Gene doping control analysis of human erythropoietin transgene in equine plasma by PCR-liquid chromatography high resolution tandem mass spectrometry

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Abstract:

Gene doping involves the misuse of gene materials to alter athlete's performance which is banned at all times in both human and equine sports. Quantitative polymerase chain reaction (qPCR) assays have been used to control the misuse of transgenes in equine sports. Our laboratory recently developed and implemented duplex as well as multiplex qPCR assays for transgenes detection. As our continuous efforts in advancing gene doping control, in this work we have developed for the first time a sensitive and definitive PCR-liquid chromatography high resolution tandem mass spectrometry (PCR-LC-HRMS/MS) method for transgene detection, thus achieving an estimated limit of detection below 100 copies/mL for human erythropoietin (hEPO) transgene in equine plasma. The method involved magnetic-glass-particle-based extraction of DNA from equine plasma prior to PCR amplification with 2'-deoxyuridine 5'-triphosphate (dUTP), followed by treatments with uracil DNA glycosylase and hot piperidine for selective cleavage to give small oligonucleotide fragments. The resulting DNA fragments were then analysed by LC-HRMS/MS. The applicability of this method has been demonstrated by successful detection of hEPO transgene in blood samples collected from a gelding that had been administered with hEPO. This novel approach not only serves as an orthogonal method for transgene detection, but also paves the way to development of generic PCR-LC-HRMS/MS method for detection of multiple transgenes.

1. INTRODUCTION

Gene therapy involves the introduction of genetic material to a patient for the purpose of treating disease. Since the National Institutes of Health (NIH) conducted the initial clinical study on gene therapy in 1989, approximately 2,600 clinical trials encompassing various indications have been launched worldwide [1]. With the advancements in gene therapy techniques, gene doping has emerged as a potential means of altering human and equine sport performance [2–6]. For controlling the possible misuse of gene therapy in sports, regulations have been established by the World Anti-Doping Agency (WADA) [7], Fédération Équestre Internationale (FEI) [8], and International Federation of Horseracing Authorities (IFHA) [9] concerning gene doping.

With the growing concerns of gene doping threatening the integrity of sport, analytical methods have been developed for anti-gene doping using various technologies, including polymerase chain reaction (PCR) [10–15], sequencing [16,17], clustered regularly interspaced short palindromic repeats (CRISPR) [18,19], and mass spectrometry (MS) [20–24]. Specifically, real-time and digital PCR assays have been utilised to identify transgenes in different biological matrices [10–15]. The PCR-based detection method achieves its specificity by using primers and/or probe hybridised to the exon–exon junctions in the transgenes, indicating their exogenous origin. The unique sequence in the transgene is exponentially amplified by the specific pairs of primers, and detection can be achieved in real time and post-PCR. For example, our laboratory has developed real-time quantitative PCR (qPCR) assays for the detection of performance-enhancing transgenes in equine blood matrices using fluorophore-labelled probes [12]. With the rapid development of PCR-

based analysis for gene doping control, WADA [25] and the Association of Official Racing Chemists (AORC) [26] published laboratory guidelines for identification of transgenes by PCR analysis in 2021 and 2022 respectively. Although Sanger sequencing or next-generation sequencing may be used to definitively confirm the transgene sequence amplified by PCR, they are technically demanding and the instruments may not be available in conventional doping control laboratories. Alternatively, MS is recognised as a definitive technique and has been widely adopted for screening and confirmatory testing for anti-doping purposes [27–29]. The mass spectra can serve as "fingerprints" for unambiguous identification of prohibited substance(s). Therefore, MS-based technique can serve as an orthogonal approach for the unequivocal identification of transgenes.

PCR has been combined with electrospray ionisation mass spectrometry (ESI-MS) as diagnostic tool for diseases, enabling the detection of amplified DNA in clinical laboratories [30–34]. By the hyphenation of PCR and ESI-MS, sequences of interest are amplified by PCR prior to mass spectral analysis to determine the nucleotide sequence of the amplicons. However, direct analysis of intact amplicons by liquid chromatography (LC)-MS can be challenging due to the reduced ionisation efficiency of their long nucleic acid strands. Various approaches have been developed to limit the size of nucleic acids being analysed by MS. For example, Naumann et al. [20] have outlined an analytical method that incorporates single base extension primers as the detection probes following PCR amplification. The short extension primers allow transgene identification using matrix-assisted time-of-flight laser desorption/ionisation MS. The developed method has successfully identified the presence of EPO

transgene in commercialised products. Alternatively, Chowdhury and Guengerich [35] have described a primer extension assay that utilises 2'-deoxyuridine (dU) incorporated primers, followed by uracil DNA glycosylase (UDG) and hot piperidine treatments to cleave DNA into shorter segments for LC-MS analysis. Despite its applicability to primer extension assay, the cleavage at the primers alone might not be able to trim down the long amplicon sequence to a size suitable for LC-MS analysis in the context of gene doping control. Moreover, these two approaches have not dealt with the challenges associated with genomic DNA in biological samples. Hence, a novel approach capable of detecting the presence of transgene in biological samples using conventional instruments is highly desirable for gene doping control.

In this work, we have successfully developed a PCR-LC-MS-based method for the detection and confirmation of hEPO transgene in equine plasma. Essentially, the targeted transgene is amplified by conventional PCR in the presence of 2'-deoxyuridine 5'-triphosphate (dUTP). Once amplified, the incorporated uracil bases throughout the amplicons are specifically cleaved by UDG and hot piperidine treatments to generate LC-MS-detectable single-stranded DNA fragments. The detection of hEPO transgene is attained by monitoring a highly specific DNA fragment 5'-ATGGCTTCCTTCTGGGCTCCCAGAGCCCGAAGCAGAG-3' (R_1) that corresponds to the exons 4/5 junctions of the hEPO transgene. To demonstrate the applicability of this technique in equine doping control, post-administration plasma sample collected from a horse that had been intramuscularly administered with rAAV2/8 carrying the hEPO transgene was analysed by the

developed method. To our knowledge, this is the first LC-HRMS/MS method coupled with PCR to confirm the presence of transgene in equine plasma.

2. MATERIALS AND METHODS

2.1. Materials

The MagNA Pure 96 DNA and Viral NA Large Volume kit was purchased from Roche (Basel, Switzerland). Primers for hEPO (hEPO-F and hEPO-R) and oligonucleotide reference standards (F_1, R_1, and R_{14}) were procured from Tech Dragon (Hong Kong, China). The sequences for these are provided in Tables 1 and 2, respectively. Q5U Hot Start High-Fidelity DNA Polymerase, Q5U reaction buffer, and Monarch® PCR & DNA Cleanup Kit (5 µg) were obtained from New England BioLabs (Ipswich, USA). QIAquick PCR & Gel Cleanup Kit was purchased from QIAGEN (Germantown, USA). Nuclease-free water, dNTP/dUTP mix, nuclease-free sodium acetate solution (3 M at pH 5.2), uracil-DNA glycosylase (UNG), Tris (1M at pH 7.0, RNase-free grade), Tris (1M at 8.0, RNase-free grade), dithiothreitol (DTT), and triethylamine (TEA, sequencing grade) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Piperidine (ReagentPlus®), molecular biology-grade isopropanol and absolute ethanol were from Sigma-Aldrich (St. Louis, MO, USA). LiChropur grade of 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and methanol were obtained from Merck (Darmstadt, Germany). Deionised water was generated from an in-house water purification system (Milli-Q, Molsheim, France).

Table 1. Primers used for PCR.

Primer name	Sequence (5' to 3')	Priming region	Amplicon size (bp)
hEPO-F	CAC TGT CCC AGA CAC CAA	Exon 3	240
hEPO-R	ATG GCT TCC TTC TGG GCT C	Exon 4/5 junction	

Table 2. Sequences of oligonucleotide reference standards.

Oligonucleotide name	5'-end	Sequence (5' to 3')	3'-end	Region	Length (bp)
F_1	5'OH	CAC TGT CCC AGA CAC CAA AG	3'Phosphate	Exon 3	20
R_1	5'OH	ATG GCT TCC TTC TGG GCT CCC AGA GCC CGA AGC AGA G	3'Phosphate	Exon 4/5 junction	37
R_{14}	5'Phosphate	CCG ACA GCA GGG CCA GGC CC	3'Phosphate	Exon 4	20

2.2. Plasmid DNA as the reference material

The coding sequence for hEPO (NCBI Reference Sequence: NM_000799.4) was synthesised and cloned into pAAV-TRE plasmid by VectorBuilder (Chicago, IL, USA) [13]. The nucleotide sequence was verified through two independent Sanger sequencing experiments. Additionally, the concentration was determined spectrophotometrically using the Thermo Fisher Scientific Nanodrop one (Wilmington, DE, USA). To calculate the theoretical number of copies of transgene-carrying plasmids, the following formula was used:

$$Number\ of\ copies = x \times 6.022 \times 10^{23}/(n \times 660 \times 10^{9})$$

where x is the amount of transgene-carrying plasmids in ng, 6.022×10^{23} is the Avogadro's constant in mol^{-1}, n is the number of base pairs in the transgene-

carrying plasmid, and 660 is the average molar mass of one base pair of double-stranded DNA in g/mol.

2.3. DNA extraction from equine samples

Magnetic-glass-particle (MGP)-based DNA extraction from equine plasma was carried out using the MagNA Pure 96 DNA and Viral NA Large Volume kit in a MagNA Pure 96 System (Basel, Switzerland), following our previously published protocol [12]. A 500-µL aliquot of plasma sample was transferred to the MagNA Pure 96 System, and an elution volume of 50 µL was selected. Briefly, the samples were lysed to release DNA, and proteins were denatured and digested using Proteinase K solution. Subsequently, the released DNA was bound to the surface of MGP and separated as DNA-MGP complexes from the lysate. After multiple washing steps, the DNA was eluted from the MGPs. The extracted DNAs were then proceeded to further analyses.

2.4. PCR amplification with dUTP

Amplification of the extracted DNAs and incorporation of dU in the amplicons were achieved by PCR in the presence of dUTP. The PCR reactions were performed in the Applied Biosystems ProFlex 3×32-well PCR system (Waltham, MA, USA). Each reaction mixture contained 1× Q5U reaction buffer, Q5U Hot Start High-Fidelity DNA Polymerase (1 U), dATP, dGTP, and dCTP (200 µM each), dUTP (400 µM), and the forward and reverse primers for hEPO (1 µM each). Six microlitres of extracted DNA was added to make up a final

reaction volume of 50 µL. The thermal-cycling profile started with initial denaturation at 98 °C for 2 min to activate the polymerase. Then, amplification of DNA was achieved by a series of three stages, denaturation at 98 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 15 s. These stages were repeated 50 times to exponentially produce the targeted sequence. The ramp rates for heating and cooling were 6.0 °C. PCRs were prepared in duplicate for each sample.

The PCR products were purified to remove primers, nucleotides, enzymes, and other impurities using the QIAquick PCR & Gel Cleanup Kit according to the manufacturer's instructions. In brief, 90 µL of PCR sample was treated with 450 µL of Buffer PB and acidified with 10 µL of sodium acetate solution (3 M at pH 5.2). Subsequently, the mixtures were applied to QIAquick column and then washed with Buffer PE. An additional centrifugation step was applied to remove residual wash buffer. Elution was performed using 50 µL of nuclease free water. The eluates were collected in microcentrifuge tubes before UDG and piperidine treatments.

2.5. Amplicon cleavage by UDG and piperidine treatments

To cleave the dU-containing amplicon, UDG and piperidine treatments were adapted from the procedure previously reported by Chowdhury and Guengerich [35]. UDG treatment was performed on the eluates from the PCR purification to hydrolyse uracil from amplicon and create abasic sites. Each reaction mixture contained UDG (6 U), Tris buffer at pH 7.7 (50 mM), and DDT (2 mM). Forty-five microlitres of purified PCR product was added to make up a

final reaction volume of 100 µL. The reaction mixtures were incubated at 37 ˚C for 10 h. To cleave the DNA at the abasic sites created by the previous UDG treatment, piperidine was added to the reaction mixtures at the final concentration of 0.48 M and incubated at 95 ˚C for 1 h.

The reaction mixtures from UDG and piperidine treatments were purified and enriched using the Monarch® PCR & DNA Cleanup Kit according to the manufacturer's instructions. Briefly, the samples (105 µL) were treated with 200 µL of DNA Cleanup Binding Buffer and 600 µL of ethanol. After that, the mixtures were applied to the column and washed twice with DNA Wash Buffer. Subsequently, the samples were eluted using 20 µL of nuclease free water and transferred into plastic autosampler vials for LC-HRMS/MS analysis.

2.6. LC-HRMS/MS analysis

Ion-pair reversed-phase LC-HRMS/MS analysis was performed on a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) coupled to a Thermo Scientific™ Q Exactive™ Plus mass spectrometer (Bremen, Germany) equipped with a heated electrospray ionisation (HESI-II) source. Chromatographic separation was performed on a Thermo Scientific™ DNAPac™ RP HPLC column (Waltham, MA, USA; 10 cm × 2.1 mm ID; 1.7 µm particle size) at 35 ˚C. The mobile phases were freshly prepared prior to analyses. Each mobile phase contained 15 mM TEA and 40 mM HFIP. The mobile phase A was prepared in water, while the mobile phase B was prepared in 50% methanol. The TEA acted as an ion-pairing agent, and the HFIP functioned as a MS-compatible buffering acid. A linear gradient was run at a

constant flow rate of 300 µL/min, with 2% mobile phase B at t = 0 min and held for 30s, increased to 60% B at t = 10 min, further increased to 98% B at t = 10.1 min and held for 1.9 min until t = 12 min. Mobile phase B was returned to 2% at t = 12.1 min, and stabilised until t = 14 min prior to next injection. The injection volume was 5 µL.

The HESI-II source was set at 300 ˚C and equipped with a high-flow metal needle insert. The sheath gas and auxiliary gas pressure were set to 50 and 10 arbitrary units, respectively. The sweep gas pressure was set to 2 arbitrary units with curtain plated installed. The ion spray voltage was approximately -3.0 kV, and the capillary temperature was set to 350 ˚C. The S-Lens RF level was 40%. Full-scan spectra were acquired using a resolution of 35,000 (full width at half maximum, FWHM at *m/z* 200) in negative mode. The maximum injection time was 120 ms, and the automatic gain control (AGC™) was set at 3e6. The scan range was *m/z* 740 to 1500. The product ion mass spectra were acquired using DIA mode with a mass resolution of 35,000 (FWHM at *m/z* 200) with an isolation window of 1 *m/z*. The maximum injection time was set to 200 ms, and the automatic gain control (AGC™) was set at 2e5. Nitrogen (>99.995% purity) was utilised as the higher-energy collisional dissociation (HCD) collision gas. A normalised collision energy of 15% was used for fragmentation of the DNA fragment precursor ion. The sample tray of the autosampler was kept at 10°C.

Data processing was performed using the Thermo Scientific Xcalibur (Version 4.4) and TraceFinder (Version 5.1) software with a mass tolerance window of ± 5 ppm. Mongo Oligo Mass Calculator [36] and Aom^{2}S [37] were used to interpret the MS/MS data.

2.7. Method validation

The PCR-LC-HRMS/MS assay for hEPO transgene in equine plasma was validated in terms of specificity, estimated limit of detection (LoD), estimated limit of confirmation (LoC), and method precision.

2.7.1. Specificity

The specificity of the method was evaluated through *in silico* and *in vitro* analyses. The *in silico* specificity was checked by conducting a Blast search with the DNA fragment R_1 against the human and horse genome databases. The *in vitro* specificity was determined by analysing different negative equine plasma samples (n = 30). The plasma samples were analysed together with R_1 standard solution at 250 nM, no template control (NTC), positive template control (PTC), plasma blank, and spiked plasma control at 2000 copies/mL of hEPO transgene. The product ion chromatograms of the plasma samples were compared to those of the oligonucleotide reference standard and control samples to observe any significant interferences.

2.7.2. Estimated LoD and LoC

LoD and LoC were estimated by analysing plasma samples fortified with hEPO transgene at 1×10^2, 5×10^2, 1×10^3, 5×10^3, 1×10^4, 1×10^5, and 1×10^6 copies/mL. The estimated LoD was taken to be the lowest concentration

among those evaluated that gave a signal-to-noise (S/N) ratio greater than 3:1 in the product ion chromatogram. The estimated LoC represented the lowest fortified concentration evaluated that returned three product ions (S/N ≥ 3) with their relative abundances and retention times matched those from the corresponding oligonucleotide standard in accordance with the limits stipulated in the AORC Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry [38].

2.7.3. Method precision

Spiked samples prepared at 2000 copies/mL of hEPO transgene were used to measure the precision of the developed method. The precision was evaluated by analysing seven replicates of each spiked sample on four different days. Peak areas, relative abundances (with reference to the most abundant product ion), and retention times of the four characteristic product ions in the spiked samples were each examined to establish the precision, expressed as the pooled relative standard deviations (%RSD).

2.8. Method applicability

The applicability of the developed method was demonstrated by analysis of the post-administration plasma sample collected one day after an intramuscular injection of rAAV2/8-hEPO at a total dose of 3×10^{13} genome copies to a Thoroughbred gelding. All experimental procedures were approved by the Animal Research Ethics Committee of the Hong Kong Jockey Club

(ERC/035/2020) as reported previously [12]. After collection, the blood tubes were centrifuged at 1,500 *g* for 10 min at 15°C for plasma isolation, and the resulting plasma samples were aliquoted into microcentrifuge tube and stored at −70°C freezer.

3. RESULTS AND DISCUSSION

3.1. Generation of DNA fragments

Figure 1 provides a schematic diagram of a bottom-up approach used for the identification of transgene by PCR-LC-HRMS/MS. Direct analysis of intact amplicons by LC-HRMS/MS is challenging due to the reduced ionisation efficiency of their long nucleic acid strands. Generation of DNA fragments was achieved by digestion of uracil-incorporated nucleic acids with sequential treatments of UDG and piperidine prior to LC-HRMS/MS analysis. Essentially, this method utilised dUTP in replacement of deoxythymidine triphosphate (dTTP) for amplification reaction during PCR. dUTP was a close structural congener of dTTP and could be readily incorporated into DNA by forming deoxyuridine-deoxyadenosine base pairs during DNA amplification. Additionally, Q5U polymerase was used in this method for its ability to read and amplify templates containing uracil bases during PCR. The substitution of dUTP for dTTP in PCR resulted in uracil-containing PCR products where the sites of uracil were subsequently converted into abasic sites using UDG.

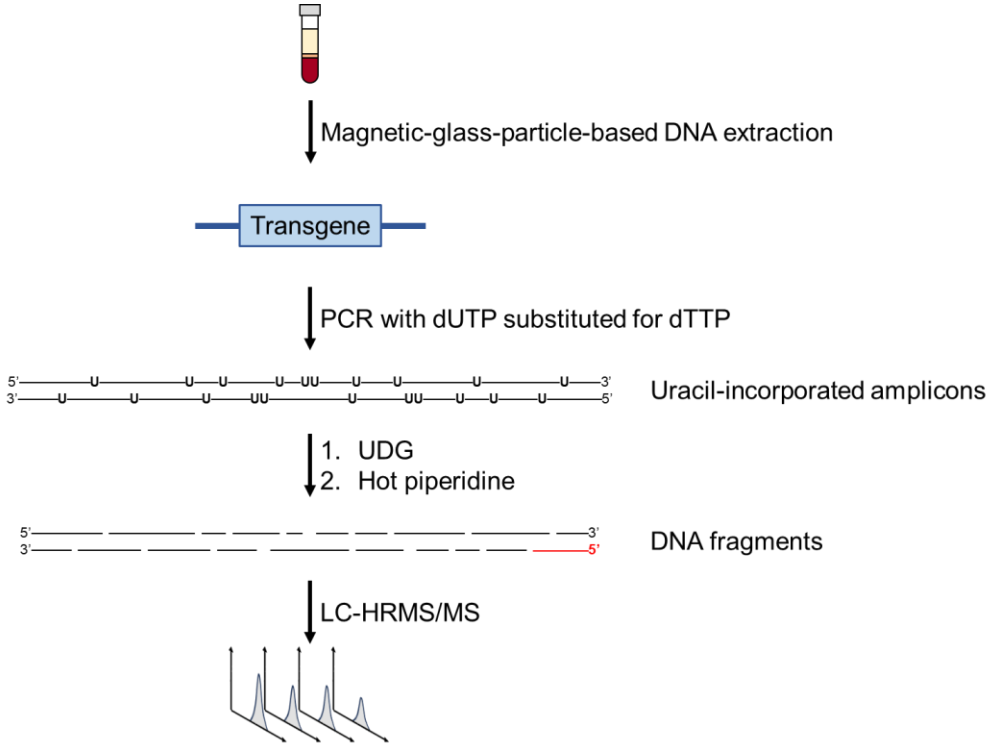


Figure 1. Schematic diagram of the bottom-up approach used for PCR-LC-HRMS/MS. Initially, DNA was extracted from equine plasma through MGP-based extraction. Subsequently, PCR with transgene-specific primers and dUTP substituted for dTTP was performed to amplify target transgene in the DNA extracts. The uracil-incorporated amplicons were cleaved by UDG and hot piperidine treatments. Finally, the DNA fragments were analysed by LC-HRMS/MS for detection of the targeted oligonucleotide specific to the transgene (highlighted in red colour).

UDG was used to selectively hydrolyse the N-glycosidic bond between the uracil and deoxyribose backbone in DNA through the 'pinch-pull-push' mechanism [39–45]. The removal of uracil from the deoxyribose created abasic sites which could be converted into a reactive open-chain aldehyde form [46]. Subsequent incubation with piperidine at 95 ˚C catalysed two successive

elimination reactions at the abasic site cleaving the amplicons (Figure 2) [47,48]. Firstly, a β-elimination reaction cleaved the 3'-phosphodiester bond at the abasic site, and released an upstream fragment with an α,β-unsaturated aldehyde and a downstream fragment with a 5'-phosphate group. Then, a following δ-elimination reaction cleaved 5'-phosphodiester bond of the upstream fragment, and left a 3'-phosphate group at the upstream fragment. As a result, the sequential β- and δ-elimination reactions at the abasic sites cleaved the long DNA strand into segments, in the form of oligonucleotide that could be further analysed by LC-HRMS/MS.

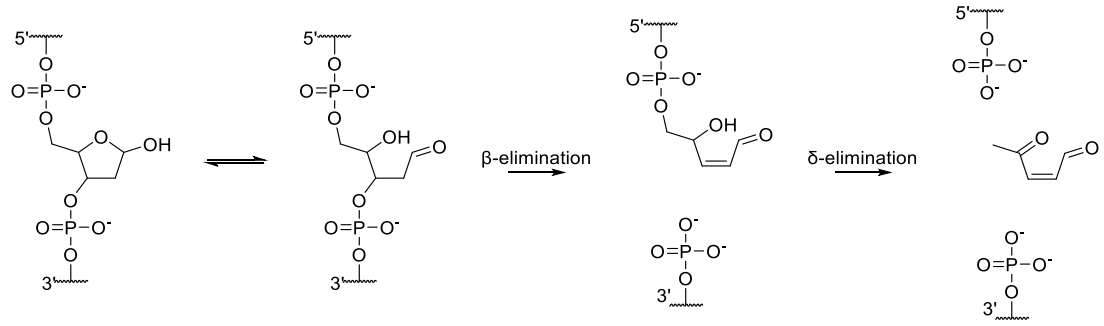


Figure 2. Strand cleavage by elimination reactions at abasic sites.

To verify the cleavage of amplicons by UDG and piperidine treatments, the undigested amplicons and their digested products were analysed by agarose gel electrophoresis (Figure 3) according to the published protocol [13]. As shown in Figure 3, the undigested PTC and positive plasma control produced a 240-bp prominent band that indicated the presence of hEPO amplicon after PCR. In contrast, no bands were observed in the corresponding digested products, indicating the absence of double-stranded DNA in the

digested samples. The agarose gel electrophoretic analysis implied the cleavage of double-stranded amplicon into single-stranded DNA fragments by sequential treatments of UDG and piperidine. On the other hand, faint bands along the lanes in both the undigested negative and positive plasma controls indicated the occurrence of non-specific amplification products in the presence of horse genomic DNA. Despite the presence of the unwanted amplification products, the inclusion of LC-MS conferred the specificity on the developed assay.

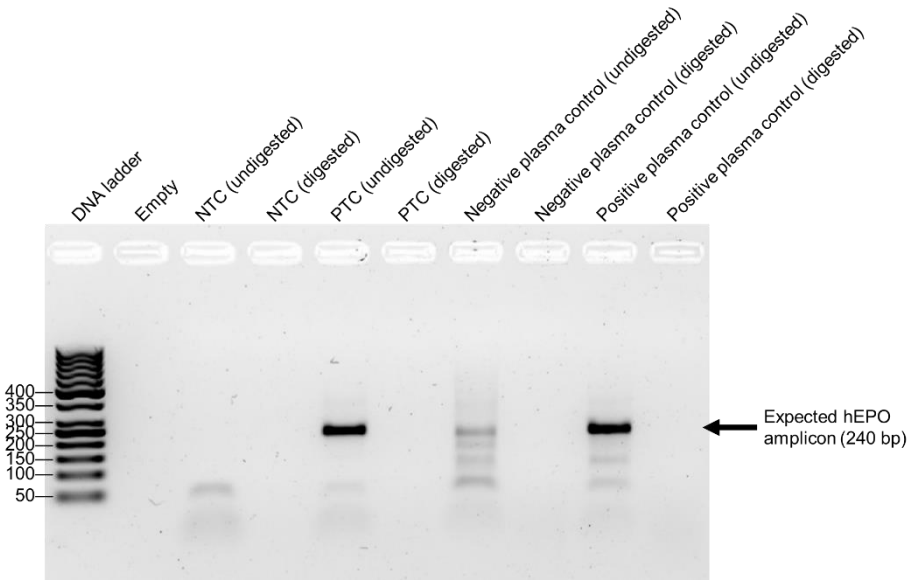


Figure 3. Agarose gel electrophoresis of samples after PCR and their corresponding digested products was performed. The NTC reaction was performed by adding water as sample to the reaction mixture prior to PCR. The PTC reaction was prepared by adding hEPO transgene plasmid to the reaction mixture to the final concentration of 1.5×10^4 copies/reaction prior to PCR. Positive plasma control was prepared at 1×10^6 copies/mL of hEPO

transgene plasmid in 0.5 mL plasma. Bands at 240 bp in PTC and positive plasma control indicated the presence of expected amplification product of hEPO transgene after PCR. Absence of these bands in digested samples indicated the cleavage of amplicon after UDG and piperidine treatments.

3.2. *In silico* analysis of amplicon cleavage by UDG and piperidine treatments

By comprehending the reactions involved in UDG and piperidine treatments, it was possible to infer the sequences and molecular formulae of the DNA fragments resulting from these treatments. Figures 4 and S1 simulated the antisense and sense strands of hEPO transgene amplicon and their resulting DNA fragments respectively. Specifically, cleavage occurred at every thymine base throughout the amplicon sequence, except the primer sequences which were not incorporated with uracil during PCR. The resulting oligonucleotides from the cleavage at both ends carried 5'- and 3'- phosphates. While the sequences connected to the primers were only cleaved at 3'-ends, it resulted in oligonucleotides carrying 5'-hydroxy and 3'-phopshate. Since the nucleic acid sequence of hEPO amplicon was known, *in silico* simulation of digestion of nucleic acid was carried out, and the sequence of the resulting oligonucleotides could be derived. The molecular formula and monoisotopic mass for each resulting DNA fragment were computed based on its base composition and respective end groups.

Figure 4. *In silico* simulation of digestion of antisense strand of hEPO transgene amplicon. Exons were represented in different colours, and the primer sequence was underlined. (A) The antisense strand of hEPO transgene (NCBI Reference Sequence: NM_000799.4) for PCR amplification (240 bp) with labelled exons and primer sequences. (B) Thymine in the sequence was replaced by uracil except that in the primer sequence, representing the integration of uracil during PCR. (C) The sequence was cleaved at uracil bases by UDG and piperidine treatments, the resulting DNA fragments, including R_1 and R_{14}, were tabulated with their respective end groups.

Based on the simulation of digestion of the hEPO transgene amplicon, three DNA fragments namely, F_1, R_1, and R_{14}, were initially chosen and analysed because of their long nucleic acid sequences. A digested plasma sample spiked with hEPO transgene was compared against the oligonucleotide reference standards (F_1, R_1, and R_{14}) in Figure 5. The extracted ion

chromatograms indicated the generation of DNA fragments from *in vitro* digestion of hEPO transgene amplicon matched with the simulated results. DNA fragment R_1 was selected for confirmatory analysis of hEPO transgene in equine plasma because of its specificity, which would be further discussed in the later section.

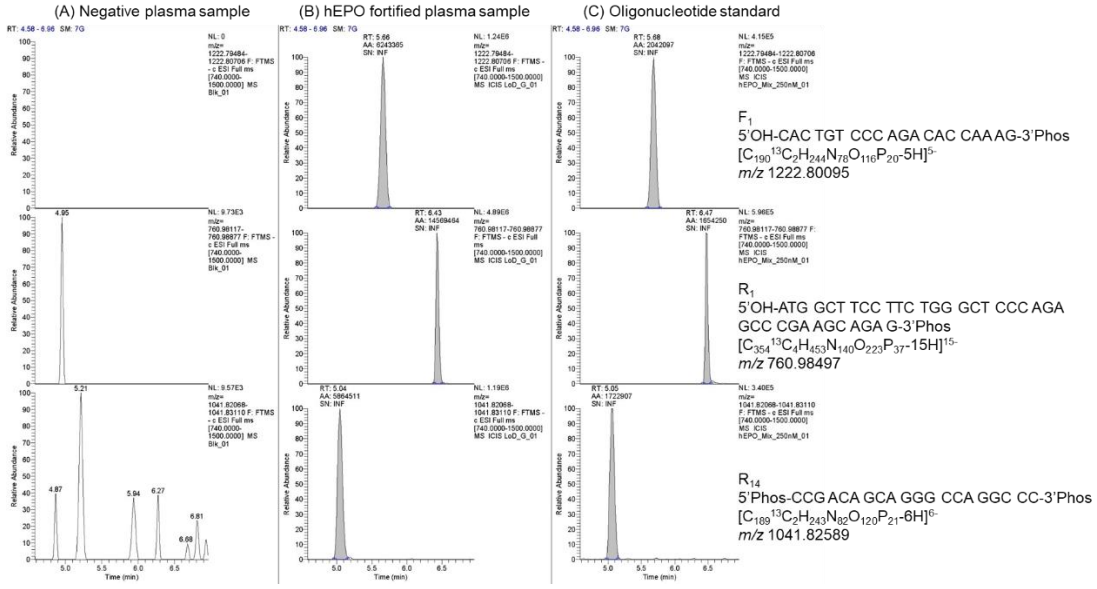


Figure 5. Full scan extracted ion chromatograms of digested products from (A) negative plasma sample, (B) plasma sample fortified with hEPO transgene plasmid at 1 × 10^6 copies/mL, and (C) oligonucleotide standard of F_1, R_1, and R_{14} at 250 nM.

3.3. Mass elucidation of MS^2 spectrum

Four product ions from fragmentation of R_1 were selected for the confirmation of hEPO transgene in the developed assay (Table 3). These product ions were generated through a selective fragmentation of

oligonucleotide which had been described by a nomenclature proposed by McLucky et al. [49] as illustrated in Figure S2. To select suitable product ions for confirmatory analysis, the oligonucleotide reference standard was firstly analysed by tandem mass spectrometry to generate a product ion spectrum. Then, online web-based applications, Mongo Oligo Mass Calculator [36] and Aom^{2}S [37], were utilised to interpret the MS/MS data. Briefly, the oligonucleotide sequence and the respective end groups of the DNA fragment were input into the applications, and the predicted fragment ions were calculated. The product ion spectra generated experimentally were then compared to the predicted results. This enabled the structural elucidation of product ions generated from the fragmentation of the targeted oligonucleotide by comprehending their sequences and sites of fragmentation (Figure 6). The molecular formula, nucleic acid sequence, and type of fragmentation for each product ion used in the developed method were summarised in Table 3.

Table 3. Precursor and product ions used for the confirmation of hEPO transgene.

Precursor ion			
DNA fragment	**Sequence (5' to 3')**	**Molecular formula**	***m/z***
R_1	5'OH-ATG GCT TCC TTC TGG GCT CCC AGA GCC CGA AGC AGA G-3'Phos	$[C_{354}{}^{13}C_4H_{453}N_{140}O_{223}P_{37}\text{-}15H]^{15-}$	760.98497
Product ions			
Type of fragmentation	**Sequence (5' to 3')**	**Molecular formula**	***m/z***
w_6	GCA GAG	$[C_{59}H_{75}N_{28}O_{38}P_7\text{-}3H]^{3-}$	665.75807
y_7	AGC AGA G	$[C_{69}H_{86}N_{33}O_{40}P_7\text{-}3H]^{3-}$	743.45516
y_6	GCA GAG	$[C_{59}H_{74}N_{28}O_{35}P_6\text{-}2H]^{2-}$	959.15758
$a_5 - B_5(C)$	ATG GC	$[C_{45}H_{55}N_{17}O_{26}P_4\text{-}H]^{-}$	1372.23818

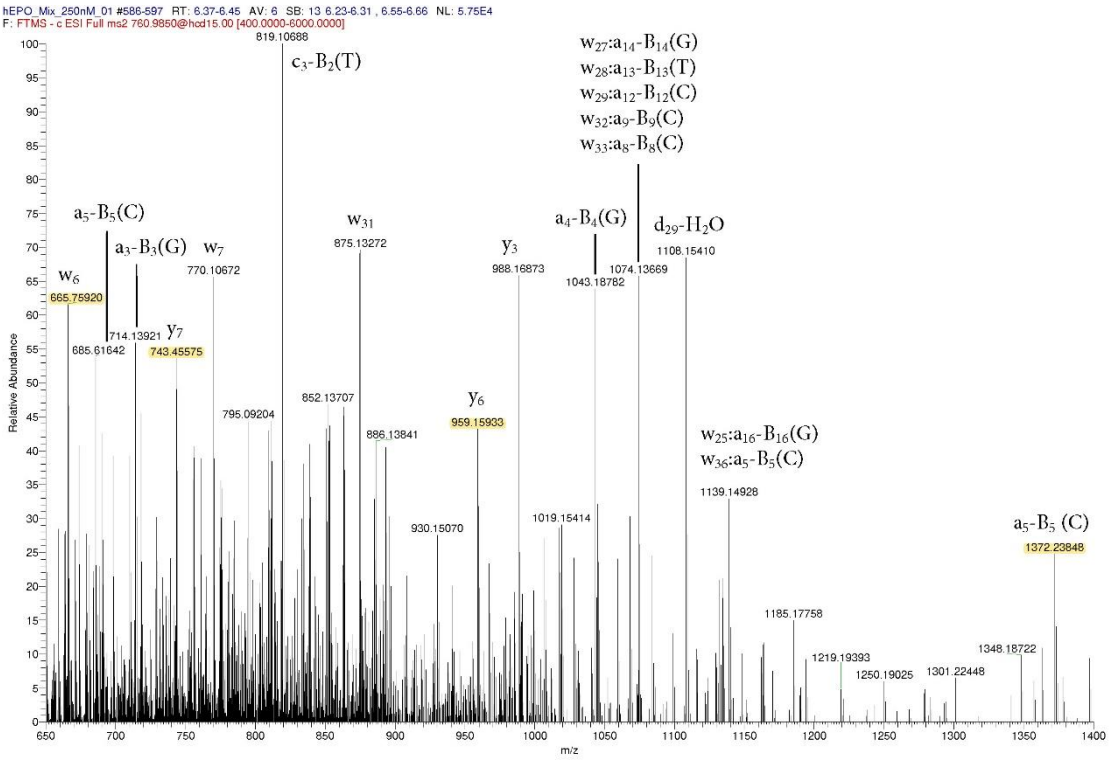


Figure 6. MS^2 spectrum of oligonucleotide R_1 reference standard. The highlighted product ions were utilised for confirmation of hEPO transgene.

3.4. Specificity using the selected DNA fragment for confirmation of hEPO

The specificity of the developed method was evaluated through *in silico* and *in vitro* analyses. A BLAST search against the human and horse genome databases displayed the *in silico* specificity of R_1 sequence to the hEPO transgene (Table S1). In terms of *in vitro* specificity, there was no significant interference observed from the plasma matrices (n = 30) at the expected retention times of the targeted ion transitions. However, it should be noted that nucleic acids consist solely of four nucleotides, resulting in a limited chemical

463 diversity, which increases the probability of generating isobaric ions that might
464 not be reliably distinguished by mass spectrometry alone [50]. The specificity
465 of this method for confirmation of hEPO in equine plasma arose from the
466 approach employed in sample preparation and analysis. In particular, the
467 primers were adapted from our published qPCR assay [13] to amplify a selected
468 segment specific to hEPO transgene. The selectivity of the primers was
469 demonstrated by conducting a Primer-BLAST search against the human and
470 horse genome databases. The nucleotide sequences of the resulting
471 amplicons from these primers were also confirmed by Sanger sequencing [13].
472 Hence, specific amplicons were produced only in the presence of hEPO
473 transgene during PCR. Moreover, the silica-membrane-based purification after
474 PCR (QIAquick PCR & Gel Cleanup Kit) was used to extract DNA fragments
475 ranging between 100 bp to 10 kb. The hEPO amplicons (240 bp) were
476 extracted while the pre-existing oligonucleotides in the sample matrix were
477 removed at this stage. Such purification procedure ensured that any detectable
478 oligonucleotides by LC-HRMS/MS were specifically generated from the
479 cleavage of amplicons. Furthermore, the presence of the targeted DNA
480 fragment was unequivocally confirmed by LC-HRMS/MS, which measured the
481 retention times and relative abundances of the characteristic product ions of the
482 oligonucleotide. This result was then compared against the corresponding
483 reference standard in accordance with the requirements outlined in the
484 Minimum Criteria for Identification by Chromatography and Mass Spectrometry,
485 as published by the AORC [38]. Finally, the DNA fragment R_1 selected in the
486 developed method was highly specific to the template sequence of hEPO,
487 which was located across the exon–exon junction of hEPO transgene. As

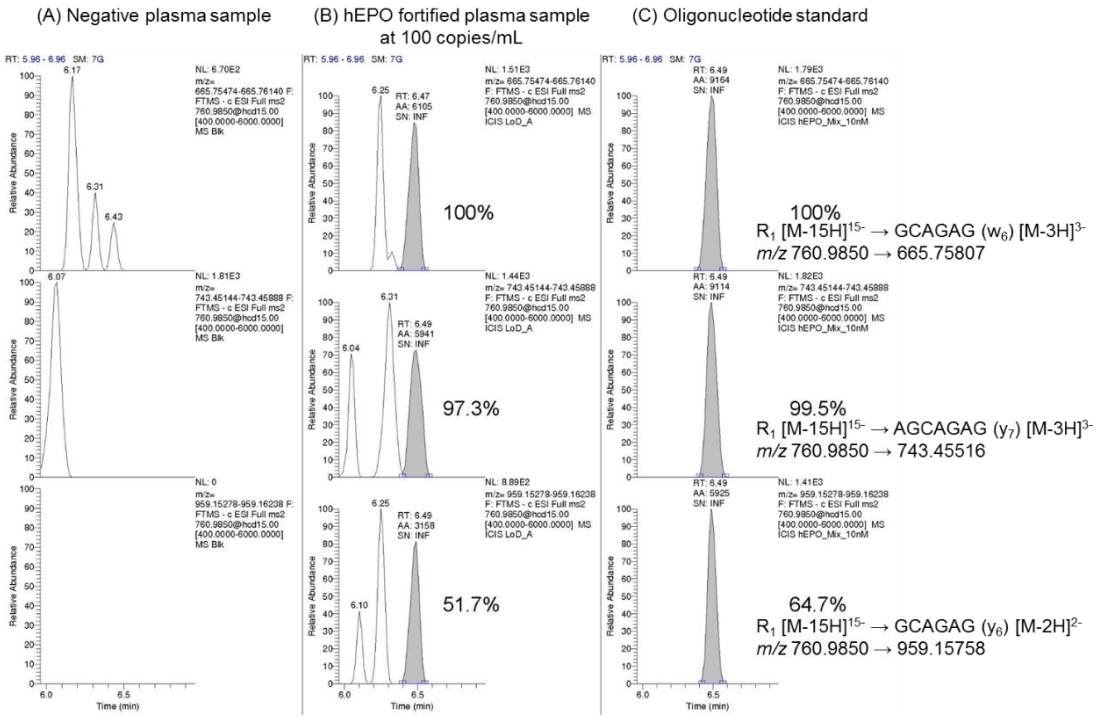
mentioned, the specificity of R_1 sequence to the hEPO transgene was shown in a BLAST search against the human and horse genome databases (Table S1). As a result, PCR, silica-membrane-based purification, LC-HRMS/MS analysis, and nucleotide sequence collectively contributed to the specificity of this method.

The utilisation of the DNA fragment R_1 in identification of hEPO transgene demonstrated an approach to design a PCR-LC-HRMS/MS assay. As mentioned above, dTTP was replaced by dUTP for amplification of targeted sequence and the uracil bases were later converted to abasic sites for cleavage of amplicons. In other words, the position and distribution of thymine bases within the sequences of targeted transgenes determined the availability of detectable oligonucleotides. However, a cleavage at every thymine base might not necessarily create any fragments specific enough for LC-HRMS/MS detection, which could either be too short or not consist of any exon–exon junctions. Nonetheless, this issue was overcome by the design of PCR assay in this study. In particular, primers synthesised without the incorporation of uracil could be used to limit the sites of cleavage by UDG. As shown in the R_1 sequence, the first 19 bases (ATGGCTTCCTTCTGGGCTC) originated from the reverse primer (hEPO-R) and the rest of the sequence was extended through PCR amplification. Despite the presence of thymine in the template sequence, uracil was not integrated into the amplicon within the primer sequence. Therefore, primers could be designed in the absence of uracil to retain a desired sequence from cleavage by UDG and piperidine treatments. Moreover, the reverse primer (hEPO-R) was located across an exon–exon junction and such characteristic could be passed on to the DNA fragment (R_1).

Hence, by manipulation of primer sequences, transgene-specific DNA fragment could be generated for PCR-LC-HRMS/MS assays.

3.5. Method sensitivity and precision

The LoD and LoC were both estimated to be below 100 copies/mL of hEPO transgene in plasma, which was the lowest concentration prepared during evaluation. Figure 7 demonstrated the product ion chromatograms for equine plasma fortified at 100 copies/mL of hEPO transgene. It was worth mentioning that there was yet a harmonised MS criteria published by AORC for identification of nucleic acids. The criteria applied to the current method was treated as the selected reaction monitoring of small molecules, implying a minimum 3 transitions with a tolerance in relative abundance within 10% absolute or 30% relative, whichever is greater [38]. Relative abundances and retention times of the selected transitions from the sample spiked at 100 copies/mL of hEPO transgene matched those from the reference standard oligonucleotide R_1. Confirmation of R_1 was achieved in two different equine plasma matrices spiked at 100 copies/mL of hEPO transgene.



532

533 **Figure 7.** Product ion chromatograms of (A) negative plasma sample, (B)
534 plasma sample fortified with hEPO transgene plasmid at 100
535 copies/mL, and (C) oligonucleotide standard of R_1 at 10 nM.

536

537 As the oligonucleotides existed in charge envelop in the ESI source,
538 utilisation of a multiplexed MS^2 technique to include the oligonucleotide
539 molecules in multiple charge states could potentially improve sensitivity
540 compared to DIA mode, which only allowed analysis of a single precursor ion.
541 During method development, multiplex MS^2 mode was employed in an attempt
542 to increase the sensitivity of MS detection. This approach enabled the selection
543 of multiple precursor ions for fragmentation and Orbitrap scanning. A
544 comparison was conducted between multiplexed MS^2 mode, using the first
545 three most abundant charge states, and DIA mode, using the most abundant
546 charge state as precursor ion, in spiked and blank plasma samples. While the

multiplexed MS^2 method was able to enhance the overall ion intensity by over 2-fold in spiked sample, it could exhibit a greater background noise in the blank samples, making it less suitable for confirmatory analysis. In addition, different combinations of mobile phase additives could influence the charge state distribution of oligonucleotides, resolution, and sensitivity in LC-MS [51–53]. Further research that accounted for these variables were necessary.

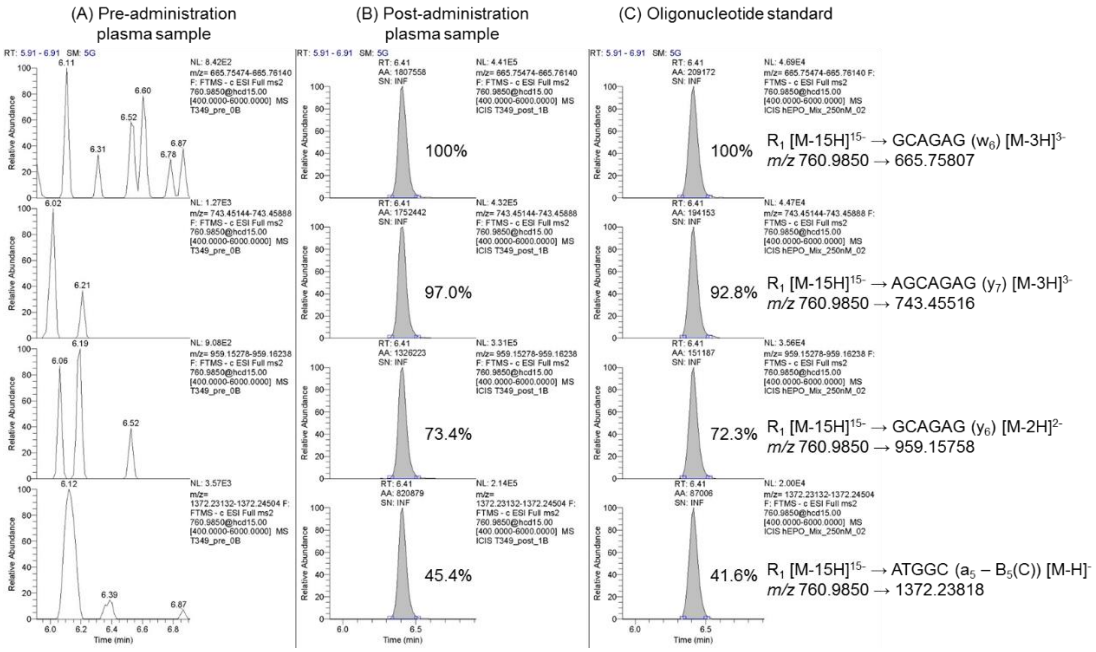
Alternatively, sensitivity of the method could be improved by optimising PCR parameters. More specifically, the developed method was an endpoint analysis which detected the final products accumulated during PCR. The number of copies of amplicons at the endpoint was a major limiting factor on the method sensitivity. While conventional PCR typically consisted of 20 to 40 thermal cycles for each run, this method applied a 50-cycle sequence to increase the amount of amplicons at the endpoint. The LoD of the method had improved 10-fold to 100 copies/mL by performing an additional 10 cycles, increasing the total number of cycles from 40 to 50. Further addition of thermal cycles might increase the method sensitivity, provided that the PCR reaction had not yet reached its plateau phase. Nevertheless, the sensitivity of this confirmatory method was considered adequate as it was comparable to that of the reported qPCR method [12].

The method precision (pooled % RSD) for relative abundances and retention times was within 6% and 0.1%, respectively. It was worth mentioning that the precision for peak areas was within 39%. The variability in peak areas could be attributed to the variable kinetics of PCR reaction. Even minor differences in reaction efficiencies among samples could result in substantial deviations in PCR end products after multiple cycles of reactions, which was a

practical limitation of endpoint PCR analysis [54,55]. Nonetheless, the method precision, in terms of relative abundances and retention times, was considered adequate for qualitative analysis.

3.6. Application of PCR-LC-HRMS/MS on post-administration equine plasma sample

Figure 8 displayed the confirmation of oligonucleotide R_1 in equine plasma collected one day after the intramuscular injection of rAAV2/8-hEPO. The post-administration sample's retention times and relative abundances of the four characteristic product ions for R_1 matched those from a reference oligonucleotide standard within the prescribed limits in the "AORC Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry" [38]. The presence of hEPO transgene in the post-administration sample was previously identified by qPCR in our published work [12]. The current method successfully confirmed the presence of oligonucleotide R_1 in the post-administration plasma sample. The result demonstrated the applicability of this method using R_1 to confirm the presence of hEPO transgene in authentic samples. However, as an end point detection, the current method was limited to qualitative analysis of hEPO transgene. For quantitative analysis, it might be more preferable to carry out a qPCR analysis. Nonetheless, the current method fulfilled its purpose in gene doping control for confirmatory detection of "zero-tolerance" transgene in equine sample.



595

596 **Figure 8.** Product ion chromatograms of (A) pre-administration plasma
597 sample, (B) post-administration plasma sample collected after
598 rAAV2/8-hEPO administration, and (C) oligonucleotide standard
599 of R_1 at 250 nM.

600

601 ## 4. CONCLUSIONS

602 A PCR-LC-HRMS/MS assay has been developed and utilised for detection
603 of hEPO transgene in equine plasma with adequate specificity and sensitivity.
604 The confirmation of hEPO transgene was conducted using a specific DNA
605 fragment (R_1) produced from UDG and piperidine treatments of hEPO amplicon.
606 The LoC and LoD of hEPO transgene in equine plasma was 100 copies/mL.
607 This developed method successfully confirmed the presence of R_1 in plasma
608 from a gelding with rAAV2/8-hEPO administration. To our knowledge, this is
609 the first LC-HRMS/MS method coupled with PCR to confirm the presence of
610 transgene in equine plasma. This work has provided an alternative approach

to confirm the presence of transgene via LC retention times and characteristic product ions. Although the current method, being an endpoint detection, is limited to qualitative analysis of hEPO transgene, it fulfils the purpose of identifying “zero-tolerance” transgene in equine sample. More importantly, this method can be expanded to accommodate the simultaneous detection of other transgenes in equine sample.

Supplementary Materials:

Author Contributions: Mr Bruce P.N. Yuen performed all experiments. Mr Bruce P.N. Yuen, Drs Kin-Sing Wong, Yat-Ming So, Wai Him Kwok, Hiu Wing Cheung, Terence S.M. Wan, Emmie N.M. Ho, and Wing-Tak Wong interpreted the results. All authors made an intellectually significant effort to revise the draft version and approved the final version.

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Institutional Review Board Statement: All experimental procedures were approved by the Animal Research Ethics Committee of the Hong Kong Jockey Club (ERC/035/2020) as previously reported [12].

Informed Consent Statement: Not applicable.

Data Availability Statement: The data underlying this article are available from the corresponding authors upon reasonable request.

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Conflict of Interest: All authors declare no competing financial interest.

References:

1. Ginn, S.L.; Amaya, A.K.; Alexander, I.E.; Edelstein, M.; Abedi, M.R. Gene Therapy Clinical Trials Worldwide to 2017: An Update. *J. Gene Med.* **2018**, *20*, e3015, doi:10.1002/jgm.3015.
2. Tozaki, T.; Hamilton, N.A. Control of Gene Doping in Human and Horse Sports. *Gene Ther.* **2022**, *29*, 107–112, doi:10.1038/s41434-021-00267-5.
3. López, S.; Meirelles, J.; Rayol, V.; Poralla, G.; Woldmar, N.; Fadel, B.; Figueiredo, M.; Costa Padilha, M.D.; De Aquino Neto, F.R.; Gualberto Pereira, H.M.; et al. Gene Doping and Genomic Science in Sports: Where Are We? *Bioanalysis* **2020**, *12*, 801–811, doi:10.4155/bio-2020-0093.
4. Cantelmo, R.A.; Da Silva, A.P.; Mendes-Junior, C.T.; Dorta, D.J. Gene Doping: Present and Future. *Eur. J. Sport Sci.* **2020**, *20*, 1093–1101, doi:10.1080/17461391.2019.1695952.
5. Wilkin, T.; Baoutina, A.; Hamilton, N. Equine Performance Genes and the Future of Doping in Horseracing: Equine Performance Genes and the Future of Doping in Horseracing. *Drug Test. Anal.* **2017**, *9*, 1456–1471, doi:10.1002/dta.2198.
6. Baoutina, A.; Alexander, I.E.; Rasko, J.E.; Emslie, K.R. Potential Use of Gene Transfer in Athletic Performance Enhancement. *Mol. Ther.* **2007**, *15*, 1751–1766, doi:10.1038/sj.mt.6300278.
7. World Anti-Doping Agency (WADA). Section M3 of the Prohibited List. Available online: https://www.wada-ama.org/sites/default/files/2022-09/2023list_en_final_9_september_2022.pdf (accessed on 4 November 2023).
8. Fédération Équestre Internationale (FEI). Acrticle 1004 (1) of the FEI Veterinary Regulations 2023. Available online: https://inside.fei.org/content/fei-veterinary-regulations-2023-0 (accessed on 4 November 2023).
9. International Federation of Horseracing Authorities (IFHA). Article 6B of the International Agreement on Breeding, Racing and Wagering and Appendixes. Available online: https://www.ifhaonline.org/default.asp?section=IABRW&AREA=2#article6 (accessed on 4 November 2023).
10. Wong, K.; Cheung, H.W.; Szeto, C.W.L.; Tsang, C.Y.N.; Wan, T.S.M.; Ho, E.N.M. A Multiplex qPCR Assay for Transgenes Detection: A Novel Approach for Gene Doping Control in Horseracing Using Conventional Laboratory Setup. *Drug Test. Anal.* **2023**, *15*, 879–888, doi:10.1002/dta.3483.
11. Tozaki, T.; Ohnuma, A.; Hamilton, N.A.; Kikuchi, M.; Ishige, T.; Kakoi, H.; Hirota, K.; Kusano, K.; Nagata, S. Low-copy Transgene Detection Using Nested Digital Polymerase Chain Reaction for Gene-doping Control. *Drug Test. Anal.* **2022**, *14*, 382–387, doi:10.1002/dta.3173.
12. Cheung, H.W.; Wong, K.; Lin, V.Y.C.; Farrington, A.F.; Bond, A.J.; Wan, T.S.M.; Ho, E.N.M. Optimization and Implementation of Four Duplex Quantitative Polymerase Chain Reaction Assays for Gene Doping Control in Horseracing. *Drug Test. Anal.* **2022**, *14*, 1587–1598, doi:10.1002/dta.3328.
13. Cheung, H.W.; Wong, K.; Lin, V.Y.C.; Wan, T.S.M.; Ho, E.N.M. A Duplex qPCR Assay for Human Erythropoietin (EPO) Transgene to Control Gene Doping in Horses. *Drug Test. Anal.* **2021**, *13*, 113–121, doi:10.1002/dta.2907.
14. Moser, D.A.; Braga, L.; Raso, A.; Zacchigna, S.; Giacca, M.; Simon, P. Transgene Detection by Digital Droplet PCR. *PLoS ONE* **2014**, *9*, e111781, doi:10.1371/journal.pone.0111781.
15. Beiter, T.; Zimmermann, M.; Fragasso, A.; Hudemann, J.; Niess, A.M.; Bitzer, M.; Lauer, U.M.; Simon, P. Direct and Long-Term Detection of Gene Doping in Conventional Blood Samples. *Gene Ther.* **2011**, *18*, 225–231, doi:10.1038/gt.2010.122.

16. Maniego, J.; Pesko, B.; Habershon-Butcher, J.; Huggett, J.; Taylor, P.; Scarth, J.; Ryder, E. Screening for Gene Doping Transgenes in Horses via the Use of Massively Parallel Sequencing. *Gene Ther.* **2022**, *29*, 236–246, doi:10.1038/s41434-021-00279-1.
17. De Boer, E.N.; Van Der Wouden, P.E.; Johansson, L.F.; Van Diemen, C.C.; Haisma, H.J. A Next-Generation Sequencing Method for Gene Doping Detection That Distinguishes Low Levels of Plasmid DNA against a Background of Genomic DNA. *Gene Ther.* **2019**, *26*, 338–346, doi:10.1038/s41434-019-0091-6.
18. Yi, J.; Kim, M.; Ahn, J.H.; Kim, B.; Son, J.; Sung, C. CRISPR/deadCas9-Based High-Throughput Gene Doping Analysis (HiGDA): A Proof of Concept for Exogenous Human Erythropoietin Gene Doping Detection. *Talanta* **2023**, *258*, 124455, doi:10.1016/j.talanta.2023.124455.
19. Paßreiter, A.; Naumann, N.; Thomas, A.; Grogna, N.; Delahaut, P.; Thevis, M. How to Detect CRISPR with CRISPR – Employing SHERLOCK for Doping Control Purposes. *The Analyst* **2022**, *147*, 5528–5536, doi:10.1039/D2AN01318E.
20. Naumann, N.; Paßreiter, A.; Thomas, A.; Krug, O.; Walpurgis, K.; Thevis, M. Analysis of Potential Gene Doping Preparations for Transgenic DNA in the Context of Sports Drug Testing Programs. *Int. J. Mol. Sci.* **2023**, *24*, 15835, doi:10.3390/ijms242115835.
21. Smith, J.; Strasser, L.; Guapo, F.; Milian, S.G.; Snyder, R.O.; Bones, J. SP3-Based Host Cell Protein Monitoring in AAV-Based Gene Therapy Products Using LC-MS/MS. *Eur. J. Pharm. Biopharm.* **2023**, *189*, 276–280, doi:10.1016/j.ejpb.2023.06.019.
22. Lam, A.K.; Zhang, J.; Frabutt, D.; Mulcrone, P.L.; Li, L.; Zeng, L.; Herzog, R.W.; Xiao, W. Fast and High-Throughput LC-MS Characterization, and Peptide Mapping of Engineered AAV Capsids Using LC-MS/MS. *Mol. Ther. - Methods Clin. Dev.* **2022**, *27*, 185–194, doi:10.1016/j.omtm.2022.09.008.
23. Paßreiter, A.; Thomas, A.; Grogna, N.; Delahaut, P.; Thevis, M. First Steps toward Uncovering Gene Doping with CRISPR/Cas by Identifying SpCas9 in Plasma via HPLC–HRMS/MS. *Anal. Chem.* **2020**, *92*, 16322–16328, doi:10.1021/acs.analchem.0c04445.
24. Tozaki, T.; Karasawa, K.; Minamijima, Y.; Ishii, H.; Kikuchi, M.; Kakoi, H.; Hirota, K.; Kusano, K.; Nagata, S. Detection of Phosphorothioated (PS) Oligonucleotides in Horse Plasma Using a Product Ion (m/z 94.9362) Derived from the PS Moiety for Doping Control. *BMC Res. Notes* **2018**, *11*, 770, doi:10.1186/s13104-018-3885-5.
25. World Anti-Doping Agency (WADA). Laboratory Guidelines—Gene Doping Detection Based on Polymerase Chain Reaction (PCR). Available online: https://www.wada-ama.org/en/resources/laboratory-guidelines-gene-doping-detection-based-polymerase-chain-reaction-pcr (accessed on 4 November 2023).
26. Association of Official Racing Chemists (AORC). AORC Guideline for the Minimum Criteria for Identification of Transgenes or Vectors by Polymerase Chain Reaction (PCR) Analysis. Available online: https://www.aorc-online.org/members/ (accessed on 4 November 2023).
27. Wong, J.K.Y.; Wan, T.S.M. Doping Control Analyses in Horseracing: A Clinician's Guide. *Vet. J.* **2014**, *200*, 8–16, doi:10.1016/j.tvjl.2014.01.006.
28. Thevis, M.; Thomas, A.; Schänzer, W. Current Role of LC-MS(/MS) in Doping Control. *Anal. Bioanal. Chem.* **2011**, *401*, 405–420, doi:10.1007/s00216-011-4859-9.
29. Maurer, H.H. Hyphenated Mass Spectrometric Techniques—Indispensable Tools in Clinical and Forensic Toxicology and in Doping Control. *J. Mass Spectrom.* **2006**, *41*, 1399–1413, doi:10.1002/jms.1112.
30. Özenci, V.; Patel, R.; Ullberg, M.; Strålin, K. Demise of Polymerase Chain Reaction/Electrospray Ionization-Mass Spectrometry as an Infectious Diseases Diagnostic Tool. *Clin. Infect. Dis.* **2018**, *66*, 452–455, doi:10.1093/cid/cix743.
31. Brinkman, C.L.; Vergidis, P.; Uhl, J.R.; Pritt, B.S.; Cockerill, F.R.; Steckelberg, J.M.; Baddour, L.M.; Maleszewski, J.J.; Edwards, W.D.; Sampath, R.; et al. PCR-Electrospray

Ionization Mass Spectrometry for Direct Detection of Pathogens and Antimicrobial Resistance from Heart Valves in Patients with Infective Endocarditis. *J. Clin. Microbiol.* **2013**, *51*, 2040–2046, doi:10.1128/JCM.00304-13.

32. Wolk, D.M.; Kaleta, E.J.; Wysocki, V.H. PCR–Electrospray Ionization Mass Spectrometry. *J. Mol. Diagn.* **2012**, *14*, 295–304, doi:10.1016/j.jmoldx.2012.02.005.
33. Kaleta, E.J.; Clark, A.E.; Johnson, D.R.; Gamage, D.C.; Wysocki, V.H.; Cherkaoui, A.; Schrenzel, J.; Wolk, D.M. Use of PCR Coupled with Electrospray Ionization Mass Spectrometry for Rapid Identification of Bacterial and Yeast Bloodstream Pathogens from Blood Culture Bottles. *J. Clin. Microbiol.* **2011**, *49*, 345–353, doi:10.1128/JCM.00936-10.
34. Ecker, D.J.; Sampath, R.; Massire, C.; Blyn, L.B.; Hall, T.A.; Eshoo, M.W.; Hofstadler, S.A. Ibis T5000: A Universal Biosensor Approach for Microbiology. *Nat. Rev. Microbiol.* **2008**, *6*, 553–558, doi:10.1038/nrmicro1918.
35. Chowdhury, G.; Guengerich, F.P. Liquid Chromatography-Mass Spectrometry Analysis of DNA Polymerase Reaction Products. *Curr. Protoc. Nucleic Acid Chem.* **2011**, *47*, doi:10.1002/0471142700.nc0716s47.
36. Rozenski, J. Mongo Oligo Mass Calculator v2.06. Available online: http://mass.rega.kuleuven.be/mass/mongo.htm (accessed on 4 November 2023).
37. Ortiz, D.; Gasilova, N.; Sepulveda, F.; Patiny, L.; Dyson, P.J.; Menin, L. Aom ^{2} S: A New Web-based Application for DNA/RNA Tandem Mass Spectrometry Data Interpretation. *Rapid Commun. Mass Spectrom.* **2020**, *34*, e8927, doi:10.1002/rcm.8927.
38. Association of Official Racing Chemists (AORC). AORC Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry. Available online: https://www.aorc-online.org/documents/aorc-ms-criteria-aug-2016/aorc-ms-criteria-aug-16.pdf (accessed on 4 November 2023).
39. Bellamy, S.R.W.; Krusong, K.; Baldwin, G.S. A Rapid Reaction Analysis of Uracil DNA Glycosylase Indicates an Active Mechanism of Base Flipping. *Nucleic Acids Res.* **2007**, *35*, 1478–1487, doi:10.1093/nar/gkm018.
40. Cao, C.; Jiang, Y.L.; Stivers, J.T.; Song, F. Dynamic Opening of DNA during the Enzymatic Search for a Damaged Base. *Nat. Struct. Mol. Biol.* **2004**, *11*, 1230–1236, doi:10.1038/nsmb864.
41. Wong, I.; Lundquist, A.J.; Bernards, A.S.; Mosbaugh, D.W. Presteady-State Analysis of a Single Catalytic Turnover by Escherichia Coli Uracil-DNA Glycosylase Reveals a “Pinch-Pull-Push” Mechanism. *J. Biol. Chem.* **2002**, *277*, 19424–19432, doi:10.1074/jbc.M201198200.
42. Parikh, S.S.; Putnam, C.D.; Tainer, J.A. Lessons Learned from Structural Results on Uracil-DNA Glycosylase. *Mutat. Res. Repair* **2000**, *460*, 183–199, doi:10.1016/S0921-8777(00)00026-4.
43. Stivers, J.T.; Pankiewicz, K.W.; Watanabe, K.A. Kinetic Mechanism of Damage Site Recognition and Uracil Flipping by *Escherichia Coli* Uracil DNA Glycosylase. *Biochemistry* **1999**, *38*, 952–963, doi:10.1021/bi9818669.
44. Parikh, S.S. Base Excision Repair Initiation Revealed by Crystal Structures and Binding Kinetics of Human Uracil-DNA Glycosylase with DNA. *EMBO J.* **1998**, *17*, 5214–5226, doi:10.1093/emboj/17.17.5214.
45. Slupphaug, G.; Mol, C.D.; Kavli, B.; Arvai, A.S.; Krokan, H.E.; Tainer, J.A. A Nucleotide-Flipping Mechanism from the Structure of Human Uracil–DNA Glycosylase Bound to DNA. *Nature* **1996**, *384*, 87–92, doi:10.1038/384087a0.
46. Doetsch, P.W.; Cunningham, R.P. The Enzymology of Apurinic/Apyrimidinic Endonucleases. *Mutat. Res. Repair* **1990**, *236*, 173–201, doi:10.1016/0921-8777(90)90004-O.

47. Haldar, T.; Jha, J.S.; Yang, Z.; Nel, C.; Housh, K.; Cassidy, O.J.; Gates, K.S. Unexpected Complexity in the Products Arising from NaOH-, Heat-, Amine-, and Glycosylase-Induced Strand Cleavage at an Abasic Site in DNA. *Chem. Res. Toxicol.* **2022**, *35*, 218–232, doi:10.1021/acs.chemrestox.1c00409.
48. McHugh, P.J.; Knowland, J. Novel Reagents for Chemical Cleavage at Abasic Sites and UV Photoproducts in DNA. *Nucleic Acids Res.* **1995**, *23*, 1664–1670, doi:10.1093/nar/23.10.1664.
49. Mcluckey, S.A.; Van Berkel, G.J.; Glish, G.L. Tandem Mass Spectrometry of Small, Multiply Charged Oligonucleotides. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 60–70, doi:10.1016/1044-0305(92)85019-G.
50. Schürch, S. Characterization of Nucleic Acids by Tandem Mass Spectrometry - The Second Decade (2004-2013): From DNA to RNA and Modified Sequences: TANDEM MS OF NUCLEIC ACIDS. *Mass Spectrom. Rev.* **2016**, *35*, 483–523, doi:10.1002/mas.21442.
51. Strezsak, S.R.; Pimentel, A.J.; Hill, I.T.; Beuning, P.J.; Skizim, N.J. Novel Mobile Phase to Control Charge States and Metal Adducts in the LC/MS for mRNA Characterization Assays. *ACS Omega* **2022**, *7*, 22181–22191, doi:10.1021/acsomega.2c00185.
52. Roussis, S.G.; Rodriguez, A.A.; Rentel, C. Determination of Individual Oligonucleotide Impurities by Small Amine Ion Pair-RP HPLC MS and MS/MS: N − 1 Impurities. *J. Chromatogr. B* **2021**, *1169*, 122611, doi:10.1016/j.jchromb.2021.122611.
53. Sutton, J.M.; El Zahar, N.M.; Bartlett, M.G. Oligonucleotide Anion Adduct Formation Using Negative Ion Electrospray Ion-Mobility Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2021**, *32*, 497–508, doi:10.1021/jasms.0c00380.
54. Fraga, D.; Meulia, T.; Fenster, S. Real-Time PCR. *Curr. Protoc. Essent. Lab. Tech.* **2014**, *8*, doi:10.1002/9780470089941.et1003s08.
55. VanGuilder, H.D.; Vrana, K.E.; Freeman, W.M. Twenty-Five Years of Quantitative PCR for Gene Expression Analysis. *BioTechniques* **2008**, *44*, 619–626, doi:10.2144/000112776.