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A quick one-tube nested PCR-protocol for EPO transgene detection

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The practice of doping threatens fair competition in sports. With the very recent reports on successful gene therapies for several diseases, the likelihood for abuse of gene transfer techniques in elite sports is rapidly increasing. It is therefore very important to develop valid detection techniques for transgenic DNA (tDNA) with ultimate sensitivity and specificity. To date, three slightly different procedures have been reported to reliably detect tDNA with sufficiently high sensitivity. Two utilize a real-time PCR-based approach and one uses a primer-internal, intron-spanning PCR approach (spiPCR). The specificity and sensitivity of these techniques, however, is still a matter of debate.

Based on spiPCR, here we present a novel one-tube nested PCR approach that minimizes the chances for cross-contamination and shows increased sensitivity compared to non-nested PCR techniques. To further reduce the occurrence of false-positives based on cross-contamination, a multi-functional 19bp extended erythropoietin standard (*EPO*) was cloned which can be easily differentiated from transgenic *EPO* DNA (t*EPO*) and can be used as an internal or external positive control in PCR-based applications.

We found that one-tube nested PCR is superior in terms of sensitivity and specificity compared to conventional PCR, and shows similar sensitivity compared to real-time based PCR assays. Although it did not reach sensitivity of spiPCR, the one-tube nested PCR technique described here is less laborious, less expensive and much faster than spiPCR. This technique might therefore be useful as a pre-screening tool for gene doping in the future. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: erythropoietin (EPO); one-tube nested PCR (OTN PCR); gene doping; SpiPCR; antisense PCR

Introduction

Somatic gene therapy has enabled scientists to treat diseases using transgenic DNA (tDNA).[1] Although important for its potential to treat life-threatening disease, gene transfer technology could easily be misused in order to improve physical performance, particularly by elite athletes.[2] To deter athletes from using gene transfer technology as an innovative doping strategy, novel detection methods have to be developed and valid test systems must be established.

Currently, there are four studies describing two different PCR-based approaches to directly detect circulating transgenic erythropoietin DNA elements (t*EPO*).[3–6] Both procedures are based on the specific PCR detection of intronless *EPO* cDNA fragments after DNA extraction from whole blood samples as described for the first time in 2006 (patent pending, http://www.wipo.int/patentscope/search/en/WO2007124861). One approach uses real-time PCR[5,6] whereas the second method is based on a single-copy, primer-internal, intron-spanning PCR approach (spiPCR)[3,4] where detection is based on a nested PCR and agarose gel electrophoresis. The spiPCR approach shows the highest sensitivity, and has already shown high specificity for six potential top gene-doping candidates in athletes *in vitro* (*EPO, FST, GH-1*, *IGF-1* and *VEGF-A/D*) and for *VEGF-A in vivo*.[4] However, the two-step PCR makes the process more laborious and theoretically at increased risk for crossover contamination compared to a one-step PCR approach.[4,7]

Here, we present a new approach for the detection of transgenic *EPO*, which not only matches the high specificity of the spiPCR but also minimizes the probability for cross contamination by using a one-tube nested PCR assay. In this one-tube nested PCR (OTN-PCR) approach (originally described by Erlich *et al*. in 1991 as a 'drop-in, drop-out' PCR method[8]) both primer pairs are present in the reaction tube in the initial step. Thus, no manipulation of the reaction mixture is needed during the course of amplification, which minimizes the risk for sample cross-contamination. Generally, the outer primer set is longer, or has a higher GC content than the inner set. Using sufficiently high annealing temperatures in the first stage, inner primer annealing is prevented while outer primer annealing and extension proceed. Using a reduced annealing temperature in the second stage, the inner primers anneal and the shorter amplicon is produced while annealing of the outer primers is prevented.[8]

Limiting the concentration of outer primers in PCR reactions has been described as a way to improve one-tube nested PCR efficiency.[8] In this study, an OTN-PCR method was combined with limited outer primer concentration, and optimized in order to achieve the best possible amplification-product for t*EPO* DNA fragments targeted by four primers in a single PCR run.

A recently described technique named antisense PCR could be a promising tool to further improve OTN-PCR efficiency, and was also tested in this study.[9] As described by Brisco *et al*., outer primers are used to create the longer nested-PCR fragment at high annealing temperatures. They are subsequently captured by the addition of antisense oligonucleotides targeting the outer primers at lower temperatures during the second round of PCR, when the shorter fragment is amplified.[9]

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Another consideration in gene doping analysis is the use of an appropriate positive control. Therefore, we constructed an elongated *EPO* cDNA-standard carrying an insertion located in the amplicon between the inner primers. This CG-rich insertion contains two restriction enzyme cutting sites and one potential probe-binding site. In case of cross contamination, the internal standard could be differentiated from the target t*EPO* DNA by length and RFLP using agarose gel electrophoresis, and also by melting curve and probe binding using a real-time based assay.

Here we describe the design and procedure of a modified one-tube nested PCR approach for *in vitro* detection of t*EPO* with high specificity and reduced vulnerability for cross-contamination.

Materials and methods

DNA extraction from whole blood

DNA was extracted from 10 ml of whole blood using a salting out procedure initially described by Miller *et al.*[10] To exclude any gender-specific bias due to altered primer annealing influencing PCR efficiency, DNA from male and female subjects were pooled. The concentration of DNA was measured on a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and diluted to 250 ng/µl in TE- buffer (10 mM Tris–HCl containing 1 mM EDTA, pH 8.0). Finally, 750 ng DNA was spiked with defined amounts of a lab-internal *EPO* standard, as described later.

Construction of a modified *EPO* standard

In order to differentiate potential cross-contamination from transgenic *EPO*-DNA, an internal *EPO* standard was created which can be discriminated from the native *EPO*-cDNA by length, melting temperature, probe binding and RFLP using either the restriction enzyme *Fse*I or *Sbf*I. Using a TOPO PCR2.1 vector (Invitrogen) carrying a 685 bp human *EPO*-cDNA fragment (NM_000799.2| *EPO*, mRNA position 57–714) a 19 bp insertion carrying an *Fse*I and *Sbf*I recognition site was introduced at cDNA position 379 by PCR. This resulted in an elongation of the native *EPO* cDNA fragment changing the sizes from the outer and inner amplicons from 450 bp and 109 bp to 469 bp and 128 bp, respectively. PCR was performed using the High Fidelity Kit (Qiagen, Hilden, Germany) according to the manual, using 50 ng of TOPO PCR2.1 *EPO* cDNA vector, for 25 cycles at 55°C annealing temperature.

The *Fse*I sites, which were located at 5′ ends of both primers, served as sticky ends for re-ligation after digestion of the PCR product with *Fse*I and *Dpn*I and gel purification. After transformation, clones were first tested by colony PCR. Subsequently, positive candidates were mini-prepped (Qiagen, Hilden) and sanger-sequenced on an ABI PRISM® 310 genetic analyzer. Correct plasmids were *Eco*RI digested, the 19 bp prolonged *EPO* standard was gel-purified and its concentration was measured on a NanoDrop® ND-3300 Fluorospectrometer (Thermo Scientific, Wilmington, DE, USA). Copy numbers were calculated using Finnzymes' DNA copy number calculation program (http://www.finnzymes.com/java_applets/copy_number_calculation.html) and standards with defined copy numbers were prepared by serial dilutions in TE- buffer (range: 10^3, 10^2, 10, 5, 2, and 1 copy /5µl) using low retention plastic ware.

To check for proper differentiation of the native from the standard *EPO* DNA fragment on a 2.5% agarose gel, both PCR-products were visualized in their uncut form and again after *Fse*I and *Sbf*I digestion (Figure 2a).

Real-time PCR

Real-time PCR using SYBR® Green as fluorescent reporter could not be used for OTN-PCR because of high levels of background noise, potentially originating from re-annealing genomic DNA. However, real-time PCR (without genomic DNA) was performed to optimize for primers, their concentrations, and the cycling conditions. To assure optimal PCR efficiency and reliability, a standard curve was assayed using a 10 x serial dilution (10^6 – 10 copies) of the lab-internal *EPO* standard. Additionally, 10^3 copies of native and standard *EPO*-fragments were amplified and the two amplicons were differentiated using melting curve analysis.

Real-time PCR analysis was carried out on an MyiQ single-colour detection system (BioRad, München, Germany) using the following thermal cycler conditions: 95°C for 5 min followed by 40 cycles of a three-step reaction consisting of denaturation at 94°C for 15 s, annealing at 52.5°C for 25 s, and extension at 72°C for 30 s. Fluorescence was measured during the 72°C step. Samples were run in triplicates with a final volume of 15 µl per single reaction, including 5 µl of standard, 7.5 µl QuantiFast SYBR Green PCR Kit (Qiagen GmbH, Hilden, Germany), and primers at a final concentration of 0.5 µM. Formation of the expected PCR product was confirmed by melting curve analysis and agarose gel electrophoresis.

One-tube nested PCR (OTN-PCR)

Four primers were selected from the human *EPO* cDNA sequence (NM_000799.2) fulfilling the criteria of spi- and OTN-PCR.[4,8] As indicated in Figure 1, all primers were intron-spanning and

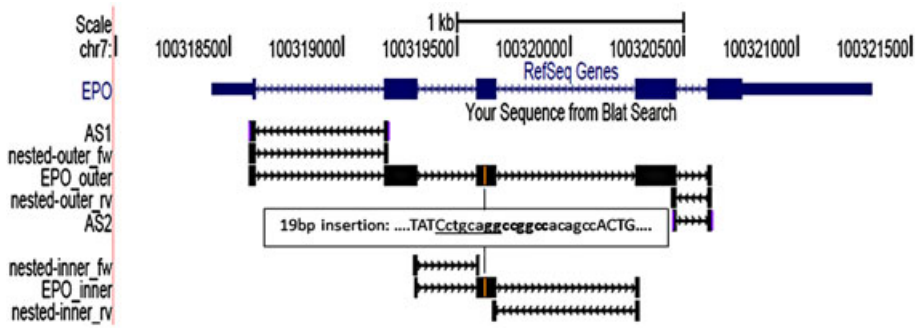

Figure 1. Genomic organization of human *EPO* on chromosome 7 (chr7: nt 100,318,000-100,322,000; GRCh37/hg19). Localisation and orientation of outer-, antisense-, and inner primers with respective amplicons are indicated. Tiny lines represent intronic, bulky lines exonic regions. Orientation of the fragments is described by the arrows. Position and sequence of the artificial 19bp insertion is displayed in the box (exonic nucleotides given in capital letters, inserted nucleotides in lowercases; *Fse*I site is indicated in bold characters; *Sbf*I site is underlined).

selectively binding at the exon junctions, with outer primers annealing at significantly higher temperatures than the inner primers (Table 1). ENSEMBL, NCBI, and UCSC databases were used to exclude polymorphic regions as primer binding sites. All primers were purchased from Eurofins MWG Operon (Ebersberg, Germany).

PCR was performed using Promega GoTaq Green Master Mix (Promega GmbH, Mannheim, Germany) in a final volume of 25 μl using a serial dilution of *EPO* standard as a template (range: 10^3, 10^2, 10, 5, 2, and 1copies/5 μl) in a background of 750 ng human genomic DNA. Outer primers were added at a final concentration of 10 nM, inner primers at a concentration of 500 nM. After an initial denaturation step at 95°C for 3.5 min, OTN-PCR was performed for 15 cycles at 94°C/15 sec for denaturation, 62.5°C/60 sec for primer annealing, and 72°C/45 sec for primer extension followed by 40 cycles at 89°C/15 sec for denaturation, 52.5°C/25 sec primer annealing, and 72°C/30 sec extension with a final extension step for 7 min at 72°C. Conventional PCR was performed under the same conditions as described for the second stage of OTN-PCR. SpiPCR was performed as described elsewhere.[4] Amplicons were analyzed by electrophoresis of 20 μl of the reaction mixtures in ethidium bromide-stained 2.5 % agarose gels. All experiments were done in triplicate.

One-tube nested antisense PCR (OTNAS-PCR)

Antisense primers were designed to hybridize to the 3′ end of the outer primers (Figure 1) at lower temperatures. At their 5′ and 3′ ends, antisense oligonucleotides carried short (3 nucleotide) tags. These tags were designed to create a 3-base mismatch, blocking extension of the oligonucleotide in both directions.[9] Antisense oligonucleotides were added at concentrations equimolar to the outer primers, and PCR was run with the same cycling conditions as described for the OTN-PCR.

Results

Here we present our efforts to further optimize t*EPO* DNA detection. We and others have already shown the detectability of very small amounts of t*EPO* DNA using either a nested PCR or real-time PCR approach. The nested PCR based approach has shown the highest sensitivity, but holds the potential for cross-contamination. We therefore tried to establish a one-tube nested PCR approach that maintains high sensitivity while limiting the vulnerability to cross-contamination. Additionally, a lab-internal *EPO* standard was created which can be discriminated from *EPO* cDNA or putative t*EPO* DNA by length before and after restriction enzyme digestion with *Fse*I or *Sbf*I on an agarose gel (Figure 2a), and also by melting curve analysis (Figure 2b). As depicted in Figure 2a, the amplification product of the native 109 bp *EPO* fragment can be clearly differentiated from the elongated 128 bp *EPO* standard. Digestion of the 128 bp *EPO* standard with *Fse*I or *Sbf*I restriction enzymes resulted in RFLP-fragments of 67/61bp for *Fse*I and 73/55bp for *Sbf*I, whereas the native 109 *EPO* fragment remained uncut. Figure 2b demonstrates that native and standard *EPO* fragments can be differentiated by melting curves; native *EPO* has a Tm of 85.5°C, while the artificial *EPO* fragment has a Tm of 88.15°C. Figure 2c displays the high efficiency and linearity of the PCR conditions for the inner *EPO* fragment (Correlation Coefficient: .999; PCR efficiency: 98.6%).

The melting temperature, concentrations, and annealing times of primers in the first and second stage of PCR were optimized for this OTN-PCR approach. Because both primer pairs are present initially and no further manipulation of the reaction mixture is needed during the course of amplification, the risk of sample cross-contamination is minimized. To further increase PCR efficiency, OTN-PCR was supplemented with antisense oligonucleotides targeting the outer primers.

The use of outer primers at a limiting concentration of 10 nM showed the best results in our test system. Usage of outer primers at higher concentrations (300 nM) revealed lower sensitivity and also by-products, possibly originating from heteroduplex DNA when more than 10^3 copies t*EPO* standard was added (data not shown). As indicated in Figure 3, OTN-PCR showed increased sensitivity for lab-internal *EPO* standard spiked to 750 ng of human genomic DNA in a serial dilution (10^3, 10^2, 10, 5, 2, 1 copies) compared to a PCR using only the inner primer set for 40 cycles (Figure 3: line a *vs* b). Whereas conventional PCR could only detect ~1000–100 copies of EPO standard (Figure 3a), OTN-PCR continued to detect a PCR-product for a reaction spiked with as little as 5 copies of EPO standard (Figure 3b).

Table 1. Primer sequences, annealing temperature, and amplicon length. *EPO*-specific exonic boundaries are highlighted by capital/lowercase letters. For Antisense primers, regions targeting the 3′-end of outer primers are indicated in lowercase with capital letters representing 5′- and 3′-tags to inactivate primer prolongation. For cloning primers *Fse*I sites used for ligation are indicated in bold characters; *Sbf*I site which was introduced by the reverse cloning primer is underlined.

Primer sequences and concentration: 5′ – 3′	**Annealing temperature**	**Amplicon length in bp tDNA vs. internal standard**
Nested-outer_forward (10nM): gagatgggggtgcacgAATGTCC	62.5°C	450/469
Nested-outer_reverse (10nM): tggaggggagatggcttccttCTG		
Nested-Inner_forward (500nM): gagaatatCACGACGGGCTGT	52.5°C	109/128
Nested-inner_reverse (500nM): tgctgcccgacCTCCATC		
Antisense-outer_forward (AS1): CGTggacattcgtCGT	≤ 62°C	n.a.
Antisense-outer_reverse (AS2): CGGcagaaggaagcGTA		
Cloning primers	**Annealing temperature**	**Amplicon length in bp**
Internal standard_forward (500nM): TAT**GGCCGGCC**ACAGCCACTGTCCCAGACACCAAAGTT	55°C	4649
Internal standard_reverse (500nM): TAT**GGCCGGCC**TGCAGGATATTCTCATTCAAGCTGCAGTGTTC		

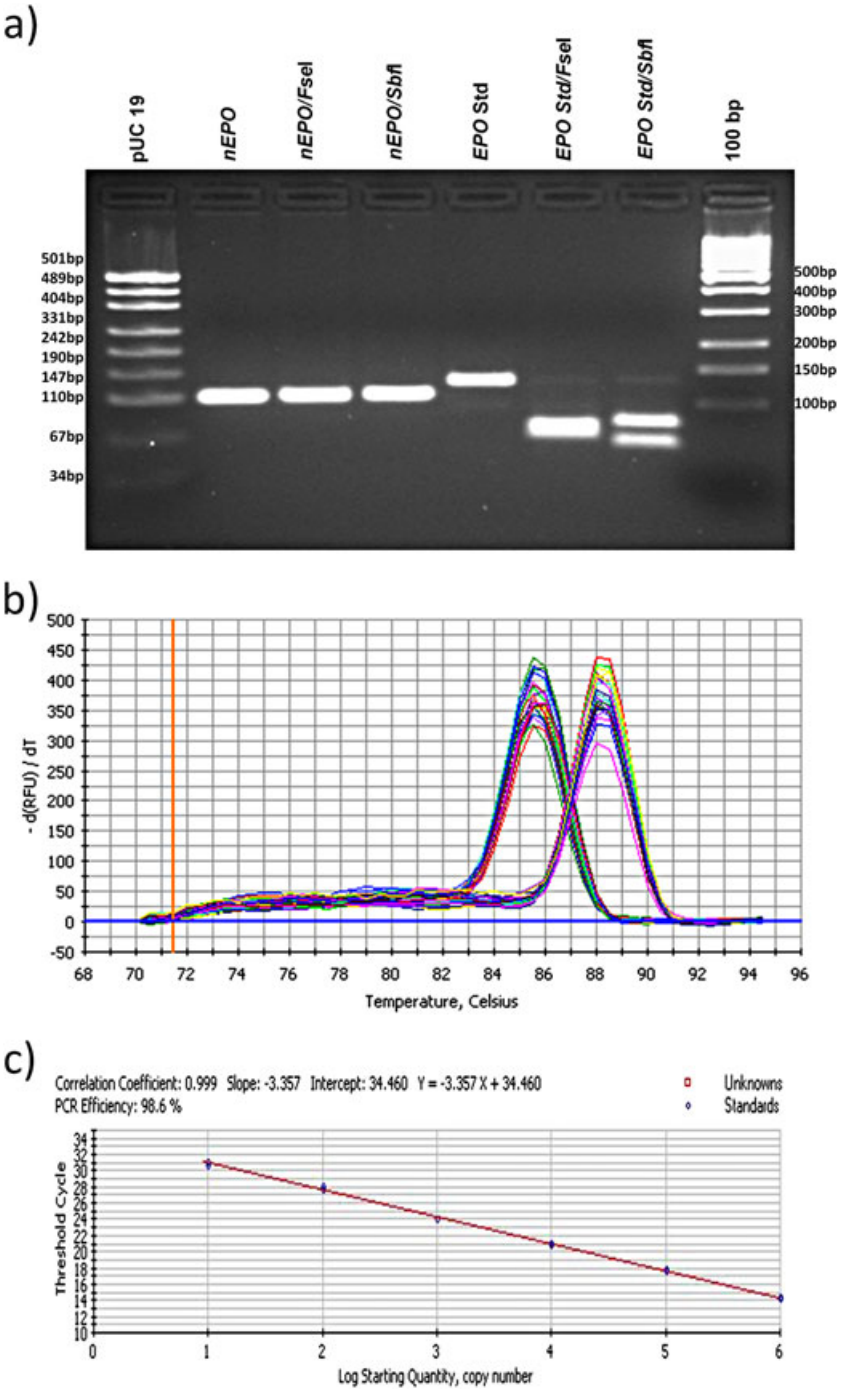

Figure 2. Comparison of native and elongated *EPO* PCR-products. a) Native *EPO* (109bp) *vs* elongated *EPO* standard (128bp) before and after *Fse*I and *Sbf*I restriction. Native *EPO* fragment remained uncut, whereas the artificial *Fse*I and *Sbf*I sites in the standard led to fragments of 67/61bp and 73/55bp, respectively; b) melting curves of native (left) and artificial *EPO*-fragment (right); c) standard curve for the amplification of the inner amplicon of the lab-internal *EPO* standard (10-fold serial dilution ranging from 10^6 to 10 copies).

It is noteworthy that the reduction of the melting temperature from 95°C to 89°C in the second stage of OTN-PCR considerably improved PCR results (data not shown).

Recently, the addition of antisense oligonucleotides into OTN-PCR was reported to affect PCR efficiency.[9] Therefore, different sets of antisense primers targeting the 3'end of outer primers were tested in different concentrations. Running a gradient-PCR (temperature range: 50°C–69°C) using outer and antisense primers at equimolar concentrations (300 nM), we observed massive inhibition of PCR-product at annealing temperatures below 64°C (data not shown). As the best PCR-product was obtained at 66.5°C using sense and antisense oligonucleotides, OTNAS-PCR was performed at this temperature, despite that the outer primer set shows optimal annealing without the addition of antisense oligonucleotides at 62.5°C. Supplementation of the reaction mixture with the inner primers following the initial OTN-protocol at an annealing temperature of 66.5°C in the first stage of PCR, however, resulted in poor product amplification due

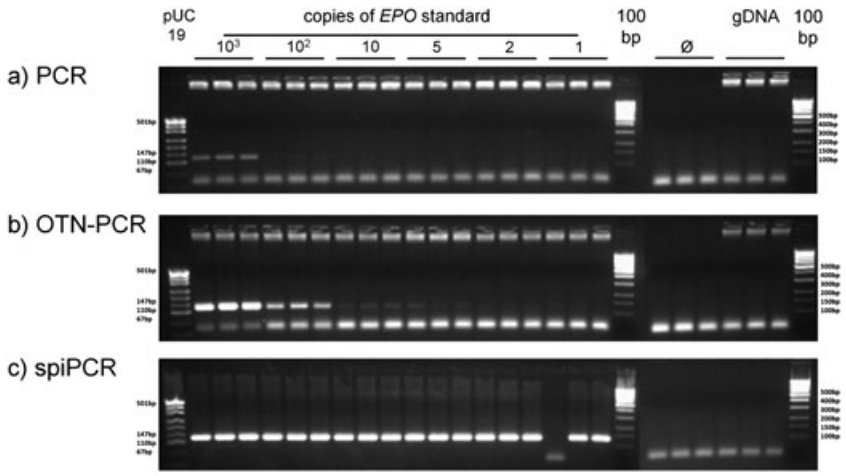

Figure 3. Comparison of *EPO* standard PCR products generated by PCR (a), OTN-PCR (b), and spiPCR (c). Triplicates of 750ng of genomic DNA were spiked with a serial dilution of *EPO* standard copies as indicated. OTN-PCR shows a detection limit of about 5 copies *EPO* standard spiked in 750ng genomic DNA. SpiPCR reveals a clear 'yes or no' message for the presence of *EPO* standard. Absence of one PCR product in one triplicate for 1 copy *EPO* standard spiked to 750ng DNA in the spiPCR can be explained by the Poisson distribution also known as the 'Poisson Law of small numbers'–a quite common phenomenon when rare events are investigated.

to, or accompanied by unspecific by-products (data not shown). Use of primers in various ratios or trials to adjust annealing time did not improve the result. Use of longer antisense oligonucleotides resulted in complete suppression of amplification, whereas shortening them did not reveal sufficient suppression.

When we compared the OTN-PCR to spiPCR (Figure 3c) only the latter created a clear 'yes or no' message for the presence or absence of *EPO* standard DNA in a background of 750ng genomic DNA.

Discussion

The concept of gene therapy grew out of the important development in the early 1970s of a novel approach in medicine that promised to treat human disease by attacking underlying genetic defects.[2,11] The first clinical gene therapy trials were carried out in the late 1980s, and at that time it was predicted that gene therapy would become a feasible treatment for serious diseases in just a matter of years. Although gene therapy represents a promising tool in the treatment of severe diseases, unapproved and uncontrolled transfer and integration of DNA into living beings not only threatens the health of athletes and the ethics of sports, it may also endanger third parties and society as a whole.

Different laboratories have already demonstrated their technical ability to detect minute amounts of putative t*EPO*-DNA in a high background of genomic DNA.[3–6] Nested PCR and real-time-based approaches have been developed for such detection. Here, we present another method for t*EPO* detection that transfers the benefits of nested PCR into a one-tube nested PCR system. This system minimizes the workflow and the potential for cross-contamination, while maintaining high specificity and sensitivity.

As illustrated in Figure 3, OTN-PCR shows higher sensitivity compared to a single PCR where amplification is performed using only the inner primer set under the same conditions. Comparing the results of conventional PCR (Figure 3a) to those achieved with OTN-PCR (Figure 3b), conventional PCR can only detect 100–1000 copies *EPO* standard in a background of 750 ng DNA whereas OTN-PCR can detect as few as 5 copies of *EPO* standard spiked to 750 ng genomic DNA. As indicated (Figures 3a–3c), the clearest detection of t*EPO* was only achieved using spiPCR. However, the detection limit of OTN-PCR almost reached sensitivity compared to real-time-based approaches. In this context it is notable that OTN-PCR is more laborious and currently it unlikely represents an acceptable alternative for real-time-based approaches. However, this study investigated the feasibility for utilizing spiPCR methodology in a one-tube nested PCR assay. Therefore, critical criteria for OTN-PCR were extrapolated from the pre-existing spiPCR procedure. Primer length, GC-content and annealing temperatures, and melting temperature of outer and inner amplicon as a whole, should differ from each other significantly.[8,12] Unfortunately, these criteria could not be strictly followed for t*EPO* detection, but could be assayed for other not yet explored gene-doping candidates. As we have shown, OTN-PCR clearly improves upon single-run PCR efficiency. However, application of real-time PCR using SYBR®Green was impossible because of high background noise produced by re-annealing of genomic DNA and formation of primer dimers, especially when antisense oligonucleotides were added to the reaction.

Nevertheless, OTN-PCR (possibly incorporating antisense primers) could represent a helpful tool and may improve real-time assays using probes for detection of other transgenic sequences in the future.

Interestingly, reduction of melting temperature from 94°C to 89°C in the second stage of PCR in order to preserve Taq-polymerase activity improved our results. As melting temperature in the first stage of PCR is still performed at 94°C, it is possible to pre-amplify tDNA which is integrated into the host cell genome and episomal tDNA. Lowering melting temperature in the second stage minimizes melting of genomic DNA. This in turn decreases false priming of oligonucleotides and amplification of unwanted DNA fragments, saves Taq polymerase activity, and preserves the buffering capacity of PCR solution. Consequently, shorter tDNA elements are preferentially and more efficiently amplified.

In conclusion, OTN-nested PCR is a tool enabling the detection of low amounts of t*EPO*-DNA with a detection limit of about five copies per test sample. Compared with spiPCR it minimizes

workflow and thus time and costs, but shows reduced sensitivity. When compared OTN-PCR to real-time based approaches, OTN-PCR at its present stage is more laborious and it does not show decisive superior sensitivity. Its principle, however, may find routine application in tDNA detection and very likely it will help to further improve real-time assays using probes for amplicon detection.

Constructing a lab-internal *EPO* control standard, we could show that it can be easily differentiated from putative t*EPO* DNA by length before and after restriction enzyme digestion, and also by melting curve analysis. Therefore, this standard may find routine application when spiPCR is used to minimize potential cross-contamination originating from PCR positive-controls, as well as in real-time PCR-based approaches.

In the development of tDNA detection methods, high sensitivity should not be the only consideration. The whole process, from blood draw and DNA extraction to tDNA-PCR detection, harbours a multitude of potential cross-contamination dangers which have to be considered and tested for on a regular basis. Therefore, to validate the quality of tDNA detection, the whole procedure starting from taking a blood sample under field conditions to PCR results, should be validated in an inter-lab comparison in the future in order to establish comprehensive tDNA test procedures.

We hope that the development of methods to detect tDNA will discourage elite athletes from using gene transfer technology to enhance physical performance, thereby violating fair sports. The continued improvement of such technologies will ensure that uncontrolled use of gene transfer technology will neither endanger the health of athletes' health nor potentially the health of third parties.

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The University of Tübingen, Germany, has a patent pending for the 'Detection of transgenic DNA' (PCT/EP2007/003385; http://www.wipo.int/pctdb/en/wo.jsp?WO=2007124861) that relates to the detection of transgenic DNA in a living being and to a kit for performing such a method. A free use without charge of the patent pending procedure has been granted to the World Anti-Doping Agency for the purpose of doping analysis in sports.

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