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Design and storage stability of reference materials for microfluidic quantitative PCR-based equine gene doping tests

Teruaki TOZAKI[1][#]*, Aoi OHNUMA[1][#], Mio KIKUCHI[1], Taichiro ISHIGE[1], Hironaga KAKOI[1], Kei-ichi HIROTA[1], Kanichi KUSANO[2] and Shun-ichi NAGATA[1]

[1]Genetic Analysis Department, Laboratory of Racing Chemistry, Tochigi 320-0851, Japan
[2]Equine Department, Japan Racing Association, Tokyo 106-8401, Japan

One method of gene doping in horseracing is administering of exogenous genetic materials, known as transgenes. Several polymerase chain reaction (PCR)-based methods have been developed for detecting transgenes with high sensitivity and specificity. However, novel designs for reference materials (RMs) and/or positive template controls (PTCs) are necessary for simultaneous analysis of multiple transgene targets. In this study, we designed and developed a novel RM for simultaneously detecting multiple targets via microfluidic quantitative PCR (MFQPCR). Twelve equine genes were selected as targets in this study. A sequence region including primers and probes for quantitative PCR was designed, and a 10 bp sequence was inserted to allow the RM to be distinguished from the original transgene sequences. The sequences of individual detection sites were then connected for 12 genes and cloned into a single plasmid vector. We performed fragment size analysis to distinguish between the PCR products of the original transgene sequence and those of the RM, enabling identification of RM contamination. PTCs diluted to 10,000, 1,000, 100, and 10 copies/μl with horse genomic DNA from RM were stably stored at 4°C for 1 year. As digital PCR enabled absolute quantification, the designed substances can serve as an RM. These findings indicate that the RM design and storage conditions were suitable for gene doping tests using MFQPCR.
Key words: *gene doping, microfluidic quantitative polymerase chain reaction, positive template control, reference material, transgene*

The International Federation of Horseracing Authorities (https://www.ifhaonline.org/) has updated the International Agreement on Breeding, Racing, and Wagering to prohibit gene doping [18]. Gene doping in horseracing is classified as the administration of gene doping substances to postnatal animals or the generation of genetically modified racehorses by editing of zygotes. One method in the former category involves the administration of an exogenous gene, known as a 'transgene', to postnatal horses [21].

Polymerase chain reaction (PCR)-based detection

methods have been developed to detect transgenes in human and equine sports [3, 7, 16, 19]. Quantitative PCR methods using a hydrolysis probe can specifically detect administered transgenes cloned into plasmids or adeno-associated virus vectors [9, 21]. Recently, the World Anti-Doping Agency published laboratory guidelines for PCR-based detection of transgenes for gene doping control in human sports [25]. A necessary step in developing a gene doping test is method validation [5, 8, 11], for which a reference material (RM) and/or positive template control (PTC) are required [4, 26]. RM is defined as a material that is sufficiently homogeneous and stable with respect to one or more specified properties and has been established to be suitable for its intended use in a measurement process, whereas a PTC is a substance diluted from the RM.

In a gene doping test, a PTC constructed from a diluted RM should be used. When detecting a single transgene, the full gene sequence should be cloned into a plasmid vector to establish an RM, which should then be diluted for use as

a PTC [21]. However, when targeting multiple transgenes simultaneously, preparing an RM or PTC individually is complicated. We recently developed a simultaneous detection method for 12 transgenes (24 assays in total) using microfluidic quantitative PCR (MFQPCR) as a gene doping test for use in horseracing [19]. However, a novel RM is required for simultaneous analysis of multiple targets.

An advantage of PCR-based detection methods is their high sensitivity [16]. Analysis of the PTC is necessary for confirmatory detection of gene doping [25]. However, PCR-based detection methods have a potential risk of false positives due to PTC contamination [14, 17], as PCR theoretically enables the amplification of a single copy. In this study, we developed an RM for MFQPCR-based gene doping tests that simultaneously detects multiple target genes. We also examined the contamination risk associated with the developed RM and its long-term storage stability.

Materials and Methods

Ethical considerations

Blood samples were collected from Thoroughbreds at the Miho and Ritto Training Centers and Equine Research Institute of the Japan Racing Association after obtaining approval from the Animal Care Committee of the Laboratory of Racing Chemistry (Utsunomiya, Tochigi, Japan, approval number 20-4).

Blood collection and DNA extraction

Blood was collected from 264 Thoroughbreds into BD Vacutainer spray-coated K_2EDTA tubes (BD Biosciences, Franklin Lakes, NJ, U.S.A.). Plasma was separated by centrifugation at $1,500 \times g$ for 10 min. The separated plasma samples were stored at $-30°C$. The separated plasma samples from the 264 horses were used for MFQPCR detection. Among these samples, two plasma samples (1.5 m*l*) were spiked with either 150 or 1,500 copies of Control_A_MSTN as positive detection models (Table 1), and two samples were spiked with either 150 or 1,500 copies of Control_C_SET1 as contamination models of PTCs (Table 1). DNA was extracted from the 1.5 m*l* plasma samples using a Custom NEXTprep cfDNA Auto Kit (1.5 m*l*; PerkinElmer, Waltham, MA, U.S.A.) with a chemagic 360 instrument (PerkinElmer). The extract was dissolved in Milli-Q water to a final volume of 50 μl.

Target genes

The following 12 equine genes were selected as targets: creatine kinase, muscle (*CKM*), erythropoietin (*EPO*), fibroblast growth factor 2 (*FGF2*), follistatin (*FST*), growth hormone 1 (*GH1*), insulin-like growth factor 1 (*IGF1*), myostatin (*MSTN*), phosphoenolpyruvate carboxykinase

Table 1. Preparation of spiked samples for microfluidic quantitative PCR detection

Spiked sample	Content of plasma (m*l*)	Content of spiked control
Sample 1	1.5	150 copies of Control_A_MSTN
Sample 2	1.5	1,500 copies of Control_A_MSTN
Sample 3	1.5	150 copies of Control_C_SET1
Sample 4	1.5	1,500 copies of Control_C_SET1
Samples 5–264	1.5	None

Samples 1 and 2: positive detection models of single transgene. Samples 3 and 4: contamination models of positive template controls.

1 (*PCK1*), pyruvate dehydrogenase kinase 4 (*PDK4*), peroxisome proliferator activated receptor delta (*PPARD*), vascular endothelial growth factor (*VEGF*), and zinc finger and AT-hook domain containing (*ZFAT*). These genes were the same as those used in our previous study [19].

Design of primers and probes for transgene detection

We used the pre-amplification primers and quantitative PCR primers and probes designed in our previous study, with minor modifications [19]. As shown in Fig. 1A, each gene had two primer/probe sets, SET1 and SET2. Forward and reverse primers targeting different exons and TaqMan-MGB probes targeting exon/exon junctions were designed and synthesised. Finally, 12 primer-probe sets for SET1 detections and 12 primer-probe sets for SET2 detections were prepared and used for the 12 transgenes. The SET1 assay for the *MSTN* transgene was labelled as MSTN_SET1; this labelling system was used for each gene. The designed probes and primers have not been listed, as this may prevent their use in actual gene-doping tests. Sequence information of the probes and primers will be provided through a confidentiality agreement with the corresponding author.

Design of reference materials

RM Control_A, with an open reading frame (DNA sequence with exons aligned without gaps) and untranslated region, was prepared as described in our previous study [19] for each of the 12 transgenes. The RM for the *MSTN* transgene was labelled as Control_A_MSTN; this labelling system was used for each gene.

Control_C_SET1 and Control_C_SET2 were novel RMs designed in this study. They contained only the detection site of each transgene between the forward and reverse primers used for pre-amplification. Additional 10-bp sequences were inserted into the region between the qPCR probe and primer sites (Fig. 1B). Sequences designed to enable the individual detection of each gene were then connected. These sequences were synthesised by Fasmac (Atsugi, Japan) and cloned into pUCFa (r-Amp$^+$, ColE1_ori$^+$). The
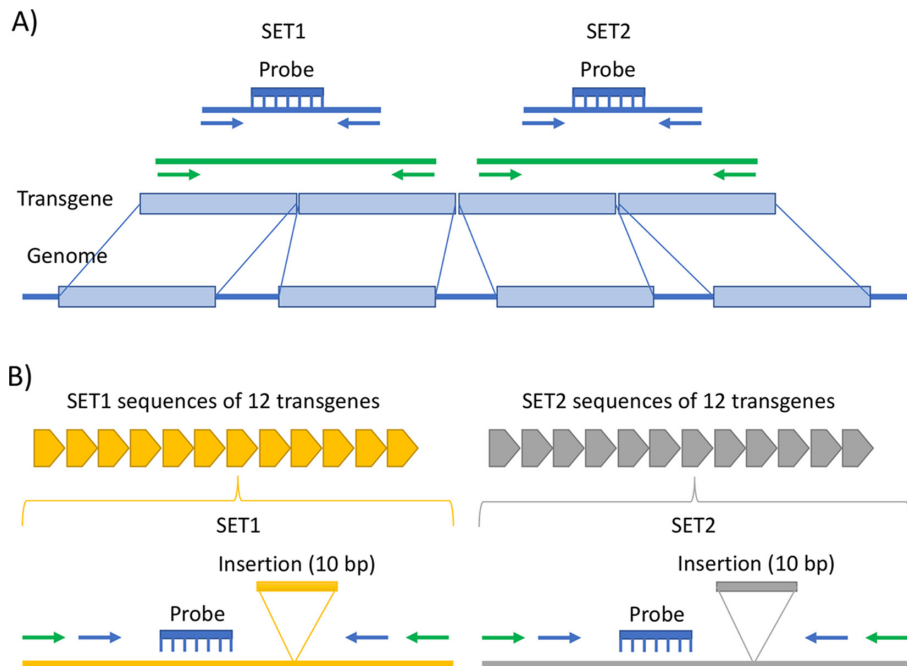
Fig. 1. Design of reference materials for gene doping tests. Each gene had two detection sites, SET1 and SET2. Primers for pre-amplification (green arrows), primers for quantitative detection (blue arrows), and a hydrolysis probe (blue bar) were designed (A). Reference materials were constructed for 12 transgenes, and SET1 and SET2 were designed separately. The detection sites for each transgene were connected into one sequence and then cloned into a plasmid. The detection sites included an additional 10-bp sequence (B).

cloned plasmid was transformed into JM109 competent cells (Takara Bio, Kusatsu, Japan) and cultured in LB medium (Amp$^+$). The plasmids were extracted from the transformed JM109 cells, purified using a Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, U.S.A.), and dissolved in Milli-Q water. The cloned sequences were confirmed by Sanger sequencing.

Storage and dilution of positive template controls

Purified Control_C_SET1 and Control_C_SET2 were diluted to 5, 10, 30, 100, 1,000, and 10,000 copies in 2.75 μl of Milli-Q water with 10 ng/μl of equine genomic DNA. The copy concentration was measured and adjusted using digital PCR, as described below. These samples were used as PTCs for MFQPCR detection.

In addition, 10, 100, 1,000, and 10,000 copies/μl of the PTCs in Milli-Q water with or without 10 ng/μl of equine genomic DNA were prepared and stored at 4°C for 1 year and used for storage stability experiments using digital PCR, as described below.

MFQPCR detection

MFQPCR detection was performed under previously described conditions with minor modifications [19]. Pre-amplification was conducted using 2.75 μl of sample solution (6 diluted PTCs and extracts from 86 unspiked plasmas and 2 plasmas spiked with Control_A in Fig. 2A; 6 diluted PTCs and extracts from 88 unspiked plasmas in Fig. 2B; and 6 diluted PTCs and extracts from 86 unspiked plasmas and 2 plasmas spiked with Control_C in Fig. 2C) with pre-amplification primer pools for SET1 and SET2. The PCR conditions for pre-amplification were the same as those used in our previous study, except for the number of PCR cycles, which was 14 in this study. MFQPCR was performed under the conditions described in a previous study [19], except with a different sample volume, using a 192.24 Dynamic Array IFC for Gene Expression (Fluidigm, South San Francisco, CA, U.S.A.). In this study, undiluted pre-amplified PCR products were used as samples for MFQPCR detection. All qPCR operations and data analyses were conducted using the Biomark Data Collection software and Real-Time PCR Analysis software (Fluidigm), respectively. Two NTCs (no template controls) were prepared for quality control.

Fragment analyses

A 15 μl reaction sample containing 2.5 μl of diluted Control_A and Control_C, 1.5 μl of 10X PCR buffer (Mg^{2+}-free; Takara Bio), 0.9 μl of 25 mM MgCl$_2$ solution (Takara Bio), 1.2 μl of 2.5 mM dNTP (Takara Bio), and 0.075 μl of TaKaRa Taq (5 U/μl, Takara Bio) was amplified using GeneAmp PCR System 9700 (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Enzyme activation was performed

at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. After a final extension at 72°C for 10 min, the amplicons were stored at 4°C until analysis.

Fragment patterns were determined using a DNA 1K Reagent Kit assay with the DNA Extended Range LabChip of LabChip GX Touch 24 (PerkinElmer) in accordance with the manufacturer's recommended procedure. Each marker was measured 6 times, and then means and standard deviations were calculated. An NTC was prepared for quality control.

Digital PCR analysis

Digital PCR analysis was performed according to the conditions described in our previous study [21]. A 22-*µl* reaction sample was prepared that contained 2.2 *µl* of

sample solution (Control_C diluted to 10,000, 1,000, 100, and 10 copies), 11 *µl* of 2X digital PCR Supermix (no dUTP; Bio-Rad, Hercules, CA, U.S.A.), 0.2 *µl* of 100 *µ*M forward primer, 0.2 *µl* of 100 *µ*M reverse primer, and 0.6 *µl* of 10 *µ*M TaqMan-MGB probe. A 20-*µl* droplet was created using an automated droplet generator (Bio-Rad) and then PCR amplified using a T100 thermal cycler (Bio-Rad). Enzyme activation was conducted at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 sec and annealing/extension at 60°C for 1 min. The amplicons were stored at 12°C after 10 min of enzyme deactivation at 98°C. DNA concentrations were measured in the samples using a QX200 droplet reader (Bio-Rad). An NTC was prepared for quality control. The linearity of diluted Control_C (10,000, 1,000, 100, and 10 copies) was calculated using the least-squares method.
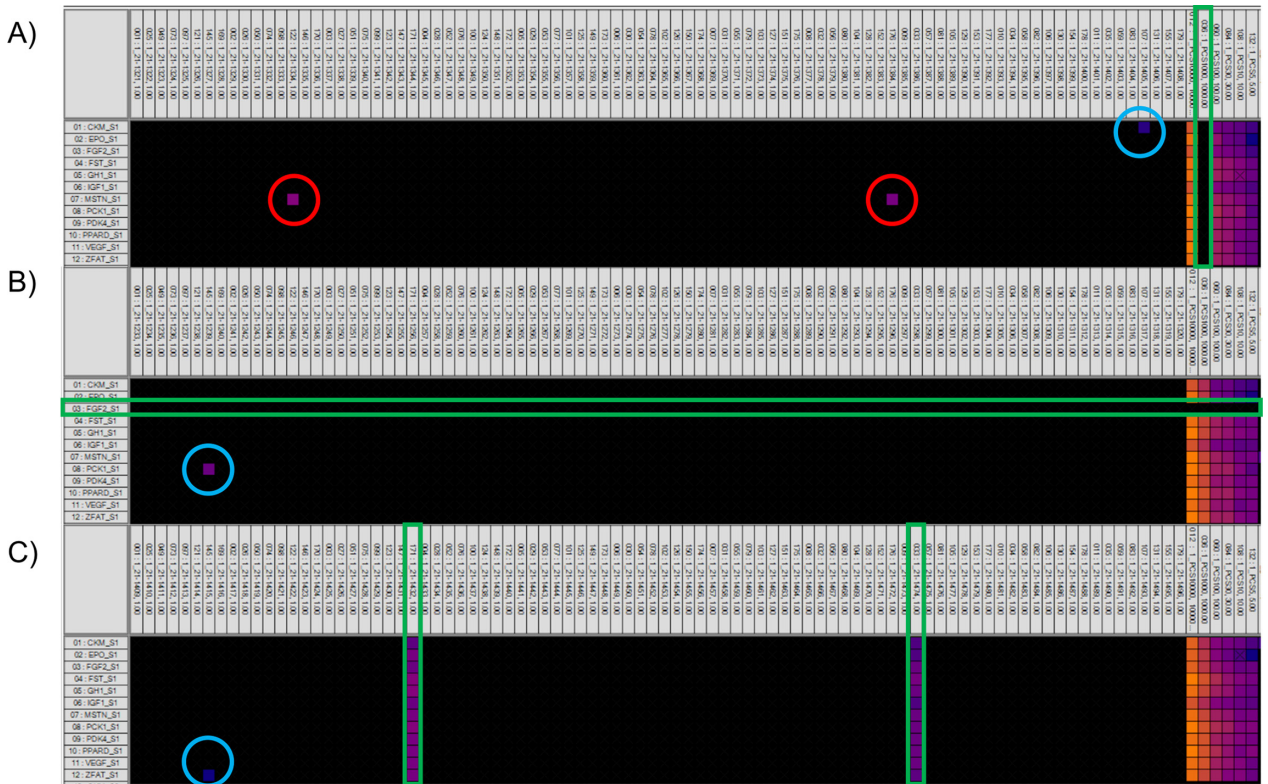

Fig. 2. MFQPCR SET1 assay using the newly designed Control_C_SET1 for simultaneous transgene detection. The vertical axes in each section (A–C) represent SET1 assays of *CKM*, *EPO*, *FGF2*, *FST*, *GH1*, *IGF1*, *MSTN*, *PCK1*, *PDK4*, *PPARD*, *VEGF*, and *ZFAT*. The horizontal axes represent the equine plasma samples (88 samples each) and Positive template controls (PTCs). The right side of the horizontal axes represents PTCs (10,000, 1,000, 100, 30, 10, and 5 copies). Orange indicates a low cycle threshold (Ct) value (high copy concentrations). Dark purple indicates a high Ct value (low copy concentrations). Black indicates non-amplification. Two plasma samples were spiked with Control_A_MSTN as positive detection models of a single transgene (red circles, samples 1 and 2 in Table 1), which was detected by microfluidic quantitative PCR (A). Delivery errors of PTC (1,000 copies) and FGF2 assay solution are shown as green rectangles (A and B). Two plasma samples were spiked with Control_C_SET1 (green rectangles, samples 3 and 4 in Table 1) as models of PTC sample contamination (C). Blue circles represent non-specific amplification (A–C).

Results

Detection of RMs via MFQPCR analysis

MFQPCR analysis indicated that 5, 10, 30, 100, 1,000, and 10,000 copies of Control_C_SET1 and Control_C_SET2 as PTCs were effectively amplified and detected in the 24 assays (12 genes × 2 assays [SET1 and SET2]), whereas extracts from unspiked plasma were not amplified.

In addition, amplification signals in samples spiked with Control_A_MSTN (150 or 1,500 copies in 1.5 m*l* plasma) were detected in both the MSTN_SET1 and MSTN_SET2 assays. The red circles in Fig. 2A indicate the signals detected in the MSTN_SET1 assay, whereas the blue circles in Fig. 2 indicate the three weak signals observed in the SET1 assay. No amplification signals were observed in the same samples via the SET2 assay, suggesting that the weak amplification signals were non-specific signals.

Among the large number of MFQPCR analyses performed, several errors in liquid transfer were observed in the integrated fluidic circuits (IFCs; Fig. 2A, 2B). When an IFC liquid transfer error occurs, no chambers in the same row produce signals. As shown in Fig. 2A (green rectangle), Control_C_SET1 at 1,000 copies failed to produce an amplification signal in any of the SET1 assays. As shown in Fig. 2B (green rectangle), no PTCs produced amplification signals in any of the FGF2_SET1 assays. In contrast, as shown in Fig. 2C (green rectangle), all SET1 assays of samples spiked with 150 or 1,500 copies of Control_C_SET1 produced positive results, indicating contamination.

Discrimination between true-positive samples and RMs

Using forward and reverse primers for MFQPCR analysis, the sizes of the PCR products amplified from the Control_A and Control_C templates were compared using a LabChip GX Touch 24. PCR products from Control_A and Control_C were differentiated by the insertion sequence of 10 bp for all markers (Fig. 3, Table 2). The assays distinguished between the amplification product lengths of Control_A as gene-doping substances and Control_C.

PTC stability

The concentrations of Control_C_SET1 and Control_C_SET2 were adjusted to 10,000, 1,000, 100, and 10 copies/μl and refrigerated for 1 year. Concentration was measured by digital PCR using SET1 and SET2 assays for *CKM*, *FST*, *MSTN*, and *PPARD*. The mean of four measurements was considered as the concentration value.

Control_C_SET1 and Control_C_SET2 diluted with 10 ng/μl genomic DNA were stably detected for 1 year. However, the quantitative values of Control_SET1 and Control_SET2 diluted with Milli-Q water tended to decrease after 6 months in samples diluted to 1,000, 100, and 10 copies/μl (Fig. 4).

The coefficient of variation (CV) of each sample at each time point was calculated for each of the four concentrations (Fig. 5). The CVs of 10,000, 1,000, and 100 copies/μl reached approximately 20%, whereas that of 10 copies/μl reached 36%, suggesting that quantification of low copy numbers was difficult (Fig. 5). In addition, at 10 copies/μl, the CV of Control_C diluted in genomic DNA solu-
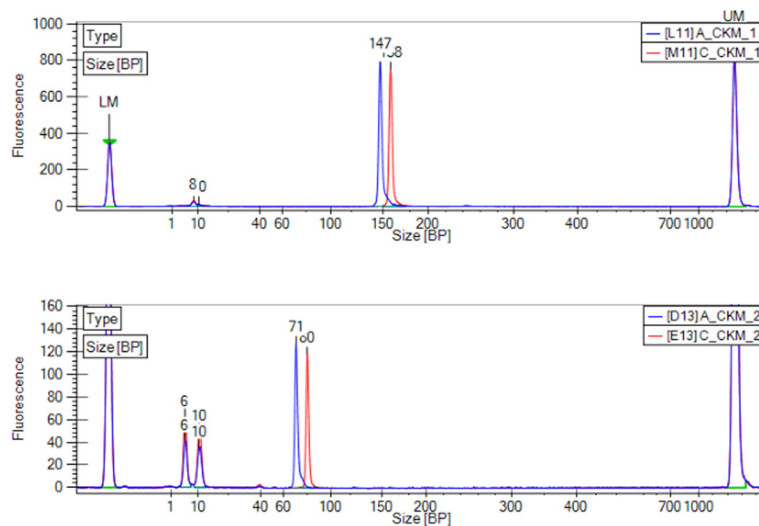

Fig. 3. Electrophoretic images of Control_A and Control_C amplified by PCR via a LabChip GX Touch 24. *CKM* was amplified using quantitative polymerase chain reaction (PCR) primers. The upper and lower charts present the results for SET1 and SET2, respectively. Blue represents Control_A, and red represents Control_C.

Table 2. Size discrimination between Control_A and Control_C by LabChip24

| | SET1 | | | | SET2 | | | |
| | Control_A | | Control_C | | Control_A | | Control_C | |
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CKM	148.1	0.31	158.1	0.35	69.8	0.73	78.2	0.41
EPO	115.2	0.25	127.2	0.44	126.9	0.29	136.1	0.26
FGF2	87.2	0.20	95.8	0.13	83.9	0.27	91.4	0.13
FST	72.3	0.08	81.6	0.16	109.3	0.38	120.0	0.45
GH1	107.5	0.50	116.7	0.32	110.6	1.49	116.5	1.85
IGF1	126.9	0.50	139.9	0.87	83.3	1.91	92.8	0.26
MSTN	75.2	0.16	85.8	0.82	78.9	0.13	88.5	0.11
PCK1	82.5	0.31	93.0	0.34	98.8	0.13	108.7	0.13
PDK4	77.3	0.34	85.7	0.39	77.7	0.30	86.2	0.15
PPARD	94.7	0.16	104.3	0.26	89.3	0.10	99.5	0.22
VEGF	72.0	0.26	82.2	0.12	82.8	0.57	90.8	0.35
ZFAT	82.5	0.29	93.6	0.59	118.9	0.21	130.3	0.60

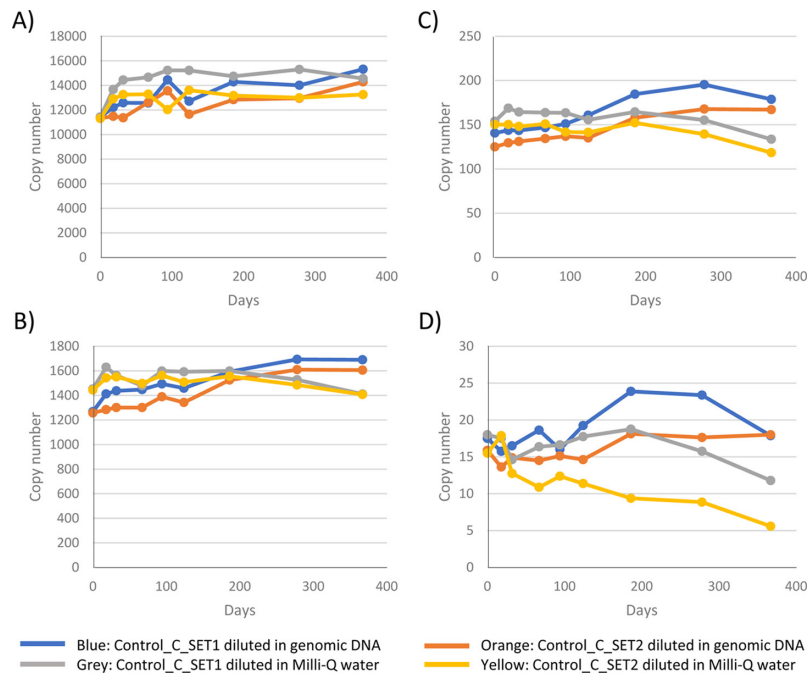Unit: base pairs (bp). SD: standard deviation.

Fig. 4. Quantitative values of Control_C over the course of 1 year. Positive template control (PTC) solutions of 10,000 (A), 1,000 (B), 100 (C), and 10 (D) copies were quantified over the course of 1 year. Blue, Control_C_SET1 diluted in genomic DNA; orange, Control_C_SET2 diluted in genomic DNA; grey, Control_C_SET1 diluted in Milli-Q water; yellow, Control_C_SET2 diluted in Milli-Q water.

tion ranged from 8.5% to 25%, whereas that of Control_C diluted in Milli-Q water ranged from 5.8% to 36%. Notably, the CVs at the last two measured time points were large, indicating that variation gradually increased over time for Control_C diluted in Milli-Q water (Fig. 5).

The linearity of diluted Control_C was calculated immediately after sample preparation and at 1, 3, 6, and 12 months (Fig. 6). The linearity of all Control_C samples was generally maintained (R^2: 0.999–1.0000), excluding Control_C_SET2 diluted in Milli-Q water (R^2=0.9983 at 3 months, R^2=0.9962 at 6 months, and R^2=0.9949 at 12 months).
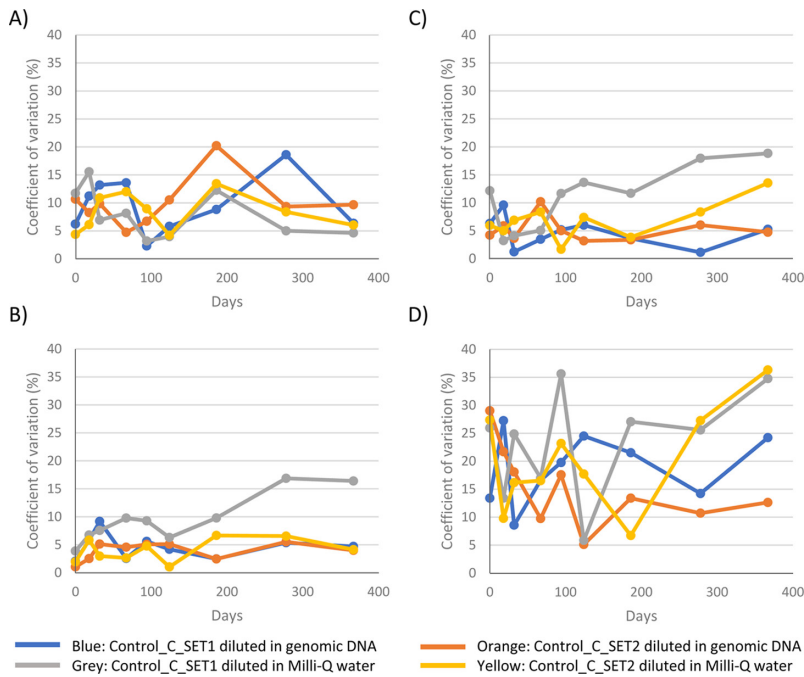
Fig. 5. Coefficients of variation (CVs) of samples containing Control_C over the course of 1 year. Positive template control (PTC) solutions of 10,000 (A), 1,000 (B), 100 (C), and 10 (D) copies were quantified over the course of 1 year. Blue, Control_C_SET1 diluted in genomic DNA; orange, Control_C_SET2 diluted in genomic DNA; grey, Control_C_SET1 diluted in Milli-Q water; yellow, Control_C_SET2 diluted in Milli-Q water.

Fig. 6. Linearity of diluted positive template controls (PTCs). Linearity was calculated immediately (A) and after 3 (B), 6 (C), and 12 (D) months. Blue, Control_C_SET1 diluted in genomic DNA; orange, Control_C_SET2 diluted in genomic DNA; grey, Control_C_SET1 diluted in Milli-Q water; yellow, Control_C_SET2 diluted in Milli-Q water.
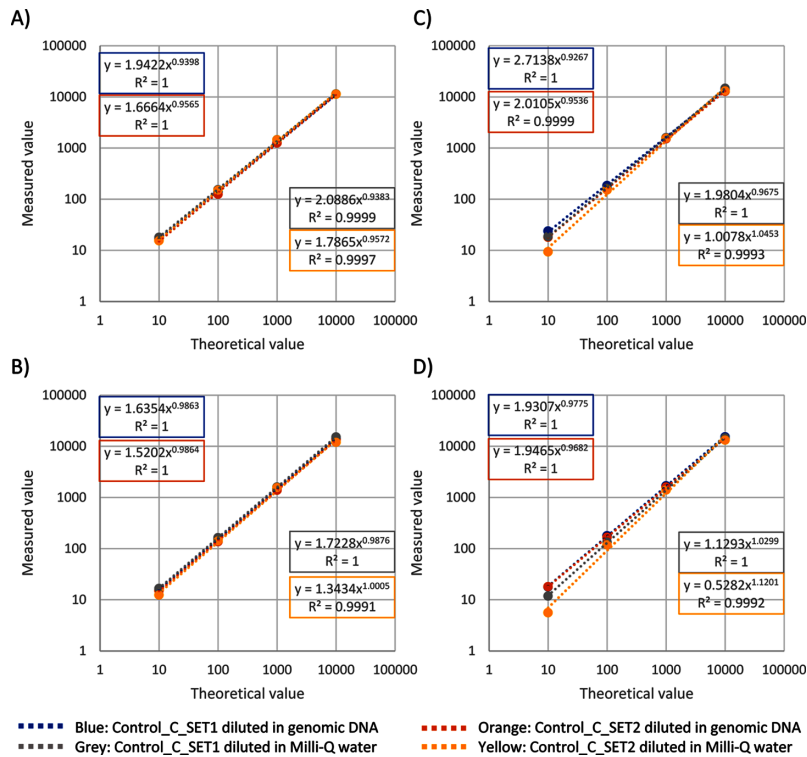
Discussion

In quantitative PCR-based gene doping tests, a PTC and NTC are required for positive determination [25]. In addition, to determine the limit of detection for method validation of a gene doping test, it is necessary to prepare low concentrations and/or serial dilutions of the PTC [14]. As we had previously developed a simultaneous detection method for 12 transgenes using MFQPCR [19], in this study, we aimed to develop an RM for multiple targets.

When a single gene is targeted in a gene doping test, an RM can be prepared as a single target [21]. However, when multiple genes are targeted, the preparation of RMs becomes complicated. In one procedure, an RM for each gene is cloned into a plasmid vector and individually quantified so that equal contents of each RM are present [19]. However, it is difficult to mix individually prepared RMs with the same number of copies, particularly those with low copy numbers (10 copies or less). This leads to variations in the PTC copy numbers, resulting in uncertain identification and quantification of gene doping substances. In fact, in a previous study, we mixed and diluted individually prepared PTCs, but the detection of mixed PTCs in the low copy number region was unstable [19]. Although not necessarily problematic when positive results are obtained by amplification and non-amplification, detection instability in the low copy number region is an issue when the limit of detection has been established. We concluded that the Control_C template designed in this study was suitable as an RM for simultaneous detection using MFQPCR because it had multiple detection sites in the single plasmid vector, rendering individually quantified and mixed PTCs unnecessary.

Although RMs are traditionally constructed from RNA extracted from equine tissues, we prepared RMs via artificial synthesis. The whole-genome sequence of horses, excluding the Y chromosome, has been determined, and the reference sequence, EquCab3.0, is currently available [13, 24]. A variant database of 101 Thoroughbreds is now available [20]. Therefore, RMs can be easily designed using genomic information and artificially synthesised. For example, in our study, Control_C comprised the quantitative PCR detection sites of 12 target genes.

One advantage of Control_C was that it could be quantified by multiple assays (12 assays maximum). Quantitative bias caused by assay differences, such as amplification efficiency differences, can be suppressed to a low level. In this study, we found that a more accurate PTC copy number could be quantified using the mean of four assays. These quantification procedures may be suitable for RM quantification [4, 26]. As digital PCR enables absolute quantification of target templates [10, 23], quantification of Control_C via multiple assays is an effective method for validating gene doping tests.

An advantage of MFQPCR analysis is the ability to analyze multiple markers simultaneously [12, 15]; however, the device used for it is very sensitive, making it necessary to monitor errors originating from the analytical instrument. Particular attention should be paid to IFC liquid delivery errors, and Control_C may be useful for identifying such errors. In one assay, no amplification signals were detected, whereas PTCs were detected in the other assays. Here, errors in the delivery of primer/probe mixtures were suspected rather than errors in the delivery of control substances.

PTCs are necessary for gene doping tests and PCR-based detection of viruses, such as COVID-19 [6, 22]. However, the use of PTCs comes with the potential risk of sample contamination. Because PCR theoretically enables amplification of a single copy, positive controls are needed to determine whether the detected signals originate from the sample or from contamination. An advantage of Control_C is that its PCR product size differs from that of the original gene. In addition, because it is unlikely that all tested genes will have been doped simultaneously, detected signals are more likely to have arisen from contamination than from a positive sample if Control_C is detected in all assays. The number of false-positive results can be reduced by using these procedures.

In real-time PCR, controls must be prepared from the RM for each gene doping test [25], either by diluting high-concentration RMs for each test or by preparing diluted controls for long-term storage. The former method is generally used for quantitative operations requiring strict dilutions because a high concentration is more stable than a low concentration [1]. The latter method is easier to perform, but the stability of PTCs with low copy numbers is a concern. Although it has been reported that PTCs with high copy numbers were stably stored for over 1 year [2], in this study, we demonstrated that even PTCs with low copy numbers could be stably stored for approximately 1 year by dissolving them in genomic DNA. This enables diluted controls to be prepared for long-term storage prior to gene doping tests.

The high CV observed at a concentration of 10 copies/μl was attributed in part to the process of dispensing the sample stock into the well of the PCR plate. Theoretically, a signal can be detected from one copy; however, because it is difficult to dispense exactly one copy, approximately five copies may be the detection limit. In addition, particularly in Milli-Q water, adsorption to the storage container may lead to variation.

In conclusion, we determined that even at 10 copies/μl, the newly designed PTC could be stored under refrigeration

for up to 1 year when dissolved in a solution containing the genome. In addition, by absolute quantification via digital PCR, we determined that the prepared PTC can be used as an RM. The results indicate that our RM design concept is suitable for use in MFQPCR-based gene doping tests and their developmental validation.

Conflict of Interest

There are no competing interests, including patents, products in development, or marketed products, to declare in relation to this work.

Acknowledgments

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