

Sequence variation in primer targets affects the accuracy of viral quantitative PCR

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Abstract

This study was designed to assess the effect of sequence mismatches between primers and their targets on viral quantitative PCR. Numerous primers were constructed incorporating various mismatches with a target sequence on the BKV T antigen gene. When using these primers in standard Taqman two-step cycling conditions, as few as two mismatches in a single primer increased cycle threshold values and significantly influenced the calculation of viral load.

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1. Text

Quantitative PCR is now routinely used by many laboratories worldwide for a broad range of applications, including gene expression studies and microbial quantification. However, accurate quantification using PCR can be significantly curtailed if appropriate steps are not taken to control for sources of error. Error can be derived from differences in the efficiency of the nucleic acid isolation process, the presence of PCR inhibitors in samples and variation in reverse transcription efficiency in the case of RNA targets (Niesters, 2002; Bustin and Nolan, 2004). In this study, we investigated sequence variation in the primer binding sites as a source of error for quantitative PCR.

A Taqman real-time PCR assay, previously described and targeting the BKV large T antigen gene, was used as the basis for this study (Tag-TM; Hirsch et al., 2001). To assess the effect of primer mismatches on quantitative results, we developed numerous primers consisting of various mismatches to the T antigen gene targets. In total, 18 different

primers were used. These included the original forward and reverse primers published by Hirsch et al. (2001) as well as an additional 16 modified primers, including 14 forward and 2 reverse primers (Table 1). Briefly, each modified primer was developed by substituting a base or bases with a different base, thus creating mismatches with the BKV T antigen targets. Overall, these primers contained between one and five mismatches and were designed to assess the impact of mismatches in the 5' and 3' ends of the primer as well as mismatches spread throughout the primer. The modified primers were developed with the aid of Primer Premier Version 5.0 (Premier Biosoft International, CA, USA) to ensure a similar T_m and to limit the formation of primer secondary structures. All modified primers were the same length as the original respective forward or reverse primers. An attempt was made to maintain the same G/C content in each primer; however, for some primers, this was not possible as would result in the production of 3' secondary structures.

Primers pairs were tested in various combinations (Table 2) using standard two-step Taqman cycling conditions. Briefly, PCR amplification and detection was performed using a 25 µL reaction volume containing 12.5 µL of Taqman Universal PCR Master Mix (Applied Biosystems,

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Table 1
Primers used in the study

Designation	Sequence (5'–3')	Reference
Forward primers		
Hirsch-BK-F	GAAGCAACAGCAGATTCTCAACA	Hirsch et al. (2001)
BK-F1	GAAGCAACAGCAGATTCTCAACG	This study
BK-F2	GAAGCAACAGCAGATTCTCAAGA	This study
BK-F3	GAAGCAACAGCAGATTCTCACC	This study
BK-F4	GAAGCAACAGCAGATTCTCTACA	This study
BK-F5	GAAGCAACAGCAGATTCTGAACA	This study
BK-F6	GAAGCAACAGCAGATTCTTGACA	This study
BK-F7	GAAGCAACAGCAGATTCTTCCA	This study
BK-F8	GAAGCAACAGCAGATTCTCACA	This study
BK-F9	GAAGCAACAGCAGATTCTCAATG	This study
BK-F10	GAAGCAACAGCAGATTCTCACAT	This study
BK-F11	TTCCGAACAGCAGATTCTCAACA	This study
BK-F12	TTCCGAACAGCAGATTCTCAACA	This study
BK-F13	GAAGCAAGAGCAGATTGTCAACA	This study
BK-F14	GAACCAAGAGCAGATTGTCAACA	This study
Reverse primers variations		
Hirsch-BK-R	AGCAGGCAAGGGTTCTATTACTAAAT	Hirsch et al. (2001)
BK-Rev1	AGCAGGCAACGGTTATATTACTAAAT	This study
BK-Rev2	AGCACGCAACGGTTATATTACTAAAT	This study

Underlined bases indicate mismatches with the BKV DNA.

Australia), 0.4 μ M of forward and reverse primers, 0.16 μ M of Taqman probe (fam-AAGACCCTAAAGACTTTCCCTCTGATCTACACCAGTTT-bhq1; Hirsch et al., 2001) and BKV DNA extracted from a suspension of BKV virus (ATCC 45024). Four 10-fold dilutions of BKV DNA ranging from 100,000 to 100 copies per reaction were tested in duplicate using each primer pair. Amplification and de-

tection were performed on an ABI7500 instrument (Applied Biosystems, USA) with the following conditions: initial hold at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. It should be noted that the primer concentration used in the PCR reactions was the optimal primer concentration for the original primers and that the modified primers may have different optimal concentrations. Nevertheless, the

Table 2
Forward and reverse primers combinations used to test the BKV dilutions

Primers forward	Reverse	Average C_t value and calculated copy number ^a				Amplification efficiency ^b
		Dilution 1 (10E5)	Dilution 2 (10E4)	Dilution 3 (10E3)	Dilution 4 (10E2)	
Hirsch-BK-F	Hirsch-BK-R	21.14 (1.0 × 10E5)	24.87 (1.0 × 10E4)	27.89 (1.0 × 10E3)	31.25 (1.0 × 10E2)	1.99
BK-F1	Hirsch-BK-R	21.16 (1.1 × 10E5)	24.82 (8.7 × 10E3)	27.75 (1.2 × 10E3)	31.18 (1.1 × 10E2)	2.00
BK-F2	Hirsch-BK-R	21.78 (7.1 × 10E4)	25.29 (6.3 × 10E3)	28.43 (7.2 × 10E2)	31.61 (7.9 × 10E1)	2.03
BK-F3	Hirsch-BK-R	21.03 (1.2 × 10E5)	24.55 (1.0 × 10E4)	27.80 (1.1 × 10E3)	31.17 (1.1 × 10E2)	1.99
BK-F4	Hirsch-BK-R	21.02 (1.2 × 10E5)	24.53 (1.1 × 10E4)	27.61 (1.3 × 10E3)	31.11 (1.1 × 10E2)	1.99
BK-F5	Hirsch-BK-R	21.07 (1.2 × 10E5)	24.51 (1.1 × 10E4)	27.90 (1.0 × 10E3)	31.03 (1.2 × 10E2)	2.00
BK-F6	Hirsch-BK-R	21.34 (9.6 × 10E4)	25.07 (7.3 × 10E3)	28.55 (6.6 × 10E2)	31.67 (8.1 × 10E1)	1.95
BK-F7	Hirsch-BK-R	24.32 (1.2 × 10E4)	28.17 (8.6 × 10E2)	31.10 (1.2 × 10E2)	34.92 (8.3 × 10E0)	1.94
BK-F8	Hirsch-BK-R	29.35 (3.7 × 10E2)	32.80 (3.6 × 10E1)	36.11 (3.6 × 10E0)	39.70 (3.1 × 10E–1)	1.96
BK-F9	Hirsch-BK-R	29.86 (2.7 × 10E2)	33.05 (3.0 × 10E1)	36.67 (2.4 × 10E0)	40.50 (2.1 × 10E–1)	1.91
BK-F10	Hirsch-BK-R	29.40 (3.7 × 10E2)	32.79 (3.5 × 10E1)	36.03 (3.7 × 10E0)	40.10 (2.3 × 10E–1)	1.92
BK-F11	Hirsch-BK-R	20.99 (1.2 × 10E5)	24.30 (1.2 × 10E4)	27.68 (1.2 × 10E3)	31.37 (9.4 × 10E1)	1.95
BK-F12	Hirsch-BK-R	21.42 (1.0 × 10E5)	24.92 (9.1 × 10E3)	28.34 (8.6 × 10E2)	31.55 (9.5 × 10E1)	1.98
BK-F13	Hirsch-BK-R	23.98 (1.7 × 10E4)	27.65 (1.4 × 10E3)	30.96 (1.4 × 10E2)	34.70 (1.1 × 10E1)	1.92
BK-F14	Hirsch-BK-R	28.48 (7.8 × 10E2)	32.64 (4.4 × 10E1)	36.08 (4.1 × 10E0)	39.82 (3.1 × 10E–1)	1.85
Hirsch-BK-F	BK-Rev1	26.37 (3.3 × 10E3)	29.98 (2.7 × 10E2)	33.40 (2.8 × 10E1)	37.10 (2.1 × 10E0)	1.91
Hirsch-BK-F	BK-Rev2	34.69 (1.0 × 10E1)	38.36 (8.0 × 10E–1)	42.67 (4.5 × 10E–2)	48.27 (1.1 × 10E–3)	1.67
BK-F13	BK-Rev1	28.80 (6.3 × 10E2)	32.65 (4.4 × 10E1)	35.87 (4.8 × 10E0)	39.16 (4.9 × 10E–1)	1.96
BK-F14	BK-Rev2	41.85 (8.0 × 10E–1)	45.87 (7.7 × 10E–3)	ND	ND	1.75

Overall, there was considerable variation in the C_t values, amplification efficiencies and copy numbers obtained using the different primer combinations; ND: not detected.

^a The Hirsch-F and Hirsch-R primer pair was used to generate the standard curve used for calculating the copy numbers for each BKV dilution obtained using the other primer pairs.

^b The amplification efficiency for each primer pair was calculated independently of the results obtained from other primer pairs and was calculated using the equation; amplification efficiency = 10E–1/slope.

same concentration was used for all primers so as not to introduce another variable in the experiments.

Following PCR amplification, the average cycle threshold (C_t) value was determined for each concentration of BKV target using each primer pair. Also, the amplification efficiency for each primer pair was calculated using the formula $10E-1/\text{slope}$ (Table 2). Further, the unmodified primer pair combination (Hirsch-F and Hirsch-R; Table 1) was used to generate a standard curve, which was then used to calculate the copy numbers for each BKV dilution obtained using the other primer pairs (Table 2). These copy numbers were then compared to the actual copy numbers added to each reaction.

Overall, there was considerable variation in the C_t values, amplification efficiencies and copy numbers obtained using the different primer combinations. A single mismatch in the 3' end of the forward primer (Primers BK-F1 to BK-F5; Table 1) had little impact on the accuracy of the assay irrespective of the position of the mismatch (Table 2). Two mismatches in the 3' end (primers BK-F6 to BK-F9; Table 1) introduced considerable error, particularly where the mismatches comprised two of the three terminal bases. In fact, the use of forward primers BK-F8 and BK-F9 increased C_t values by more than eight cycles compared to the unmodified forward primer and calculated copy numbers were 2–3 logs less than the actual number of copies of BKV added to each reaction (Table 2). Similar erroneous values were obtained with three mismatches in the extreme 3' end of the forward primer (BK-F10; Tables 1 and 2). In contrast, up to five consecutive mismatches in the 5' end of the forward primer had no discernable effect on viral quantification or amplification efficiency (primers BK-F11 and BK-F12; Tables 1 and 2).

Mismatches that were spread across the primer but were away from the 3' end also affected quantification. Two mismatches along a single primer delayed amplification by three to five cycles and underestimated viral load by 1–2 logs (primer combinations BK-F13/Hirsch-BK-R and Hirsch-BK-F/BK-Rev1; Tables 1 and 2). Three mismatches along a single primer delayed amplification by 7–13 cycles and underestimated viral load by 2–4 logs (primer combinations BK-F14/Hirsch-BK-R and Hirsch-BK-F/BK-Rev2; Tables 1 and 2). The introduction of error was additive, where both the forward and reverse primers contained mismatches. For example, C_t values were increased by approximately 20 cycles and viral load was underestimated by about 5 logs, where both the forward and reverse primers contained three mismatches (primer combination BK-F14/BK-Rev2; Table 2). A similar additive effect was noted, where both the forward and reverse primers contained two mismatches (primer combination BK-F13/BK-Rev1; Table 2).

Interestingly, the mismatches in the primers had little impact on the quality of amplification curves. In fact, all primer combinations except those including primers BK-F11 or BK-F12 provided similar linear amplification curves for all BKV dilutions, even where the quantitative values were grossly underestimated (Fig. 1). In contrast, the linear amplification curves obtained using forward primers BK-F11 and BK-F12

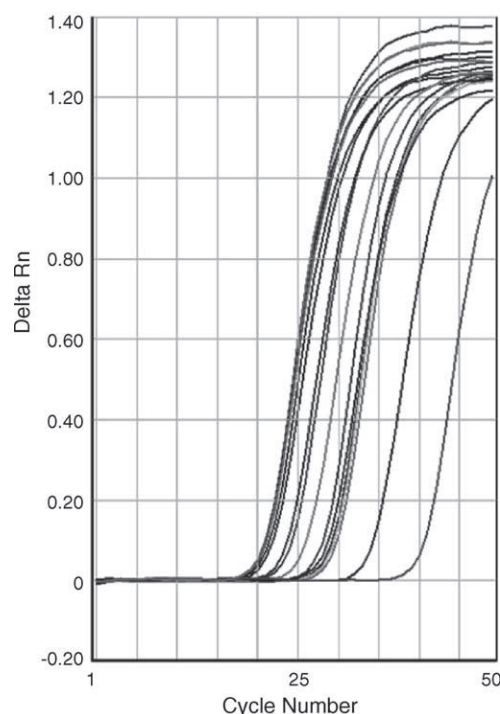


Fig. 1. Quantification curves for all primer combinations except those including primers 5K-F11 and BK-F12. Target concentration was $10E5$ /reaction.

deviated from those of the unmodified primers (Fig. 2); however, the quantitative results obtained using these primers remained accurate (Table 2).

Unexpectedly, several of the BKV dilutions provided amplification curves exceeding cycle 40. Theoretically, such high C_t values are indicative of an initial concentration at or below one copy per reaction, thus specimens providing such C_t values are generally viewed with caution. However, the lowest concentration of BKV used in this study was 100 copies per reaction. This shows that mismatches in the primer binding sites can produce reliable amplification past cycle 40. Therefore, any specimens repeatedly providing such high C_t values may warrant further investigation to determine if sequence variation exists in primer targets.

Overall, the above results show that sequence mismatches in the primer targets can introduce significant error into quantitative PCR. This has considerable implications for viral quantification studies, particularly where the target virus exhibits considerable sequence variation between strains and subtypes. Compounding this problem is that sequence information on databases such Genbank is often limited and not sufficiently representative of the sequence variation associated with the strains and subtypes of some viruses. This considerably limits the ability to identify highly conserved primer and probe targets for quantitative PCR.

The results also show that error derived from sequence variation may be difficult to detect. Visual inspection of the quantification curves provided no indication that the quantitative data was erroneous (Fig. 1). In addition, some primer

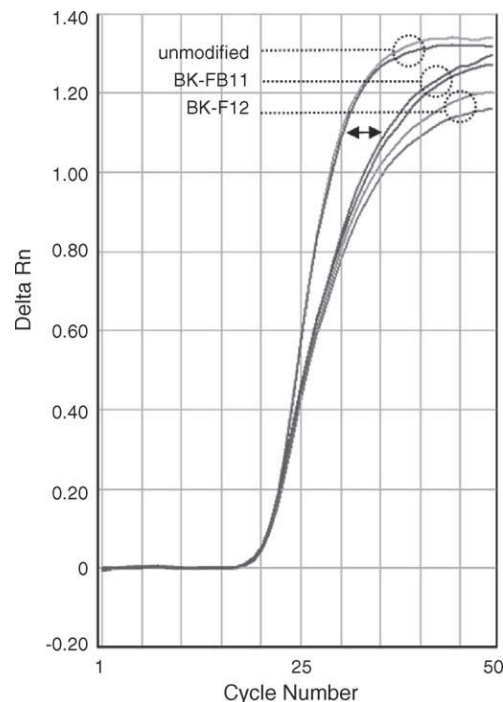


Fig. 2. Quantification curves for primer combinations Hirsch-BK-F/Hirsch-BK-R, BK-F11/Hirsch-BK-R and BK-F12/Hirsch-BK-R. Primers BK-F11 and BK-F12 provided similar C_t values to the unmodified forward primer but linear amplification phases deviated. Target concentration was $10E5$ /reaction.

combinations (BK-F8/Hirsch-BK-R and BK-F13/BK-Rev1) provided high amplification efficiencies even though the quantitative data were underestimated by more than 2 logs (Table 2). Thus, testing each specimen in a dilution series and comparing the amplification efficiency with that of the standard may not reliably detect the effects of sequence variation. Sequencing the primer targets and determining if mutations are present would be more reliable, though this is time consuming, would involve considerable expense and would not indicate the extent of error if mismatches were identified. We have found it useful to test specimens in duplicate using two separate quantitative assays targeting different genes. The C_t values provided by the assays are then compared to identify if discrepancies exist.

It should be noted that numerous other factors might influence the overall impact of primer mismatches on the accuracy of quantitative PCR. These include the T_m and nucleotide composition of the primer, the PCR annealing temperature, the reaction mix composition and the particular nucleotides that are subject to mismatch. Therefore, the above results may only be representative of PCR assays using similar protocols. In fact, we recently showed that a single base mismatch in the extreme 3' end of a primer introduced an error of approximately 3 logs for a local BKV strain in one of our three-step real-time BKV PCR assays (data not published). In that assay, the particular design and T_m of the primer may have compounded the effect of the sequence mismatch. In any event, such results differ considerably from the single mismatch results presented in this study and show that the effects of sequence variation in the primer targets are variable. Notwithstanding the findings presented here, we have found the Tag-TM assay to be reliable for quantifying BKV DNA. To date, we have not found sequence variation in the Tag-TM primer targets of any local BKV strains and this assay was only chosen to investigate the effects of sequence variation because our laboratory's interest in the quantification of BKV virus.

In summary, our results indicate that sequence variation in primer targets can have a negative impact on the accuracy of viral quantitative PCR. In particular, primers containing two or more mismatches at or near the 3' end of the primer can significantly underestimate viral load when using standard Taqman two-step cycling conditions, whereas mismatches at the 5' end are less likely to introduce error.

References

- Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J Biomol Tech* 2004;15:155–66.
- Hirsch HH, Mohaupt M, Klimkait T. Prospective monitoring of BK virus load after discontinuing sirolimus treatment in a renal transplant patient with BK virus nephropathy. *J Infect Dis* 2001;184: 1494–5.
- Niesters HG. Clinical virology in real time. *J Clin Virol* 2002;25:3–12.