Establishing a novel single-copy primer-internal intron-spanning PCR (spiPCR) procedure for the direct detection of gene doping

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ABSTRACT

So far, the abuse of gene transfer technology in sport, so-called gene doping, is undetectable. However, recent studies in somatic gene therapy indicate that long-term presence of transgenic DNA (tDNA) following various gene transfer protocols can be found in DNA isolated from whole blood using conventional PCR protocols. Application of these protocols for the direct detection of gene doping would require almost complete knowledge about the sequence of the genetic information that has been transferred. Here, we develop and describe the novel single-copy primer-internal intron-spanning PCR (spiPCR) procedure that overcomes this difficulty. Apart from the interesting perspectives that this spiPCR procedure offers in the fight against gene doping, this technology could also be of interest in biodistribution and biosafety studies for gene therapeutic applications.

Key Words: Gene doping, gene therapy, gene transfer, direct detection, spiPCR, transgenic DNA

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INTRODUCTION

The World Anti-Doping Agency (WADA) gives the following description for the forbidden method gene doping in its upcoming 2009 Prohibited List: *"The transfer of cells or genetic elements or the use of cells, genetic elements or pharmacological agents to modulat[e] expression of endogenous genes having the capacity to enhance athletic performance, is prohibited."*

Modulation of the expression of endogenous genes is typically achieved by virtually every conventional doping substance including human or non-human peptides (1-4) and anabolic androgenic steroids (5). Accordingly, scientific articles on gene doping usually point out or take for granted that gene transfer technology has to be used in order to justify the term *gene doping* (6-16).

In the following, we will therefore use the term *gene doping* in its stricter definition that is generally approved within the scientific community - as the abuse of gene transfer technology to enhance athletic performance. According to this definition an athlete who practices gene doping incorporates "an extra" amount of genetic information (DNA or RNA) by means of gene therapeutic procedures. The added genetic information itself can be of human origin and is not the direct source of the performance enhancing effect. If the incorporated genetic information is DNA, it is called *transgenic* DNA (tDNA) and serves as a template to produce a protein within the athlete's body that is known to improve physical performance, such as erythropoietin (EPO) (17). More recently, the development of antisense RNA technology and advances in the delivery of such RNA molecules have additionally opened the possibility to specifically inhibit the production of proteins that limit or restrict physical performance on a natural basis (18).

The definition given above already implicates the three major and unique problems associated with gene doping, which are as follows:

I. The genetically modified athlete
Depending on the stability and functionality of the introduced genetic information an athlete could have a permanently genetically modified physical performance. This imposes exceptional ethical concerns (14;19).

II. Undetectability
Gene doping is regarded as principally undetectable, since the introduced gene is of human origin and the protein which mediates the performance enhancing characteristics is even built within the athlete's own body (8-10;13).

III. Safety concerns
Gene therapeutic interventions are subject to very tight safety regulations (20-22). The unapproved use of gene transfer technology in athletes may not only be of high risk to the individual, but also to others that get in contact with, or inappropriately handle substances required for gene transfer. On top of this it is well known that intensive exercise can severely influence the innate immune response (23-27). Especially in elite athletes this might have unknown consequences on the first line of immune response to gene transfer related interventions that are conducted without appropriate medical supervision.

Currently it is only assumed that athletes may already practice *gene doping* (7). Nevertheless, the subject gene doping, i.e. the consideration of candidate genes and techniques for potential abuse and its potential detection strategies, has been reviewed in 27 scientific articles during the past 5 years. Table 1 summarizes solutions for detection strategies of gene doping that have been proposed and the potential problems associated with these solutions.
Three main aspects lead to the conclusion that the abuse of gene transfer technology in sports will be very difficult to detect:

I. *Homology between gene transfer material and the normal human body (8;18)*
The material typically introduced into the body by gene transfer is frequently found in the normal human population. It is either of human origin like the tDNA itself or it contains additional non-human material and molecules that most humans are in frequent contact with - like viral proteins, viral DNA sequences or other material that is required for the transfer or the proper function of the tDNA (Table 1; lines 1 and 2).

II. *Homology of the generated protein with the natural protein (6;10;13;28)*
Following gene transfer, the human body itself produces the doping relevant protein, which may therefore be principally undetectable by direct detection methods (Table 1; line 3).

III. *Limited specificity of indirect procedures*
Indirect measurements on the level of the doping effect or on the level of the bystander effects provoked on the transcriptome or proteome in peripheral blood cells may only be of limited specificity for doping. Nevertheless, such procedures might be very important for pre-screening of samples and may contribute an important suspicious fact (Table 1; lines 4 and 5).

Table 1

Level of Detection	Type of Detection	Problem	Known Solution	Problem of the Solution
transgenic DNA (tDNA) or antisense RNA	direct	homology to the genomic DNA	genetic "bar-codes" for tDNA enforced by law	easy to bypass & unrealistic
viral vector or other gene transfer related material	direct	endemic presence	ø	ø
protein	direct	homology to the natural protein	detection of posttranslational differences	hard to detect & unlikely to occur
doping effect	indirect	arguable specificity	follow-up values, limit values	costs, effectiveness in law?
proteome and transcriptome	indirect	arguable specificity	expression profiling	costs, effectiveness in law?

In this article we describe for the first time a procedure that principally enables direct detection of gene doping on the level of human tDNA. As a basis for diagnostic discrimination, the gene sequences of human tDNA being employed by

gene transfer protocols are not 100% homologous to the human genomic DNA (gDNA), since these do not contain the intronic sequence parts of the gDNA (see Fig. 1, upper versus lower part). We have developed a method enabling detection of tDNA on a single molecule level within ordinary blood samples. Detection is based on specific amplification of tDNA even in the presence of huge amounts of gDNA by a patent pending **s**ingle-copy **p**rimer-internal **i**ntron-spanning PCR (spiPCR) procedure (PCT/EP2007/003385; http://www.wipo.int/pctdb/en/wo.jsp?WO=2007124861).

This spiPCR procedure can principally be used to directly detect gene doping using any kind of gene transfer protocol that either works with DNA or leads to the integration of tDNA into our genome. Currently, 1457 of the 1472 registrated Clinical Gene Therapy Trials use a gene transfer procedure that leads to the generation of such tDNA sequences (http://www.wiley.co.uk/genetherapy/clinical/). In this article we firstly describe a spiPCR protocol for the detection of gene transfer focusing on the first line gene doping candidate genes *EPO* and Vascular Endothelial Growth Factor-D *(VEGF-D)*. Secondly, we then discuss the perspectives of spiPCR for the application as a direct detection technique for gene doping.

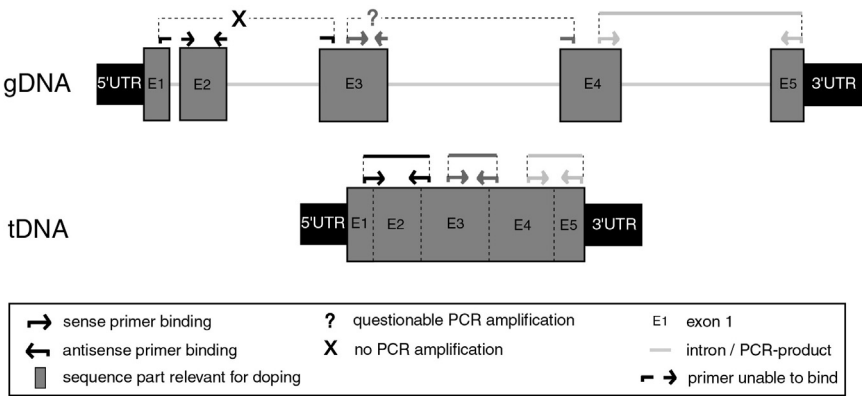

Fig. 1: In case primers for the PCR can be chosen in a way as illustrated for the black pair of primers, maximum specificity is achieved by assuring that only tDNA, but not gDNA can be primed and amplified by both primers. This principle is called primer-internal intron-spanning (pi). In the case of the dark grey primer pair, only the reverse primer shows this primer-internal intron-spanning, while the forward primer does not. In the case of the light dark primers the choice of primers is either termed intron-spanning or exon-skipping and usually is used to differentiate between gDNA and cDNA, since they can be differentiated by the size of the generated amplicons. However, in such a setting the primers can still bind and amplify both, gDNA (upper part) and tDNA (lower part). This does not only reduce specificity remarkably, but will also reduce sensitivity for the tDNA amplification.

METHODS

spiPCR-based detection of Epo and Vegf-d tDNA

The principle of spiPCR-based detection of tDNA is illustrated in Fig. 1. Whereas coding sequences of gDNA are 100% homologous to coding sequences of any tDNA, gDNA contains introns, whereas tDNA does not. This difference can be used to discriminate tDNAs abused in gene doping from "parental" gDNA sequences.

According to the above illustrated principles of tDNA detection (Fig. 1), primers have to be chosen with respect to two main points that are important for the sensitivity and specificity of the tDNA detection:

(i) Primer-internal intron-spanning: every single primer spans an intron. For this purpose, the first bases (5' end) of every forward primer are located in an exon upstream to the exon where the last bases (3' end) are located and the first bases of a reverse primer are located in an exon downstream to the exon where the last bases are located (Fig. 1, black primers). Subsequent to mRNA splicing these primers will bind only at the exon-junctions in the tDNA.

(ii) tDNA specificity: none of the primers shows a high enough homology to hybridize anywhere else in the human genome.

Selection of primers within the coding sequence of a candidate gene has therefore been done with respect to the gene specific exon-intron structure. We used the Blast Like Alignment Tool (BLAT) from the UCSC Genome browser for the alignment of reference gene coding sequences to the human genome (29). Fig. 2 shows an example for such an alignment for the locus of erythropoietin *(EPO)* on chromosome 7.

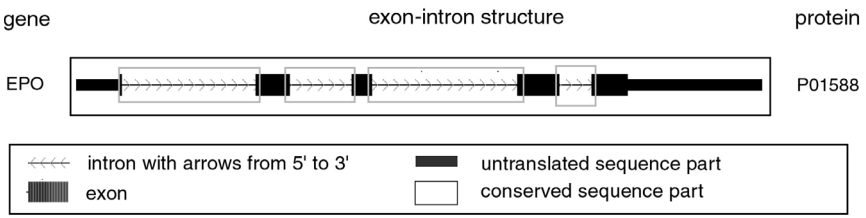

Fig. 2: BLAT alignment of the reference mRNA sequence of EPO with its gene locus. Four potential exon-intron junctions (boxes; "conserved sequence part") can be found all of which are suitable according to principle **(i)** to serve as regions where exon-intron spanning primers can be located. The "conserved sequence part" needs to be a sequence part that is conserved among various different mRNA sequences that could be translated into a functional protein.

For *Epo* a spiPCR protocol was established with an outer primer pair "EPOs1/as3" for the amplification of a 1st round 437 bp PCR product and an inner primer pair "EPOs2+3/EPOas3-II" flanking the 2nd round 289 bp product. Localization of the above mentioned primers to the *EPO* gene locus and the reference RNA sequence of Epo is shown in Fig. 3. Sequences of primers are given in

Table 2. Primers were purchased from MWG (Ebersberg, Germany). All primers are intron-spanning and are within the region that is canonical to the reference gene mRNA sequences that is known to be protein coding (black alignment in Fig. 3).

Table 2

Primer	Sequence
EPOs1 (outer forward)	5'-ATGGGGGTGCACGAATGTC-3'
EPOas3 (outer reverse)	5'-ATGGCTTCCTTCTGGGCTC-3'
EPOs2+3 (inner forward)	5'-AGAATATCACGACGGGCTGTG-3'
EPOas3-II (inner reverse)	5'-TCCTTCTGGGCTCCCAGAG-3'
vegfD_1s (outer forward)	5'-CCTCGTACATTTCCAAACAGCTC-3'
vegfD_1as (outer reverse)	5'-TCCTGGAGATGAGAGTGGTCTTC-3'
vegfD_2s (inner forward)	5'-AAGAAGATCGCTGTTCCCATTC-3'
vegfD_2as (inner reverse)	5'-AGAGTGGTCTTCTGTTCCAGCA-3'

For *Vegf-d* a spiPCR protocol was established as above with primer-internal intron-spanning primer pairs that amplify a 1st round 289 bp PCR product and a 2nd round 119 bp PCR product.

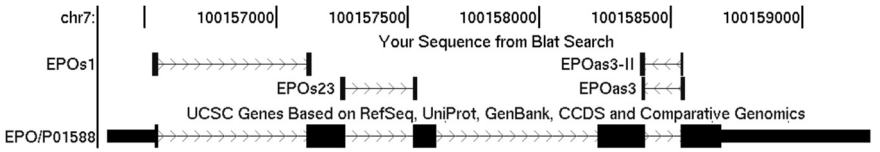

Fig. 3: Localization of EPO primers to the respective gene locus.

First and second round of the nested PCRs were prepared with Promega GoTaq® Green Master Mix (Promega, Madison, Wisconsin, USA) containing a HotStart Polymerase to avoid unspecific nucleotide incorporation prior to the first PCR denaturation step. Reactions were set up under bench top UV cabinets, using PCR-dedicated pipettes and filter tips. Preparation of PCR Master Mix, extraction and addition of DNA samples were performed in three separate areas.

The first round (outer) PCR contained Promega GoTaq® Green Master Mix, 0.3 µM of each outer primer and ~300ng genomic DNA in a total volume of 25 or 50 µl, respectively. The positive and negative controls were pipetted by adding defined copies of the tDNA standard or the same volume of nuclease-free water (Promega, Madison, Wisconsin, USA), respectively.

Amplification started with a single denaturation step of 94 °C for 3 min to activate the HotStart enzyme. To reduce unspecific amplification of by-products, a touch-down PCR protocol was used during the first six cycles of the 1st round PCR, starting with an annealing temperature of 63 °C and decreasing the annealing temperature by 0.5 °C/cycle to reach the optimum annealing temperature of 59 °C which was subsequently used for additional 14 cycles. Each cycle consisted of

denaturation at 94 °C for 20 sec, annealing for 25 sec, and elongation at 72 °C for 35 sec. Final extension was performed at 72 °C for 7 min.
The second round (inner) PCR was performed using 2.5 (5) µl of the first PCR product in a 25 (50) µl reaction mixture containing Promega GoTaq® Green Master Mix and 0.3 µM of each inner primer. PCR was performed as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 20 sec, 58 °C for 25 sec, and 72 °C for 35 sec, and a final extension for 7 min at 72 °C.
The final PCR product was analyzed on a 1.5% agarose gel and visualized by UV illumination after staining with GelRed (Biotium Inc., Hayward, CA).
Optimum annealing temperature and the specificity of the PCR-products generated during the respective PCR amplification rounds was tested separately prior to application of the spiPCR protocols.
The effectiveness of the spiPCR protocols was tested on different preparations of 300 ng total DNA from whole blood that were spiked with known copy numbers ranging from 1 - 1000 of respective tDNAs as positive controls. Unspiked DNA samples represented negative controls.
All tDNA standards were constructed by target specific PCR from cDNA libraries. For this purpose 1 kb standards were generated that included the whole locus of interest. Concentrations of the respective standard tDNAs were determined both photometrically and by photodensitometry from serial dilutions run on 1.2% agarose gels using Quantity One 1-D Analysis Software (BioRad, Germany). Copy numbers were calculated and standards with defined copy numbers were prepared by serial dilutions.

Preparation of total DNA from whole blood samples
The isolation of total DNA from 200 µl of EDTA whole blood was performed with a silica-gel-membrane based method by applying the QIAamp DNA Blood Mini Kit according to the manufacturer's instruction manual (Qiagen, Hilden; Germany) with a final elution volume of 100 µl. In some cases and for refined analysis the yielded DNA was further concentrated by an additional isopropanol precipitation step.

Construction of Ad-Vegf-d vector and in vitro gene transfer
A recombinant adenoviral vector encoding the *Vegf-d* transgene (Ad-*Vegf-d*) purposefully was purchased as a ready to use virus stock from Vector Biolabs (Philadelphia, PA), thereby simulating a ´classical gene doping initiation scenario` (i.e. vector purchase via the internet).
The virus has a backbone of the human Adenovirus Type 5 with partial deletions in the E1 and E3 domains. In this adenoviral vector, the expression of the *Vegf-d* transgene was placed under the control of a CMV promoter. The adenoviral vector was amplified on 293 cells and subsequently purified by centrifugation as described previously (30). Stocks of 10^{11} pfu/ml were stored at -80 °C.
Expression of the *Vegf-d* transgene was verified by infection of HeLa cells and detection of VEGF-D protein in culture supernatants using a commercially available ELISA system (Quantikine-Human VEGF-D Immunoassay from R&D Systems; DVED00; data not shown).
For the spiking experiments of whole blood, the transduction efficiency of U937 cells with Ad-*Vegf-d* was determined by FACS analysis 7 days post-trans-

duction using a FITC-conjugated goat anti-adenovirus antibody (Chemicon, AB1056F, 1:100). At a multiplicity of infection (MOI) of 100 (i.e. 100 plaque forming untis (pfu) / cell) 0,5% of the U937 cells stained positive at this time point.

RESULTS

The established spiPCR protocol for Epo tDNA

In spiPCR experiments we were able to detect down to 1 copy of *Epo* tDNA in the presence of 300 ng of genomic DNA (Fig. 4). No by-products were detected except primer dimers < 50 bp. All negative controls were tested negative. All spiPCR results were verified three times.

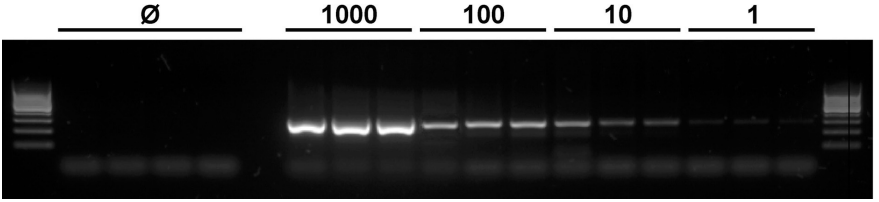

Fig. 4: Outcome for the spiPCR-protocol with the primers for *Epo*. Lanes 1-4 represent the negative controls (~300 ng gDNA), and lanes 5-16 represent ~300 ng gDNA with decreasing spike in copies of an *Epo* standard; copy number: 1000 (lanes 5-7), 100 (lanes 8-10), 10 (lanes 11-13), 1 (lanes 14-16).

The established spiPCR protocol for Vegf-d tDNA

In spiPCR experiments we were able to detect down to 1 copy of *Vegf-d* tDNA (Fig. 5). No by-products were detected except primer dimers < 50 bp. All negative controls were tested negative and all spiPCR results were verified three times.

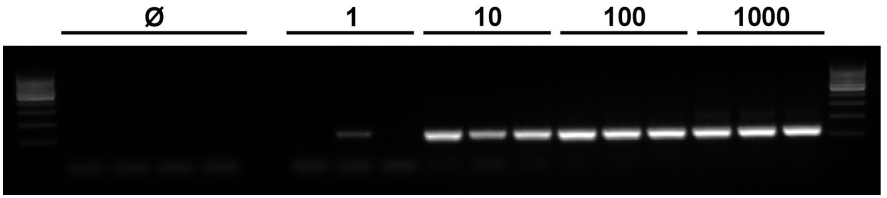

Fig. 5: Outcome for spiPCRs with the above mentioned primers for *Vegf-d*. Lanes 1-4 represent the negative controls (~300 ng gDNA), and lanes 5-16 represent ~300 ng gDNA with increasing spike in copies of a *Vegf-d* standard; copy numbers: 1 (lanes 5-7), 10 (lanes 8-10), 100 (lanes 11-13), 1000 (lanes 14-16).

Detection of Vegf-d from transduced cells in whole blood samples

The detectability of tDNA in body samples has been shown for extracellular tDNA. Extracellular tDNA is expected only shortly after viral gene transfer *in vivo* as a result of direct virus input into the blood circulation post-injection. A functional gene transfer requires transduced cells. These may be target cells in a solid tissue but also circulating blood cells that eventually got transduced. In the following experiment transduction of blood cells with recombinant adenoviral vectors was simulated by spiking blood with *Vegf-d* transduced cells at known cell numbers.

Therefore, U937 cells were infected with Ad-*Vegf-d* at MOI 10 and MOI 100. Cells were washed several times and transferred to new vials to avoid free viral particles. Transduction efficiency 7 days later was determined to be 0.1% for MOI 10 and 0.5% at MOI 100. This low transduction efficiency may be a result of a more rapid proliferation of non-transduced cells compared to transduced cells in the culture dish. Furthermore, a limited sensitivity of the detection of adenoviral proteins by immunocytochemistry might underestimate the infected cell number in our assay at least to some extent. For the further experiments U937 cells, infected with MOI 100, were used to spike blood samples with a specified number of transduced cells and the DNA was isolated directly after spiking.

The following analysis of samples was done in a blinded fashion. Among the samples tested in this way an additional internal negative control along with 9 samples that on a calculated basis had less than 0.5 transduced cells per sample volume were investigated. In addition, an internal PCR negative control from previous extractions was run in parallel to ensure that no contamination did occur during the PCR process (Fig. 6; first two lanes).

After isolation of DNA and subsequent additional precipitation with isopropanol, all PCRs were run with the total gDNA harvested from 25 µl whole blood (~ 1 µg) each and tested for the presence of *Vegf-d* tDNA. Transfected cells down to a calculated number of 2.25 cells / µl blood could be detected in blood DNA preparations (Fig. 6; lane 3).

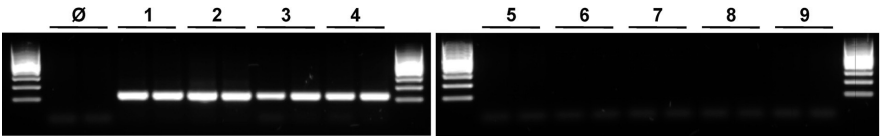

Fig. 6: One negative control (ø) and the probes 1-9 were precipitated with isopropanol and run in duplicates with a spiPCR for *Vegf-d*. The calculated number of transduced cells / µl blood was as follows: 112.5 cells in sample 2, 22.5 cells in sample 1, 12.5 cells in sample 4, 2.25 cells in sample 3. Samples 6-9 contained less than 0.25 cells / µl blood. Note that the gDNA put into the sample was derived from 25 µl of whole blood sample.

DISCUSSION

To become effective as evidence of gene doping in a court of law, direct detection techniques for gene doping have to be developed. So far, there had been only one report that suggested a solution for the direct detection of gene doping (28;30). At

the protein level it has been shown that it could be possible to discriminate between EPO proteins derived from genomic DNA (gDNA) and proteins artificially encoded by tDNA using a conventional test for doping with recombinant EPO. However, it is not yet elucidated why tDNA derived proteins can have differing post-translational modifications and under which circumstances such differences occur. Detection of gene doping on the level of the protein derived from transgenes may therefore face the problem that differences in post-translational modifications are highly variable depending on the protocol for gene transfer, the transgene delivered, the route of vector administration and the target tissue.

Help and orientation for the development of more generalisable direct gene doping detection procedures may come from clinical research in somatic gene therapy. In somatic gene therapy the tDNA sequence transferred to a patient is known and researchers are principally able to use tDNA specific sequence parts in order to design a PCR that is able to detect the tDNA. PCR is therefore routinely used for monitoring plasma and serum levels of tDNA to control for the presence of infectious vector in the blood stream during somatic gene therapy trials and related animal studies. From these tests we know that serum and plasma probes will only show tDNA for a very limited number of hours up to a few days following many different kinds of administration routes and gene transfer technologies used (8;31).

The test for presence of tDNA in whole blood is relatively rarely performed, since it is not indicative for the presence of infectious vector, but rather for a back dated transfection of blood cells. On top of this, it is technically much more difficult than testing serum and plasma probes.

Importantly, in blood cells long-term presence of tDNA was found following injection of (i) recombinant adenovirus (Ad) into the prostate of humans for 76 days (32), (ii) recombinant adeno-associated virus (AAV) into the muscle of primates for 10 months (33), (iii) recombinant adeno-associated virus into the hepatic artery to target the liver of humans for 20 weeks (34), and (iv) retroviruses into the peripheral vein of humans for > 1 year (35). In all of these cases serum or plasma probes were found to become predominantly negative within hours or a few days. While up to now most publications speculate that muscle biopsies might be necessary to detect gene doping at all, the above mentioned findings indicate that it seems to be very promising to develop a technique that is able to detect tDNA relevant for gene doping in whole blood samples. Additionally, it is known that exercise increases the turnover and redistribution of leukocytes within the human body (23;24;36), which may increase the likelihood that leukocytes, once transfected at the site of vector application, can be found in the blood stream.

The challenging technical difficulty for tests of whole blood in contrast to plasma and serum is the sensitive and specific amplification of tDNA in the presence of huge amounts of genomic DNA (gDNA). In the case of gene doping this technical difficulty is further complicated, since the non-human part of the tDNA sequence is completely unknown and highly divergent between different gene transfer procedures employing different sources of vectors.

Here we show for the first time that these technical difficulties can be overcome by employing our novel spiPCR technology. This spiPCR procedure enables direct detection of the doping relevant sequence - namely the sequence part that is

necessary to generate the protein that mediates the enhancement of physical performance. This sequence part has to be present in any gene doping attempt. Further studies are now under way to verify the specificity of this attempt to detect gene doping by spiPCR.

First, we will establish more spiPCR protocols enrolling the most important tDNA sequences that could be abused for gene doping. We will then try to develop a multiplex spiPCR that is able to detect as many as possible of these tDNA sequences at once.

Second, we will investigate the specificity of the spiPCR in normal persons and athletes known not to be genetically altered by gene transfer technology. For this purpose, probes taken from athletes and persons under different conditions including following intensive exercise will be analyzed for false positive results.

Third, the sensitivity of spiPCR will be tested in animal studies and on blood samples taken from patients that have undergone somatic gene therapy.

Apart from the interesting perspectives spiPCR offers in the fight against gene doping, this technology may also be of interest in biodistribution and biosafety studies for gene therapeutic applications. The crucial quality feature of spiPCR is the high sensitivity to detect a tDNA sequence part being relevant for gene therapy or for gene doping against a high background of gDNA.

List of Abbreviations

AAV	adeno-associated Virus
Ad	adenovirus
Ad-Vegf-d	adenoviral vector with Vegf-d
bp	base pairs
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
gDNA	genomic DNA
MOI	multiplicity of infection
spiPCR	single-copy primer-internal intron-spanning PCR
tDNA	transgenic DNA
WADA	World Anti-Doping Agency

List of genes mentioned

EPO	Erythropoietin
VEGF-D	Vascular endothelial growth factor family member D

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