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ORIGINAL ARTICLE

Establishment of two quantitative nested qPCR assays targeting the human EPO transgene

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For ethical and safety reasons it is critical to develop easily implemented assays with high sensitivity and specificity for gene doping surveillance. Two nested quantitative real-time PCR (qPCR) assays were developed that target the human EPO (hEPO) cDNA sequence in a circular form, representative of recombinant adeno-associated viral (rAAV) vector genomes found *in vivo*. Through an interlaboratory evaluation, the assays were validated and utilized in an *in vitro* blinded study. These assays are specific and extremely sensitive with a limit of detection (LOD) of 1 copy of circular plasmid DNA and a limit of quantification (LOQ) of 10 to 20 copies in the presence of 500 ng of human genomic DNA (hgDNA) extracted from WBCs. Additionally, using the two nested qPCR assays in a non-human primate study, where macaques were injected intramuscularly with a rAAV8 vector harboring a promoterless hEPO cDNA sequence, the viral vector was detected 8 to 14 weeks post-injection in macaque WBCs. The high sensitivity of the nested qPCR approach along with the capability of quantifying target DNA, make this approach a reliable tool for gene doping surveillance and the monitoring of exogenous DNA sequences.

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INTRODUCTION

In the light of evidence that some members of the sports community are seeking gene therapy drugs to increase athletic performance,[1–4] there has been a large effort placed in the development of legally defensible and easily implemented PCR-based assays that can be used to screen athletes for vector-transferred exogenous DNA. Direct DNA detection methods are necessary for gene doping surveillance, since the transgene product produced *in vivo* will be difficult to distinguish from its endogenous counterparts. The World Anti-Doping Agency (WADA) considers one form of gene doping as the transfer of polymers of nucleic acids or nucleic acid analogues which can potentially enhance sports performance.[5] Implementing analytical techniques to detect gene doping in sports will promote a doping-free environment with the highest standard of fairness, as well as promote the well-being of athletes.[2] The candidate genes that are likely to be illicitly used in gene doping include erythropoietin (EPO), growth hormone, luteinizing hormone, and growth factors (vascular-endothelial growth factor, fibroblast growth factor, and platelet-derived growth factor).

EPO is an endogenously produced glycoprotein that is the major regulator of erythropoiesis. According to its effects to increase the red blood cell mass, a plethora of erythropoiesis stimulating agents (ESAs) were developed to treat anemia, but likewise were misused by athletes to increase endurance performance.[6] A large number of pre-clinical studies were conducted to prove the efficiency of gene transfer technology for long term EPO protein expression. Using recombinant adeno-associated virus (rAAV) vectors, which were injected intramuscularly in non-human primates, long-term persistence of vector genomes and non-human primate EPO transgene expression were detected for several years.[7,8] EPO gene therapy has not reached clinical relevance yet, and its proficiency and safety is not approved. However, the misuse of gene transfer technology cannot be excluded.

For *in vivo* gene transfer the rAAV vector has become a tool of choice and in 2012 the first rAAV based gene therapeutic drug was granted market approval by the European Commission.[9] The wild-type AAV is single stranded non–pathogenic DNA virus. Twelve different serotypes are known that differ in tissue tropisms.[10,11] The small ~4.7 kb long genome of the AAV comprises life cycle relevant *rep* and *cap* genes that are flanked by inverted terminal repeat (ITR) sequences. These genes can be replaced by a transgene cassette of interest and packaged into viral capsids of choice. For gene transfer targeting muscle tissue the serotypes AAV8 and AAV9 are the leading candidates.[12]

In the past decade a lot of effort was made to gain a better understanding of vector host interactions, with the intention to optimize and extent the long-lasting therapeutic efficacy of rAAV gene transfer. Next to the evaluation of the most suitable serotype,[11] potent promoter/enhancer sequences,[13] optimal vector dose, injection site, engineered vector capsids,[14] and optimized vector cassettes were developed.[15] These steps were conducted to enhance the specificity of gene transfer and evade immune reaction that would reduce transduction efficacy and long-term transgene expression. As reviewed by Basner-Tschakarjan and Mingozzi the immune response of the host can be directed against the AAV vector capsid, vector DNA genome

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and transgene product.[16] With regard to the progress of gene therapeutic approaches reliable gene doping detection assays are of pivotal importance.

A number of different PCR based approaches were suggested for transgene detection (Table 1). These include endpoint PCR,[17] nested endpoint PCR,[18–20] quantitative real-time PCR (qPCR),[21–23] and digital droplet PCR (ddPCR).[24] The nested endpoint PCR assay, which incorporates two rounds of conventional PCR and gel electrophoresis, achieved a sensitivity of 1 copy of hEPO cDNA harbored in a linearized plasmid and in the presence of 300 ng of human genomic DNA (hgDNA) extracted from whole blood.[18] Although this PCR assay has a high level of sensitivity, it requires the additional handling step of gel-electrophoresis that involves subjective analysis and it does not enable the quantification of transgene molecules. A more automated and less subjective gene doping test will provide the governing bodies in sports with additional information that can aid in setting the time frame for testing athletes, and used as a qualitative gene doping test with binary results is sufficient for high throughput screening of athletes.

A major consideration for developing detection strategies is that transgenes will persist as episomal circles (AAV, lentiviral vectors) or integrate into the host genome (retroviral or lentiviral). Complex DNA structures such as plasmids significantly reduce the sensitivity of PCR detection.[25] Although, the digestion of complex molecules with restriction enzymes can increase the sensitivity for PCR-based assays, the use of restriction enzymes is not ideal for a gene doping surveillance test since in this setting the sequence of the illicit gene vector transfer system would be unknown. Therefore, for each target a restriction enzyme would need to be selected with the criteria that the site is present in the gene of interest and not near the primer and probe binding sites; a strategy previously used in qPCR[23] and ddPCR[24] for hEPO transgene detection. Due to the high number of PCR cycles in the nested qPCR approach described here, no digestion of the plasmid is necessary, since linear and circular DNA structures amplify with high efficiency, making this approach independent of the vector system used.

In order to address the need for a reliable gene doping test that specifically targets the hEPO cDNA with extreme sensitivity, accuracy, and ruggedness, two quantitative nested qPCR assays were developed. We chose to incorporate a nested qPCR approach since it has been previously shown to significantly increase the sensitivity and assay performance over one round qPCR assays.[26–28] The nested qPCR assays combine a first round endpoint PCR of 25 cycles, with a second round qPCR (nested qPCR) of 40 cycles. The product of the pre-amplification step is a linear molecule that is subsequently detected by qPCR. By using 5 replicates per sample in the first round PCR which are pooled and diluted, and then processed for qPCR for detection (Figures 1 and 2), these assays achieve single copy sensitivity and enable quantification of copy numbers concomitantly.

The assays were validated and the performance of each assay was confirmed by the following experiments: (1) measuring the limit of detection (LOD) and quantification (LOQ), as well as common assay parameters using a circular rAAV vector plasmid that harbors the hEPO cDNA sequence spiked into a background of 500 ng of naïve hgDNA extracted from white blood cells (WBCs) (2) implementing the assays in a blinded *in vitro* study to detect and quantify samples spiked with known amounts of the circular rAAV vector plasmid that harbors the hEPO cDNA sequence in a background of 500 ng of naïve hgDNA extracted from WBCs and (3) utilizing the assays in a non-human primate study to quantify a rAAV8 viral vector harboring a promoterless hEPO cDNA sequence in the WBCs of macaques injected in the tibialis anterior muscle (Figure 3).

The rAAV vector was selected as the gene transfer vector system in this study, as it is ideal for gene doping due to the nonpathogenic nature of the vector, transduction capability in dividing and non-dividing cells, and ability to sustain transgene expression in different tissues, as demonstrated in pre-clinical and human clinical trials.[15,29–32]

The promoterless vector cassette was designed to avoid hEPO protein expression and the possibility of an immune response that could eliminate transduced cells. The skeletal muscle was chosen as the site of injection as it is most likely the delivery site used by athletes due to the robust transduction efficiency by rAAV vectors,[29–33] physical accessibility, mass of tissue, and the accessibility to vasculature. WBCs were chosen as the sample material as they are easily accessible and it has been shown that WBCs are collaterally transduced following IM injection of rAAV vectors in macaques and exhibit long term maintenance of rAAV vectors (57 weeks post-injection).[34] Moreover, WBCs do not contain the potent PCR inhibitors found in whole blood (immunoglobulin G in plasma and hemoglobin in erythrocytes).[35,36] Compared to other body fluids WBCs are the superior sample material for long term transgene detection. Indeed, following rAAV2 gene transfer Manno *et al.* compared the detectability of viral genomes in serum, urine, semen and WBCs. Using qPCR the transgene was detectable up to 21 weeks in WBCs. In urine the transgene became undetectable after several days.[32]

The nested qPCR assays described here were designed and validated to detect and quantify low levels of hEPO cDNA harbored in a circular form (plasmid or rAAV vector[21,24]) without the need for additional sample processing. Following the administration, the linear rAAV vector genome is quickly circularized into monomeric and concatameric episomes.[37] These circular structures have been shown to persist in WBCs and in various tissues.[7,38,39] The nested qPCR assays enable ultra-sensitive detection of complex molecules such as plasmids and concatemeric episomal viral vector circles that harbor the hEPO transgene.

RESULTS

Nested qPCR assay design and optimization

As a result of the assay design the sensitivity of a nested endpoint PCR could be combined with the additional advantage of sample quantification. As described in Figure 1, the nested qPCR assay is based on the strategy to pre-amplify 5 replicates of a sample by conventional PCR. These samples are pooled and diluted 1:50 in H_2O prior to a second round qPCR (Figure 1). The establishment of a standard curve for the nested qPCR assay, which enables subsequent quantification of samples, required the optimization of different protocol steps.

For the pre-amplification round 25 cycles were chosen with the considerations that the number of pre-amplification steps would be high enough to guarantee highest sensitivity, but likewise be low enough to prevent the amount of PCR product reaching the plateau-phase. 20 cycles of pre-amplification round were not sufficient to guarantee single copy number detection of the circular plasmid containing the EPO cDNA (data not shown). Twenty five cycles of pre-amplification were proven to fulfill the requirements. Furthermore, using 5 replicates of a single test sample for the pre-amplification round, which are pooled subsequently, minimized the variability between samples. Additionally, it increased the sensitivity, accuracy and precision of the assay.

The priming strategy of the two nested qPCR assays is described in Figure 2. Both assays use the same pre-amplification primers to generate a 437 bp linear amplicon. In the second round qPCR a 114 bp amplicon (Assay #1), or a 133 bp amplicon (Assay #2) is generated.

The pre-amplification primers were designed in a previous study[19] and bind to the exon junction 1 and 2 and exon junction 4 and 5, respectively. The annealing temperatures of the primers were optimized for the PCR protocol using temperature gradient

Table 1. Description of recent PCR-based approaches for gene-doping detection

	Brief description of the method	*Advantage*	*Disadvantage*	*Assays for gene-doping detection*
End-point PCR	Conventional PCR amplification of the target. Subsequent gel electrophoresis.	Low costs	Low sensitivity	Bogani *et al.*[17]
Nested endpoint PCR	The nested PCR includes two rounds of PCR. After a pre-amplification PCR and subsequent dilution, the PCR product undergoes a second round PCR. The product is visualized after gel electrophoresis.	Increased specificity Ultra sensitivity	Laborious workflow Higher costs compared with end-point PCR No reliable quantification possible	Beiter *et al.*[18] Beiter *et al.*[19] Moser *et al.*[20]
Quantitative real-time PCR (qPCR)	The qPCR includes a fluorescent reporter molecule (DNA binding dye or a probe) in the amplification reaction. This enables the quantification of the target in 'real time'. Probes increase specificity and sensitivity.	Quantification of transgene copy numbers Cost efficient Few handling steps High sensitivity	Need of a standard curve for quantification Possibly low signal-to-noise ratio for low copy number detection	Baoutina *et al.*[21] Ni *et al.*[34] Ni *et al.*[22] Baoutina *et al.*[23]
Digital PCR (dPCR)	Digital PCR is an alternative approach to qPCR for absolute quantification of samples. dPCR systems partition a sample in a large number of small PCR partitions. For each of the partitions the end point is measured. Based on Poisson statistics the absolute number of targets can be calculated.	Absolute quantification with high accuracy and precision No need of a standard curve Sensitivity similar or better than qPCR Better tolerance to PCR inhibitors than qPCR	Possibility of false-positive events in the sample or the NTC that are not distinguishable from true positives with current digital PCR platforms	Moser *et al.*[24]
Nested qPCR	The nested qPCR assay combines a pre-amplification PCR with a subsequent qPCR round. Five replicates are pre-amplified within the mid-linear phase of PCR amplification, pooled and diluted afterwards. This enables that the samples can be used to generate a standard curve in the second round qPCR, which enables the quantification of samples.	Increased specificity as a result of the nested design and the utilization of probes Ultra sensitivity as a result of five replicates in the pre-amplification round (including 500 ng gDNA background per replicate) Possibility for quantification Possibility for the use of synthetic standards	More laborious compared with qPCR and dPCR High demand of chemicals More manual handling steps that render susceptility to technical bias or sample cross contamination	

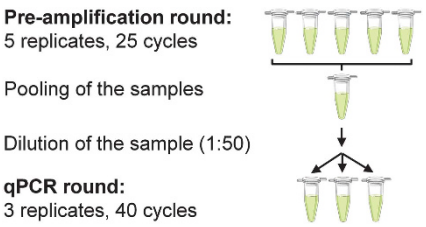


Figure 1. Setup of the nested qPCR assay. 5 replicates of a sample undergo a pre-amplification round of 25 cycles. These samples are pooled and diluted 1:50 in H_2O subsequently. 3 µl of diluted, pooled sample are tested in 3 to 7 replicates in the second qPCR round.

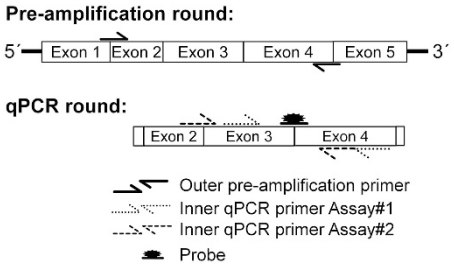
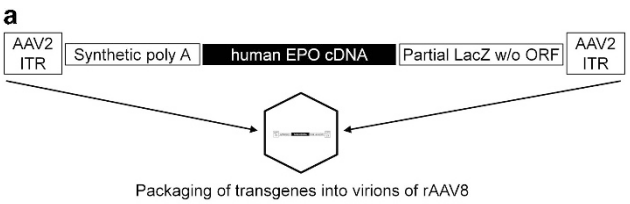


Figure 2. Priming strategy of the nested qPCR setup for the amplification of the human EPO cDNA sequence (NM_000799). In the pre-amplification round both assays use the same primer pair, which binds exon junction 1 and 2 and exon junction 4 and 5, respectively. In the qPCR round Assay#1 and Assay#2 use different primer pairs, whereas the same binding site for the probe is used.



b

	Mac 1	Mac 2	Mac 3
Injected viral genomes (vg)	1.3×10^{11}	1.3×10^{12}	Non-injected control
Injected vg/kg	$\sim 2.5 \times 10^{10}$	$\sim 2.5 \times 10^{11}$	Non-injected control
Injection site	m. tibialis anterior	m. tibialis anterior	n/a
Blood sample collection	Up to 12 weeks	Up to 26 weeks	Up to 4 weeks

Figure 3. Study design. (**a**) Diagram of packaged vector genome containing AAV2 inverted terminal repeat sequence (ITR), promoter-less human EPO cDNA (NM_000799), partial LacZ gene without open reading frame (ORF), and a synthetic poly A sequence, to overcome possible EPO cDNA transcription initiation from ITR sequence. (**b**) Injection strategy and duration of blood sample collection for macaques (Mac) 1-3.

PCR (temperature range: 50-66 °C, 40 cycles). The conditions that produced the most amount of a single PCR-product (visualized by UV illumination after gel-electrophoresis and ethidium bromide staining) were obtained at 61-63 °C (data not shown). 62 °C was chosen as the annealing temperature for the pre-amplification round. The primer pair for the second round qPCR in Assay #1 was designed previously and targets exon 3 and 4 respectively.[22] The primers in nested qPCR Assay #2 were designed in this study and target the exon-exon boundary 2 and 3 and exon 4. Both of the assays utilize a common probe designed previously.[22] An *in silico* analysis of the primer and probe sequences with the UCSC genome browser excluded non-specific binding sites in the human and macaque genomes.[40]

Table 2. LOD and LOQ of the nested qPCR assays

hEPO cDNA copy #	*Assay #1*		*Assay #2*	
	C_q *mean ± s.d.*	*% Error*	C_q *mean ± s.d.*	*% Error*
1280	16.06 ± 0.08	5.61	15.95 ± 0.11	8.70
640	17.31 ± 0.09	7.51	16.77 ± 0.09	5.26
320	18.41 ± 0.05	6.95	17.85 ± 0.12	7.89
160	19.40 ± 0.10	4.65	18.46 ± 0.13	16.29
80	20.33 ± 0.02	9.04	19.46 ± 0.15	14.24
40	21.31 ± 0.09	16.58	20.51 ± 0.10	4.97
20[a]	22.87 ± 0.04	13.64	21.72 ± 0.12	18.57
10[b]	24.13 ± 0.05	23.02	22.25 ± 0.16	14.12
5	26.33 ± 0.06	62.34	22.68 ± 0.14	58.88
2.5	26.22 ± 0.04	19.16	27.25 ± 0.21	89.51
1.25[c]	27.65 ± 0.15	34.89	25.11 ± 0.04	2.83
NTC gDNA[d]	n.d.	n/a	40.50 ± 0.61[e]	n/a
NTC H_2O	n.d.	n/a	n.d.	n/a

Abbreviations: LOD, limit of detection; LOQ, limit of quantification; n.d., not detected; n/a, not applicable. As a result of the nested qPCR approach 1280 to 1.25 copies of circular plasmid become detectable in a C_q range between 15.95 and 27.65. LOD was 1.25 copies for both assays, LOQ between 10 and 20 copies. Five replicates were used in the pre-amplification round, seven replicates were used in the qPCR round. [a]LOQ for nested qPCR assay #1. [b]LOQ for nested qPCR assay #2. [c]LOD. [d]Negative template control with 500 ng of naive hgDNA per replicate in the pre-amplification round. [e]Two out of seven replicates produced PCR signal as a result of slight background signal.

Validation of the two nested qPCR assay parameters

A standard curve was generated in each of the laboratories Mainz (Germany) and Gainesville (Florida, USA) to evaluate the specificity of each nested qPCR assay, as well as the linearity, dynamic range, LOD, and LOQ. As described in the material and methods, different Nano-Drop machines, PCR cyclers, and second round qPCR chemicals were used. A standard sample containing 1280 copies of rAAV hEPO cDNA circular plasmid was diluted sequentially 2-fold for 10 times to prepare an 11- point standard curve. The samples were subsequently spiked into a background of 500ng of naïve hgDNA. Additional negative control samples (NTCs) containing naïve hgDNA or water were included. Five replicates of each sample were PCR'd in the pre-amplification round, pooled and 7 replicates were then analyzed in the nested qPCR round. All of the samples were detected by both nested qPCR assays, thus demonstrating a 4-log dynamic range. Using the nested qPCR approach, 1280–1.25 copies of circular plasmid became detectable in a quantification cycle (C_q) range between 15.95 and 27.65 (Table 2). Additionally, samples containing 0.625 copies were reproducibly detected (data not shown). This amount was not included in LOD/LOQ determinations, since the maximum possible number of standard curve samples that can be tested in a single 96-well plate is reached when including the standard curve samples of 1280 to 1.25 copies. For nested qPCR Assay #1 the amplification efficiency was 90% with a linearity value of 0.995, LOD of 1.25 copies, and a LOQ of 20 copies of circular plasmid (Table 2). For nested qPCR Assay #2 the amplification efficiency was 111% with a linearity value of 0.996, LOD of 1.25 copies, and a LOQ of 10 copies of circular plasmid (Table 2).

The negative control samples did not generate a PCR signal for nested qPCR Assay #1. Nested qPCR Assay #2 generated slight background signals, where 2 out of the 7 negative control replicates containing hgDNA generated a PCR signal with a mean C_q of 40.50 ± 0.61 (Table 2). This corresponds to a signal-to-background spread of about 13 C_qs.

Evaluation of the two nested qPCR assays on blinded samples

A blinded study was performed to confirm the assay validation results. Known amounts of rAAV hEPO plasmid DNA were spiked into hgDNA in Nantes (France) and sent in randomly numbered tubes to Mainz (Germany) and Gainesville (Florida, USA) for nested qPCR testing. Five replicates of each sample were PCR'd in the pre-amplification round, pooled and diluted. For the second qPCR round the number of replicates was reduced from 7 to 3. The reliability of the overall assay is not influenced negatively by reducing the number of qPCR replicates, as C_q standard deviations (SDs) observed during the evaluation of the assay parameters were low (Table 2). The number of pre-amplification replicates was kept at 5 to maintain the high precision and accuracy of the assays.

The unspiked blinded sample containing just hgDNA generated no signal, thus confirming the specificity of both assays. The assays detected the target template in all 6 blinded samples containing 1000, 100, 50, 10 or 2 copies of the hEPO cDNA plasmid (Figure 4). These results provide further evidence that the assays have a 4-log dynamic range and the ability to reliably detect 2 copies of circular plasmid, which is close to the LOD of 1.25 copies for both assays (Figure 4). The calculation of the copy numbers for the blinded samples revealed higher copy number than the calculated copy number of plasmid (based on nanospectometry) spiked into the experiment.

Detection of hEPO cDNA in WBCs of transduced macaques

To further demonstrate the suitability of the nested qPCR assays for gene doping surveillance, a proof of principle non-human primate study was conducted. It is notable that the number of macaques was not selected with respect to statistical significance. Two macaques received different doses of rAAV8 vectors containing the promoterless EPO cDNA cassette. A third macaque served as a non-injected control (see Figure 3b). The vector doses (~2.5×10^{10} vg/kg for Mac 1 or ~2.5×10^{11} vg/kg for Mac 2) were selected with regard to a former study.[34] The previous study used a similar vector dosage and injection site than the present one, but the rAAV8 vector system used contained a different vector cassette (macaque EPO cDNA under control of CMV promotor). The gene transfer led to long term transgene expression and transgene detectability up to 57 weeks (latest timepoint analyzed).[34]

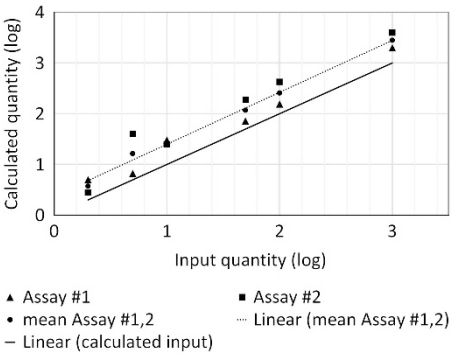


Figure 4. Expected and calculated copy numbers of the blinded samples. Seven randomized blinded samples that were spiked with 1000, 100, 50, 10, 5, 2 or 0 copies of circular plasmid were analyzed in different laboratories. All samples were detectable except the NTC which were negative in both assays. The results are in parallel with the spike-in, however both assays detected greater than the calculated copy numbers. The input quantity is plotted against the copy number calculations of Assays #1 and #2 as well as the mean results.

Before analyzing the macaque samples, we examined the possibility of primer specificity and amplification efficiency differences due to using different sources of background DNA (macaque genomic DNA versus hgDNA). We compared C_q values between samples containing 500 ng of each type of gDNA spiked with rAAV hEPO plasmid DNA. At plasmid copy numbers greater than 10 no significant differences in amplification efficiency were observed (data not shown). hgDNA was selected as the source of background DNA to prepare plasmid standard curves used to quantify the macaque samples since the assays were validated using hgDNA and the eventual application of these assays will involve human DNA samples. Additionally, the specificity of the nested qPCR assays for hEPO in the presence of macaque gDNA was examined through the use of negative control samples containing only naïve macaque genomic DNA. These samples were not detected by the nested qPCR assays (data not shown).

The hEPO cDNA sequence in the viral vector was constructed without a promoter to avoid expressing the hEPO transgene product that had the potential of eliciting an adverse immunological response in the macaques.[41] Prior to vector injection, the serum of Macaque 1 (Mac 1) and Macaque 2 (Mac 2) were screened for anti-AAV8 neutralizing factors using an *in vitro* neutralization assay. The screening results showed that both macaques were negative for anti-AAV8 neutralizing factors (Supplementary Table 1).

The maximum vector copy number detected for Mac 1 was at day 1 post-injection (p.i.): ~859 copies were detected by nested qPCR Assay #1, (Table 3) and ~1287 copies were detected by nested qPCR Assay #2 (Table 4). The transgene was detectable for up to three weeks (Tables 3 and 4). The overall quantity of hEPO cDNA measured in the WBCs of Mac 2 was higher than Mac 1, which is expected since Mac 2 received a 10-fold higher dose of vg/kg (Figure 3b). The highest template copy number was detected at 1 day p.i.: ~2772 copies (nested qPCR Assay #1; Table 3) and 7118 copies (nested qPCR Assay #2; Table 4). At 3 days p.i. there was a sharp decline in the viral vector genome copy number, followed by a gradual decrease of vector copy number that was maintained below 10 copies after 3 weeks p.i. (nested qPCR Assay #1; Table 3) or 3 days p.i. (nested qPCR Assay #2; Table 4). In Mac 2 the transgene was detectable for 14 weeks (nested qPCR Assay #1) and 8 weeks (nested qPCR Assay #2), respectively. The one step qPCR analysis (i.e. without the pre-amplification round) showed similar results, however, due to the expected lower sensitivity of the qPCR assays the viral vector was not detected at the longer points (Tables 3 and 4). The samples of Mac 2 were measured twice with Assay # 2 as the 2 copy quality control sample was negative in first pass. The results of the re-analysis were similar with regard to copy number calculation and negativity of NTCs (data not shown). All samples of the non-injected macaque, Mac 3, generated negative signals in the nested qPCR assays and the single round qPCR assay (Tables 3 and 4).

When comparing the period of transgene detectability to the previous study conducted by Ni *et al.*,[34] it was shorter than anticipated. Especially Mac 1 showed a rapid clearance of the hEPO cDNA from WBCs. In the former study a macaque that had received a similar vector dose to Mac 1 had the transgene detectable for up to 26 weeks p.i.[34]

Possible reasons for the brief duration of the viral vector genome in the Mac samples are i) degraded sample DNA, ii) presence of PCR inhibitors or endonucleases, iii) low-level expression of the hEPO that elicited an immunological response which eliminated transduced cells, and iiii) an innate immune response against the vector sequence. To address the possibility of degraded DNA, a qPCR assay previously designed to detect the endogenous macaque ε-globin gene was performed.[34] The results showed that the integrity of the genomic DNA was intact for all

Table 3. Assay #1

Time point	*Macaque 1*		*Macaque 2*		*Macaque 3*	
	Nested qPCR	*qPCR*[a]	*Nested qPCR*	*qPCR*[a]	*Nested qPCR*	*qPCR*[a]
Before injection	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
30 min p.i.	7.56 ± 0.70	5.51 ± 4.75	160.92 ± 5.70	269.15 ± 37.47	Not tested	Not tested
1 Day p.i.	859.06 ± 22.42	2811.58 ± 15.05	2772.18 ± 86.33	3609.13 ± 481.21	n.d.	n.d.
3 Days p.i.	86.64 ± 4.11	310.72 ± 51.35	224.69 ± 4.78	473.39 ± 6.46	n.d.	n.d.
10 Days p.i.	n.d.	n.d.	17.46 ± 1.00	128.94 ± 6.77	n.d.	n.d.
16 Days p.i.	0.97 ± 0.02	n.d.	8.41 ± 0.23	54.05 ± 0.00	n.d.	n.d.
3 Weeks p.i.	0.91 ± 0.06	n.d.	4.01 ± 0.08	6.71 ± 3.12	n.d.	n.d.
4 Weeks p.i.	n.d.	n.d.	1.18 ± 0.02	n.d.	n.d.	n.d.
6 Weeks p.i.	Not tested	Not tested	0.83 ± 0.02	n.d.	Not tested	Not tested
8 Weeks p.i.	Not tested	Not tested	1.79 ± 0.07	n.d.	Not tested	Not tested
14 Weeks p.i.	Not tested	Not tested	1.06 ± 0.02	n.d.	Not tested	Not tested
18 Weeks p.i.	Not tested	Not tested	n.d.	n.d.	Not tested	Not tested
26 Weeks p.i.	Not tested	Not tested	n.d.	n.d.	Not tested	Not tested

Abbreviations: n.d., not detectable; p.i., post injection. Detection of transgenic hEPO cDNA copy numbers in macaque WBC samples. Using the nested qPCR approach, the transgene could be detected over an extended period of time compared with the one round qPCR approach. [a]$n = 2$ replicates.

Table 4. Assay #2

Time point	*Macaque 1*		*Macaque 2*		*Macaque 3*	
	Nested qPCR	*qPCR*[a]	*Nested qPCR*	*qPCR*[a]	*Nested qPCR*	*qPCR*[a]
Before injection	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
30 min p.i.	1.68 ± 0.06	n.d.	45.19 ± 5.20	17.61 ± 4.82	Not tested	Not tested
1 Day p.i.	1287.74 ± 52.80	1885.12 ± 127.98	7118.21 ± 175.02	10968.80 ± 0.00	n.d.	n.d.
3 Days p.i.	72.56 ± 4.35	134.23 ± 16.35	176.19 ± 9.07	95.43 ± 5.62	n.d.	n.d.
10 Days p.i.	0.16 ± 0.01	0.26 ± 0.19[b]	2.82 ± 0.14	0.10 ± 0.07[b]	n.d.	n.d.
16 Days p.i.	0.02 ± 0.00	n.d.	0.57 ± 0.04	n.d.	n.d.	n.d.
3 Weeks p.i.	0.01 ± 0.00	n.d.	2.67 ± 0.08	0.61 ± 0.55[b]	n.d.	n.d.
4 Weeks p.i.	n.d.	n.d.	0.42 ± 0.04	n.d.	n.d.	n.d.
6 Weeks p.i.	Not tested	Not tested	1.19 ± 0.02	0.40 ± 0.18[b]	Not tested	Not tested
8 Weeks p.i.	Not tested	Not tested	1.16 ± 0.05	n.d.	Not tested	Not tested
14 Weeks p.i.	Not tested	Not tested	n.d.	n.d.	Not tested	Not tested
18 Weeks p.i.	Not tested	Not tested	n.d.	n.d.	Not tested	Not tested
26 Weeks p.i.	Not tested	Not tested	n.d.	n.d.	Not tested	Not tested

Abbreviations: n.d., not detectable; p.i., post injection. Detection of transgenic hEPO cDNA copy numbers in macaque WBC samples. Using the nested qPCR approach, the transgene could be detected over an extended period of time compared with the one round qPCR approach. [a]$n = 2$ replicates. [b]Samples are not detectable, the signals are a result from background noise.

macaque samples (Supplementary Table 2). Subsequently, a spike-in experiment was performed with the samples from Mac 1. 1000 copies of GFP plasmid were spiked into the samples and then quantified by a previously designed qPCR assay.[42] The results indicated that the samples were free of PCR inhibitors (Supplementary Figure 1a). Additionally, endonuclease activity was excluded by incubating the samples at 37 °C for 1 h prior to GFP qPCR (data not shown). The sera of Mac 1 and Mac 2 were free of hEPO protein, as evaluated by an ELISA assay with a sensitivity of 1.25 mU/ml (data not shown). To further confirm the absence of an immune response against low-level hEPO expression a Western blot analysis was performed to detect potential anti-hEPO antibodies in the sera of Mac 1 and Mac 2. No anti-hEPO antibodies were detectable up to 12 weeks p.i. (Supplementary Figures 1b and c). Subsequently, the DNA sequence of the promoterless vector insert sequence was re-analyzed and compared to the insert sequence used by Ni *et al.*[34] with regard to putative CpG islands, using EMBOSS Cpgplot software.[43] Five putative CpG islands were found in the vector cassette used in this study, compared to 2 putative CpG islands in the vector insert used in the study by Ni *et al.*[34] (Supplementary Figure 2). Furthermore, the promoterless hEPO vector cassette contained the CpG motif 5′-GTCGTT-3′, which is the strongest TLR9 recognition site in humans and macaques.[44]

DISCUSSION

Assay validation results demonstrated that the nested qPCR assays exhibited specificity, ultra-sensitivity, large dynamic range, and the ability to accurately quantify small amounts of the hEPO cDNA sequence with precision and accuracy. The assay specificity observed confirmed the predictions from the *in-silico* analysis. The LOD for both of the nested qPCR assays is comparable to the nested PCR assay previously described and enables highest sensitivity.[18] Since the assays described here utilize qPCR technology, this approach for detecting the hEPO cDNA sequence is objective and quantitative versus gel-based readouts. Moreover, it is important to note that the operators from different laboratories, which were using different PCR machines, were able to obtain a similar LOD and LOQ for the two nested qPCR assays that share the same pre-amplification method, but differ in the qPCR round reactions. These interlaboratory results demonstrate

the ruggedness of the pre-amplification round and that there is some flexibility in the qPCR design used after pre-amplification as similar sensitivities were obtained.

The LOQ determined for both nested qPCR assays was similar: 20 copies for nested qPCR Assay #1 and 10 copies for nested qPCR Assay #2 (Table 2). This is most probably due to the fact that both assays share the same pre-amplification round, generate similar sized amplicons in the nested qPCR round, and utilize primers and probe in the nested qPCR with proximal binding sites (Figure 2). Moreover, the blinded study demonstrated that both assays detected all the unknown samples, including the sample spiked with 2 copies of hEPO cDNA. Both assays detected higher copy numbers for the blinded samples than was expected from the plasmid copy number spiked in. This can be influenced by the different spectrophotometers that were used to measure the concentration of stock plasmid for the preparation of blinded samples in one laboratory and the preparation of standard curve samples in the other laboratories. Additional contributing factors may be the use of different operators and due to pipetting error and the inherent variability of pooled hgDNA from different individuals that was used as the background DNA between laboratories.

Implementing the nested qPCR assays in the non-human primate study, designed to mimic the setup and conditions of a human gene doping test setting, demonstrated that the assays have superior sensitivity to one-round qPCR. With this increased sensitivity the assays demonstrated that the rAAV8 vector harboring the hEPO cDNA sequence was cleared from the WBCs of Mac 2 14 weeks p.i. (nested qPCR Assay #1, Table 3) to 8 weeks p.i. (nested qPCR Assay #2, Table 4). Comparing these results to a previous study where a macaque was injected with a 1-log lower dose of a rAAV8 vector harboring an expressible macaque EPO sequence (detected in WBCs up to the longest timepoint analyzed: 57 weeks p.i.)[34] indicates that the unexpressed hEPO cDNA sequence harbored in the rAAV8 vector was maintained for a much shorter time in the WBCs of Mac 2.

Referring to the CpG analysis it is possible that the short-term persistence of the vector was due to an immune response against the DNA sequence of the vector cassette used in this study. Using EMBOSS Cpgplot analysis tool [43] it became obvious that the promoterless hEPO vector cassette used in this study contained 5 putative CpG islands compared to 2 putative CpG islands in the vector cassette used by Ni *et al.*[34] Moreover, the promoterless hEPO vector cassette contained the 5′-GTCGTT-3′ CpG motif, which is the strongest TLR9 recognition motif in human and macaque WBCs.[44] This motif was not present in the vector cassette used in the study by Ni *et al.*[34]

The TLR9 receptor recognizes unmethylated viral CpG motifs which initiates the activation of signaling pathways leading to the production of inflammatory cytokines. Finally, this leads to the initiation of innate and adaptive immune responses. As recently described by Faust *et al*., CpG-depleted vector DNA is less immunogenic following rAAV8 mediated gene transfer in skeletal muscle and led to attenuated T cell responses, the inhibition of MHCII expression, and promoted transgene stability.[45]

It is notable that vector was designed to avoid any kind of transgene expression, to circumvent possible immune response against the human EPO protein. Therefore, it was not possible to investigate the transduction efficiency indirectly by an increase in hematocrit or directly by transgene expression. In principle, the period of possible transgene detection goes along with the transduction efficiency. Higher transduction efficacies and lower immune reaction against the transgene and transduced cells should lead to longer transgene detectability. To make a conclusive and meaningful evaluation for the limit of transgene detectability, the transduction efficiency and transgene expression need to be evaluated.

The highest vector copy number observed was at 1day p.i. (859-1288 copies for Mac 1 and 2772-7118 copies for Mac 2). In this study and the Ni *et al.*[34] study, after 2 weeks p.i. the vector copy number dropped below ten copies. These results underscore the importance of implementing ultra-sensitive assays for the detection of the hEPO cDNA transgene, since it is probable that if gene doping occurs the transgene will be present in the blood of athletes at very low copy numbers.

There are drawbacks with the nested qPCR assays presented in this study. One issue is related to the laborious nature of the assay as it includes the preparation of replicate samples for two rounds of PCR, but these can be made less laborious by the use of automated liquid handling workstations. Other issues include the risk of laboratory contamination and cross contamination between test samples and standard samples during the setups for PCR. Some potential solutions to these contamination issues are to (i) modify the standard curve template (incorporate additional nucleotides or a restriction site within the amplicon sequence) so it can be distinguished from target DNA; a strategy that has been previously used[20] or (ii) convert these quantitative assays into qualitative assays by eliminating the use of standard samples and incorporating a synthetic and distinguishable internal threshold control (ITC) template added into each sample at a fixed copy number that is near the LOD of each assay; a strategy that has been described previously.[22]

To increase the sensitivity for transgene detection, it is typically recommended to linearize circular plasmids due to DNA melting constraints of circles. Recent qPCR and ddPCR assays have achieved a high level of sensitivity (5 copies of linearized hEPO cDNA in 400 ng of hgDNA extracted from plasma,[23] or 10 copies of linearized hEPO cDNA in 500 ng of hgDNA extracted from whole blood; no lower concentrations were tested).[24] However, the linearization of samples for doping surveillance is complicated by the fact that the sequence of the illicit vector system would be unknown before analysis. Hence, a restriction site within the target gene sequence must be found which is not near or between primer and probe binding sites. As it shown in this study we are able to achieve ultra-sensitive detection of the hEPO cDNA sequence (~1 copy of circular transgene in 500 ng of hgDNA) without the need to linearize samples before analysis.

The use of ddPCR technology for vector-transferred exogenous DNA detection poses a number of advantages over qPCR, including reproducibility and interlaboratory accuracy. Additionally, ddPCR has the advantage of not requiring standard samples to quantify unknown samples and hence reduces the risk of laboratory contamination and sample cross contamination. However, since this is a new technology there are conflicting results in the literature about whether this technique has better sensitivity and if it is more cost efficient than conventional qPCR.[46–49] The different cost matrices (instruments, reagents, and labor costs) used by various approaches makes it difficult to discern the actual cost of implementing ddPCR. The most relevant issue is that currently used digital PCR platforms can lead to false positive signals.[50,51] These false-positive signals cannot be differentiated from true-positive signals. Therefore, typical nested PCR approaches should be preferred in a setup of low-copy number detection with the need for discriminating between the presence or absence of foreign DNA.[50]

The surveillance of gene doping in sports is critical in maintaining the integrity of sports and ensuring the safety of all athletes. It is therefore imperative to implement a legally defensible test that can detect low copies of vector-transferred exogenous DNA, as well as be able to detect DNA sequences engineered to circumvent detection: insertion and deletion of DNA nucleotides within the amplicon that prevent proper annealing of primers and probes, as well as silent mutations. The nested qPCR assays presented in this study were shown to be extremely sensitive and reliable and with a common

pre-amplification round, demonstrating flexibility for a qPCR detection step. These assays were therefore shown to be suitable for the surveillance of gene doping with circular (plasmid or rAAV[21,24]) or linear vectors harboring transgenes such as the hEPO cDNA sequence. In the future this approach will be applied for the detection of engineered DNA sequences that have been manipulated to avoid detection. Lastly, we believe that the nested qPCR approach can be further extended into clinical and research settings where extremely low amounts of DNA sequences, such as infectious disease agents, transgenic plant DNA, cancer related circulating nucleic acids, gene therapy vectors and other gene doping transgenes, can be detected.

MATERIALS AND METHODS

Ethics statement

Pooled human whole blood samples used for hgDNA isolation for qPCR Assay #1 and nested qPCR Assay #1 was obtained from the Blood Bank at the University of Florida, Shands Hospital. The collection of blood samples was approved by the Institution Review Board at the University of Florida. For qPCR Assay #2 and nested qPCR Assay #2 blood was collected by venipuncture from 3 healthy subjects into EDTA blood tubes (Order number 01.1621.001, Sarstedt, Nümbrecht, Germany) and pooled subsequently. This collection of blood samples was approved by the Human Ethics Committee Baden Württemberg. For blinded samples, human whole blood samples were obtained from the Blood French Establishment of Nantes, under authorization NTS 2005-01.

Outbred cynomolgus macaques were purchased from Bioprim (Baziège, France). Macaques were housed in an enriched environment with access to toys, fresh fruits, and vegetables at the Boisbonne Center, under protocol # CEEA.2011.47 that was approved by the Institutional Animal Care and Use Committee of the Pays De Loire. This study was authorized by the Departmental Direction of Veterinary Services (Loire-Atlantique, France) and conducted with the recommendations of the Weatherall report: 'the use of nonhuman primates in research'. During the study, macaques were screened daily and hematological and biochemical parameters were tested monthly. The IM injection of rAAV vectors is considered a mild severity procedure, so during the procedure macaques were anesthetized with an IM injection of 7 mg/kg ketamine (IMALGENE, Merial, Lyon, France) and 30 µg/kg Medetomidine (Domitor, Pfizer, Paris, France).

DNA extraction from human and macaque WBCs

The whole blood samples collected from humans and macaques were used to extract gDNA from WBCs, according to a modified protocol of the Gentra Puregene Blood Kit (Cat no. 158467, Qiagen, Hilden, Germany) with slight modifications. The samples were not vortexed but mixed by pipetting several times. In order to optimize gDNA recovery, glycogen solution (Cat. no. 158930, Qiagen, Courtaboeuf, France) was added to obtain a final concentration of 200 µg/ml in isopropanol. An additional centrifugation step at 2000 × g for 1 min was also added to further remove the 70% ethanol before the gDNA was resuspended in DNA hydration solution. Subsequently, the DNA was incubated at room temperature for 10 min to 1 h and incubated at 4 °C overnight without shaking. The concentration and purity of the isolated gDNA was measured and assessed with a nanophotometer (nested qPCR Assay #1; Implen, Westlake Village, CA, USA) or a NanoDrop1000 (nested qPCR Assay #2; Thermo Scientific, Wilmington, DE, USA). Moreover, the integrity of hgDNA was determined by gel electrophoresis and ethidium bromide staining of 1 µg of hgDNA on a 0.8% agarose gel. Low molecular weight bands observed in the gel indicated DNA degradation and a high molecular weight band was considered intact DNA. The integrity of macaque genomic DNA and lack of PCR inhibitors was assessed by testing 500 ng of sample DNA using a qPCR assay targeted to the endogenous macaque ε-globin gene.[34]

Nested qPCR assay

Pre-amplification round by conventional PCR. Five replicates of a sample were pre-amplified. The outward primer pair for the pre-amplification round was designed previously.[18] The PCR reaction contained 2X GoTaq Green Master Mix (Cat. No.: M7122, Promega, San Luis Obispo, CA, USA), 900 nM primers, 500 ng of gDNA, and water to attain a final vol of 25 µl. The T100™ Thermal Cycler (nested qPCR Assay #1; Bio-Rad, Hercules, CA, USA) with an average ramp rate of 2.5 °C/s and the Eppendorf Mastercycler ep Gradient S (nested qPCR Assay #2; Eppendorf, Hamburg, Germany) with an average ramp rate of 40% were used with this optimized PCR program: 95 °C for 3 min 30 s, 25 cycles of 94 °C for 15 s, 62 °C for 1 min, and 72 °C for 45 s, followed by 72 °C for 7 min.

Nested qPCR round. The replicates of the pre-amplified sample were pooled and then diluted 1:50[19] in 500 µl of water. Afterwards, 3 µl of the pooled sample was analyzed in 7 replicates for the establishment of the LOQ in the standard curve and in triplicates for the measurement of further samples. For nested qPCR Assay #1 the PCR reaction and the PCR program used were previously described.[22] The PCR reaction was scaled up to a total reaction vol of 30 µl and the StepOnePlus™ qPCR system was the instrument used (Life Technologies, Grand Island, NY, USA). For Nested qPCR Assay #2 the sequence of the forward primer was 5′-AGAATATCACGACGGGCTGT-3′ and the reverse primer was 5′-AGGCCCTGCCAGACTTCTAC-3′ (Integrated DNA Technologies, San Diego, California, USA). The sequence of the locked nucleic acid probe was [FAM]AAG[+A]GG[+A]TG[+G]AG[+G]TCGG[BHQ1] (Sigma-Aldrich, St Louis, MO, USA). The PCR reaction mixture was prepared in 10 µl and contained 1X SsoFast™ Probes Supermix (Cat. no. 172-523, Bio-Rad, Munich, Germany), 900 nM primers, and 250 nM probe. The CFX 384 Touch Real-Time PCR detection system (Bio-Rad, Munich, Germany) was used with the following optimized PCR program: 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 63 °C for 30 s.

Nested qPCR quantification strategy

To calculate the copy number of the blinded and macaque samples, the data generated by nested qPCR was linearly fitted with the linear equation obtained from the LOD/LOQ standard curve for each of the two assays. Beforehand, the threshold-line for C_q determination of each plate (for all samples) was adjusted to an internal 100 copy quantification calibrator. Adjacent to this calibrator on each plate the following samples were included: a negative template control sample to detect possible carry over contamination from the pre-amplification round or the nested qPCR round, a 2 copy control sample to control for amplification efficiency near the LOD (assay validity), and an additional 1000 copy control sample. All control samples were prepared in a background of 500 ng of naïve hgDNA.

qPCR

The standard one round qPCR for Assay #1 was performed as in the nested qPCR Assay #1. In the one round qPCR for Assay #2 the components were scaled up to a final reaction vol of 20 µl based on the concentrations used in the nested qPCR Assay #2. The qPCR was performed without the pre-amplification step to compare the results to nested qPCR.

In vitro study to evaluate assay parameters

An 11-point standard curve was prepared from a stock of circular plasmid pSSV9-promoterless-pA-hEPO-LacZ. The DNA concentration of the stock was measured with a nanophotometer (nested qPCR Assay #1, Implen, Westlake Village, CA, USA) or a fluorospectrometer (nested qPCR Assay #2; NanoDrop 3300, Thermo Scientific; Wilmington, DE, USA). These values along with the mass of the plasmid were used to calculate the template copy number/µL. The stock of plasmid was diluted in 1X Tris-EDTA to prepare a standard of 1280 copies/8 µl. This was then diluted 2-fold sequentially 10 times. These standards were spiked into a background of 500 ng of naïve hgDNA. Five replicates of each copy number standard sample were used in the pre-amplification round. Following the pooling of replicates and dilution, 7 replicates were analyzed by nested qPCR. The LOD was designated as the template copy number distinguishable from background with a 95% confidence level. The LOQ was designated as the template copy number that produced an average error ≤20%. The LOQ was calculated using the following steps and equations: (1) the expected template copy number (x-axis) was plotted against the mean C_q value for each standard sample (y-axis) (2) the experimental data was fitted and the linear equation obtained (1-1) related the C_q value of each standard replicate sample to the experimentally derived copy number.

$$C_q = slope \cdot \ln(hEPOcopies_{\substack{\text{experimentally} \\ \text{derived}}}) + y - \text{intercept} \quad (1)$$

Equation 1 was then manipulated as shown in Equation 2 so that the experimentally derived template copy number could be extrapolated for all standard replicate samples using the C_q value obtained.

$$hEPOcopies_{\substack{\text{experimentally} \\ \text{derived}}} = 10^{\frac{y-\text{intercept}-C_q}{slope}} \quad (2)$$

The average error (%) was calculated for each standard sample according to Equation 3.

$$100 \times \frac{Mean\left|\left(hEPOcopies_{\substack{experimentally \\ derived}} - hEPOcopies_{actual}\right)\right|}{hEPOcopies_{actual}} \quad (3)$$

Blinded sample study

The DNA concentration of a stock of circular pSSV9-promoterless-pA-hEPO-LacZ plasmid was measured with a nanophotometer (Implen, Munich, Germany) and the plasmid mass was then used to calculate the template copy number. The plasmid was then diluted to prepare the blinded samples containing 500 ng of hgDNA extracted from WBC spiked with 0, 2, 5, 10, 50, 100, or 1000 copies of plasmid. The blinded samples were prepared in Nantes, France, and were sent to Operator #1 at the University of Florida and Operator #2 at the University of Mainz at ambient temperature. Following quantification of the samples by the nested qPCR assays the samples were decoded in Nantes, France.

rAAV vector production

The pSSV9-promoterless-pA-hEPO-LacZ plasmid (6893 bp) harbors a 3012 bp non-expressing cassette placed between two inverted terminal repeat sequences of AAV serotype 2. This non-coding cassette contains the complete hEPO cDNA sequence, followed by a partial Lac Z sequence (2218 bp), in which the absence of an open reading frame was confirmed (Figure 3a). To overcome possible transcription initiation from the ITR sequence, a synthetic poly A sequence was also inserted between the first inverted terminal repeat sequence and the hEPO cDNA sequence. This plasmid was generated and used by the vector core at Atlantic Gene Therapies, University Hospital of Nantes (Nantes, France) to produce the rAAV8 viral vector harboring the promoterless hEPO cDNA vector genome. Plasmid sequence and length was verified by sequencing and gel electrophoresis (data not shown). Sequencing of the vector revealed a point mutation at nucleotide 599 of the hEPO coding sequence (NM_000799). This mutation is a missense mutation (140G>R), which does not affect the expression of the resulting hEPO protein, and was not located within the regions of binding of our primers/probe sets. Vector production was carried out as previously published.[34] The absence of hEPO expression from this expression cassette was verified after *in vitro* transduction of HeLa cells using different high multiplicity of infection of rAAV8 phEPO vector (multiplicity of infection 1500 to 5000+co-infection with wild type adenovirus type 5). The supernatant was analyzed using a specific hEPO enzyme-linked immunosorbent assay (Quantikine IVD kit, Cat. no. DEP00, R&D Systems, Lille, France).

Administration of rAAV8 harboring the hEPO cDNA in macaques

At the time of injection, the 3 male macaques were ~3 1/2 years of age and weighed ~5 kg. The site of injection was pre-tattooed on the right tibialis anterior muscle at least two weeks before injection. The dose was administered in one injection with a total vol of ~400 µl. Mac 1 received a total dosage of 1.3×10^{11} viral genomes (2.5×10^{10} viral genomes/kg), Mac 2 received a dosage of 1.3×10^{12} viral genomes (2.5×10^{11} viral genomes/kg), and Mac 3 served as the non-injected control animal.

Anti-AAV8 Neutralizing factors analysis

The possibility of circulating anti-AAV8 neutralizing factors was analyzed in animal sera using an *in vitro* transduction inhibition assay. Briefly, serum dilutions were made in culture medium at dilutions 1:5, 1:10 and 1:20. The diluted sera were pre-incubated with a rAAV8-CMV-LacZ viral vector preparation for 30 min at room temperature to allow binding of potential neutralizing factors. The mixture (serum/AAV viral preparation at a multiplicity of infection of 4000) was added to HeLa cells and then incubated for 24 h at 37 °C. To facilitate rAAV-based transduction, HeLa cells were pre-infected with adenovirus type 5 (multiplicity of infection = 8) for 2 h at 37 °C. The following day, LacZ transduction (i.e. ß-galactosidase expression) was semi-quantitatively assessed by light microscopy after an X-Gal-based staining. Each assay included controls: HeLa transduction in the absence of serum, HeLa transduction in the presence of a known positive serum as well as with a neutralizing-negative serum.

In vivo hEPO expression analysis

Serum hEPO levels were measured by enzyme-linked immunosorbent assay using the Quantikine IVD kit (Cat. no. DEP00, R&D Systems, Lille, France).

Anti-hEPO response analysis

Five hundred nanograms of recombinant human EPO protein (Eprex, Janssen-Cliag) was subjected to electrophoresis on a 10% polyacrylamide SDS-PAGE gel (Cat. no. NP0315, Life Technologies, Saint-Aubin, France) and then transferred to a Hybon electrochemiluminescence nitrocellulose membrane (Cat. no. 45-000-929, GE Healthcare Life Sciences, Velizi-Villacoublay, France). The membrane was then cut into strips, and after blocking, each membrane strip was incubated with a dilution of individual macaque serum. The presence (or absence) of an anti-hEPO antibody was revealed using a secondary antibody specific for non-human primates (Rhesus macaca) IgG conjugated with peroxidase (Cat. no. 6200-05, Clinisciences, Nanterre, France). The result was obtained after chemiluminescence detection (Cat. no. 32106, Life Technologies, Saint-Aubin, France). As negative controls, the serum of each animal before rAAV injection was used. As positive controls, serum from a previous animal (House) that had developed anti-cynomolgus macaque EPO antibodies[34] was used.

CONFLICT OF INTEREST

ROS is an inventor on patents related to rAAV technology. ROS owns equity in a gene therapy company that is commercializing adeno-associated virus for gene therapy applications. To the extent that the work reported in this article increases the value of these commercial holdings, R.O.S. has a conflict of interest.

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