 ± 5.23**

Data are shown as the mean ± SD

Above the black line: The ages and BMI of women recruited into the study were analysed using one-way ANOVA with Dunnett's multiple comparison test with the women with atrophic uteri used as the control and found to be not significantly different. All groups consistent of 3 patient samples (n=3). Below the black line: To increase the power of the study, the groups were combined into control (n=9) and endometrial carcinoma (n=15) and re-analysed using Student's unpaired t-test; *p<0.01; **p<0.0001 for age and BMI, respectively.

A comparison of the raw Ct values generated from qRT-PCR for CB1 using MRPL19 or PPIA or IPO8 as individual reference genes in uniplex and duplex reactions are shown in Figure 1. In uniplex and duplex reactions the raw Ct values were very similar to each other, with the mean (± SEM) Ct values of uniplex MRPL19 being 27.64 ± 0.43 and 27.24 ± 0.61 for duplex (1.4% error), whilst they were 32.36 ± 0.45 for uniplex CB1 and 32.46 ± 0.63 for duplex CB1 (0.6% error; Figure 1, left panel). Similarly, the mean Ct values of uniplex IPO8 reactions were 27.66 ± 0.48 and 28.82 ± 0.58 for duplex IPO8 (4.2% error), whilst Ct values for uniplex and duplex CB1 were 32.36 ± 0.45 and 32.66 ± 0.56, respectively (0.9% error; Figure 1, middle panel). Unpaired Student's t-test indicated that no statistically significant different values were obtained with any of the genes tested. The Ct values for uniplex PPIA were 23.18 ± 0.46 and 23.73 ± 0.64 for duplex (2.4% error), whilst it was 32.36 ± 0.45 for uniplex CB1 and 32.97 ± 0.65 for duplex CB1 (1.9% error; Figure 1, right panel).

The expression of CB1 in the various patient samples following normalisation with the MRPL19, PPIA and IPO8 reference genes, resulting from the uniplex and duplex reactions are shown in Figure 2. When CB1 transcript levels were normalised with MRPL19 or PPIA (Figure 2, left panel and right panel, respectively) both uniplex and duplex reactions showed similar results with a statistically significant reduction in CB1 transcript levels in the malignant samples; when IP08 was used to normalise the CB1 transcript levels (Figure 2, middle panel), both uniplex and duplex showed very similar reductions of CB1 transcript levels in the malignant tissues as to that shown when MRPL19 or PPIA were used as normalisers, but in both cases (uniplex and duplex), that decrease did not reach statistical significance.

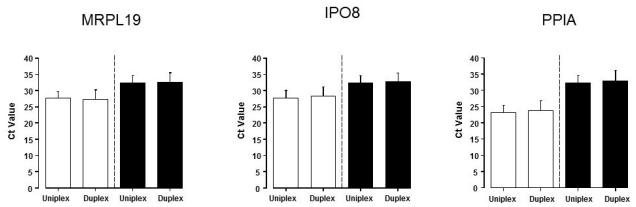

Figure 1: The effect of uniplex and duplex qPCR on raw Ct values. Each graph shows the raw Ct values obtained from qPCR with primers and probes for the indicated housekeeping genes listed above each graph (open bars) and for CB1 (filled bars) using cDNA generated from the entire patient cohort (n=24) as shown in Table 1. The data are presented as the mean ± SEM. Unpaired Student's t-test on the data indicated no significant difference between uniplex and duplex reactions.

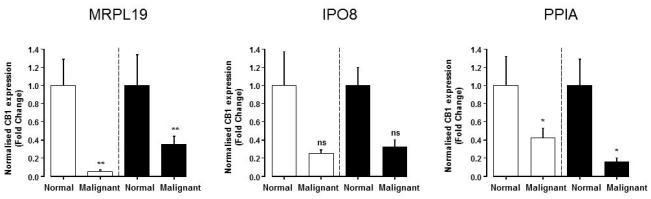

Figure 2: The effect of uniplex and duplex qPCR on normalised CB1 expression. Each graph shows the CB1 expression in normal (atrophic, secretory and proliferative endometrial samples combined; n=9) and malignant (type 1 and 2 tissue endometrial cancer combined; n=15) tissue. The names of the endogenous reference genes used for normalisation are indicated above each graph. The data are presented as the mean ± SD. Unpaired Student's t-test indicated data to be significantly different at *p<0.05; **p<0.01 or not significantly different (ns) when malignant tissue were compared to normal tissue.

When all three normalising genes were combined and the CB1 expression presented as a fold difference to that of the control, there was a significant decrease in CB1 expression to 0.29 ± 0.07-fold (mean ± SD) using the uniplex method, and a

significant decrease in CB1 expression to 0.05 ± 0.03-fold using duplex data (Figure 3). The difference in CB1 expression due to the different methodologies was not significantly different ($p > 0.05$), but in both cases the CB1 expression was significantly different ($p < 0.05$) in the malignant tissues.

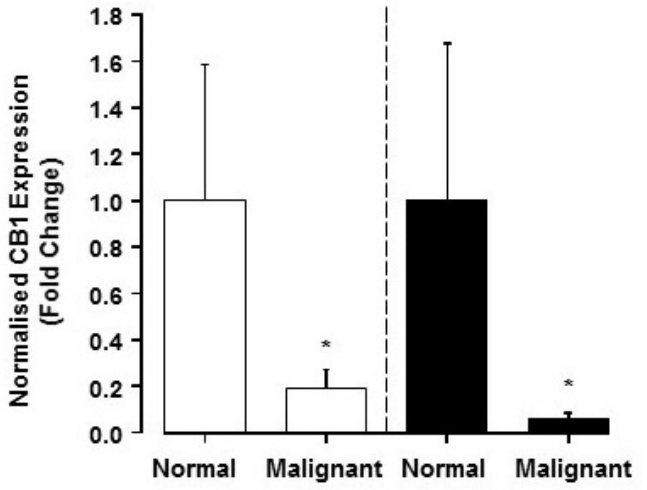

Figure 3: The effect of uniplex and duplex qPCR on CB1 expression in endometrial cancer. The graph shows the expression of CB1 transcripts in endometrial cancer (n=15) compared to that of the normal endometrium (n=9). The data have been normalised to the geometric mean of all three control reference genes and are presented as the mean ± SEM. Unpaired Student's t-test indicated data to be significantly different at *p<0.05 when malignant tissue was compared to normal tissue.

Discussion

In this study, uniplex and duplex techniques using VIC dye-labelled TaqMan® Gene Expression (endogenous control genes) primers and probes, FAM dye-labelled TaqMan® Gene Expression (CB1) primers and probes with TaqMan® universal PCR Master Mix with normal and endometrial cancer samples without preceding optimisation and validation showed that both methodologies produced similar results, suggesting that either technique can be used for such samples.

Multiplex or duplex qPCR has many advantages over uniplex qPCR [11], but it also comes with its own problems [12]. In duplex reactions, the amplification of highly expressed transcript may lead to disproportionate consumption of the master mix components (enzymes, co-factors and dNTPs) and thus may interfere with the detection threshold level of a particular transcript of interest [12]. Therefore, evaluating the optimisation of primer designs and reaction components in a particular duplex study is considered to be essential to obtain good results that are similar to uniplex experiments. To overcome this issue, many studies have resorted to using multiple uniplex approaches, primarily with SyBr green technology [13,14]. This technique, has a number of advantages and disadvantages that have been highlighted previously [14], but the most important consideration maybe the excessive use of the precious mRNA and resulting cDNA, especially when the amount of starting tissue available may be limited through the use of pipelle [15] or curettage [16], techniques often used to obtain endometrial biopsies.

In this study, three TaqMan® gene Expression Assay endogenous controls (MRPL19, PPIA and IPO8) and a gene of interest (CB1) were evaluated in both uniplex and duplex qPCR and found to be very similar. For qRT-PCR duplex assays to be successful, the Ct values generated must be equivalent to those obtained in real-time uniplex PCR [13]. The data presented (Figure 1) show that this can be achieved at the first attempt using the TaqMan® gene expression system where the endogenous control gene probes were VIC labelled and the gene of interest was FAM labelled in the presence of their universal PCR Master Mix. The Ct values obtained in the duplex and uniplex qPCR were within the generally accepted experimental error of 1 – 5% for assays (all were < 2% error). This suggests that 'mixing and matching' of genes of interest, once the levels of endogenous reference gene controls have been established, could easily be produced with the VIC and FAM labelled probes for the different target genes. Indeed, we have used this in other projects (data not shown).

For researchers interested in duplexing, the ability to achieve comparable uniplex and duplex results may be vital for maintaining accurate and reliable results, especially when the amount of sample is limited, as in the case of biopsies from patients with different grades and types of malignancy or when the tissue is particularly small, i.e. during embryonic testing or neonatal biopsy [17]. It is recommended through the MIQE (Minimum Information for publication of Quantitative real time PCR Experiments) [18], that uniplex and duplex reactions need to be validated before they are used routinely, even though this is seldom done in practice. Therefore, it is important that in the first place, the results obtained from duplex reactions should be validated against uniplex reactions setup. The data presented herein demonstrate that even without optimisation, duplex results are produced with reliability similar to that obtained with the uniplex technique.

The relative quantification of CB1 normalised against the endogenous control reference genes MRPL19, PPIA and IP08 *via* both uniplex and duplex techniques showed very similar results (Figure 2). When either MRPL19 or PPIA were used as the endogenous control for CB1 in either uniplex or duplex reactions, statistically significant reductions in CB1 levels were observed, however, although CB1 transcript levels were also reduced in the malignant tissues when normalised against IP08, and also showed similar results between the uniplex and duplex methods, these reductions did not reach statistical significance. The reason for this apparent discrepancy is probably due to the small number of samples examined (n=9 controls and n=15 for malignant tissues) or because a single housekeeping gene may be insufficient for normalisation purposes [19-21]. Indeed, when all three housekeeping genes were combined, whether as uniplex or as duplex data (Figure 3), the expression of CB1

was significantly decreased in the malignant endometrial tissue. These values are similar to those reported by us using only the atrophic uteri of postmenopausal women as our controls (22). The number of patients chosen for this study may be considered a limitation in that only 3 patient samples were chosen from each group, but this was done specifically so that the minimum number of samples for a PCR study would be available and to demonstrate the actual power of studies where samples are limiting. By using a good selection of samples that transcend the whole spectrum of possible controls and possible malignancies, we have ensured that any difference is due to disease rather than due to sampling bias. This is borne out by the analyses of BMI and ages of the patients used because only when the control group was expanded to include premenopausal women, did the difference in age and BMI reach statistical significance (as would be expected [13,14]).

In conclusion, these results support the notion that researchers and biomedical scientists can take advantage of the benefits that TaqMan® duplex qRT-PCR has over standard TaqMan® uniplex qRT-PCR, such as: higher throughput, cost savings in reagents and time, especially where the amount of sample available is of a premium.

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