Affinity Sensing for Transgenes Detection in Antidoping Control

ARTICLE *in* ANALYTICAL CHEMISTRY · OCTOBER 2009

Impact Factor: 5.64 · DOI: 10.1021/ac901445b · Source: PubMed

Anal. Chem. **2009,** *81,* 9571–9577

Affinity Sensing for Transgenes Detection in Antidoping Control

Simona Scarano,[†] Maria M. Spiriti,[‡] Genny Tigli,[†] Patrizia Bogani,[‡] Marcello Buiatti,[‡] Marco Mascini,[†] and Maria Minunni*[,†]

Dipartimento di Chimica, Università degli Studi di Firenze, 50019 Sesto Fiorentino, Italy, and Dipartimento di Biologia Evoluzionistica "Leo Pardi", Università degli Studi di Firenze, 50127 Firenze, Italy

Sports authorities fear that a new form of doping called gene doping, based on the misuse of gene therapy, represents an emerging important problem and so far no methods are available for detecting it. The World Anti-Doping Agency (WADA) has included since 2003 for the first time gene doping methods in the "Prohibited List of Substances and Methods", thus detection of this new form of doping is challenging for analytical chemists. In this work, we apply affinity-based biosensors (ABBs), in particular DNA piezoelectric sensing, for detection of target DNA sequences selected as transgenosis markers. In this work, two sequences widely used in transgenosis experiments have been identified as markers: the enhanced green fluorescence protein (EGFP) gene and the promoter of *Cytomegalovirus* (CMV). The biosensors are characterized in their analytical performances using synthetic oligonucleotides and amplified DNA obtained from purified plasmid used as a template. Finally they have been applied to transgenic human cell cultures (human embryonic kidney HEK-EGFP), transformed with the same plasmid and carrying the target markers. This represents the closest human real matrix available for our transgenes.

The possibility of either transferring genes into human cells or modulating endogenous gene expression is leading to novel and revolutionary therapeutic tools.[1−3] Gene therapy is in some way late as compared to the anticipations: clinical applications of gene therapy are faced with incessant failures and also tragic results,[4] but the success will hopefully arrive. Behind this, misuse of gene therapy, in particular in sport practice, can be foreseen. Despite repeated scandals, doping has become irresistible to many athletes and sports authorities fear that a new form of doping called gene doping, based on misuse of gene therapy, represents an "ideal doping practice" because it will be undetectable and thus much less preventable. The science and ethics of "genetically modified athletes" (GMA) is on the way. In 2003, the World Anti-Doping Agency (WADA) included for the first time gene doping methods in the "Prohibited List of Substances and Methods". The last update of the list, in 2009, defines gene doping as "the transfer of cells or genetic elements or the use of cells, genetic elements, or pharmacological agents to modulating expression of endogenous genes having the capacity to enhance athletic performance".[5−7]

Detection of this new form of doping is challenging for analytical chemists. Among gene doping analytical detection strategies,[8,9] biosensors, more specifically affinity-based biosensors (ABBs), can play an important role.[10] In particular nucleic acids-[11,12] or immuno-based[13−15] sensing can provide selective, sensitive, and reproducible responses to target analytes in a very short time. The contribution of ABBs can be envisaged both in the direct detection of selected target analytes, as well as in an indirect approach, such as the monitoring of the presence of secondary side marker effects, for example a transgenosis-induced immune response, in athletes.[8−10]

In this scenario, we aimed at developing label free DNA-based piezoelectric sensors, based on quartz crystal microbalances (QCM),[14] for detecting target sequences used as transgenosis markers in animal and human cells, in line with our previous work for genetically modified organism detection (GMOs) in different matrixes of plant origin using suitable markers.[16,17]

* To whom correspondence should be addressed. E-mail: maria.minunni@unifi.it.

[†] Dipartimento di Chimica.

[‡] Dipartimento di Biologia Evoluzionistica "Leo Pardi".

(1) Osborne, R. *Nat. Biotechnol.* **2008**, *26*, 1057–1059.
(2) Zhang, Y. P.; Deng, M. M. *Nat. Biotechnol.* **2008**, *26*, 1235–1240.
(3) Buch, P. K.; Bainbridge, J. W.; Ali, R. R. *Gene Ther.* **2008**, *15*, 849–857.
(4) Thrasher, A. J.; Gaspar, H. B.; Baum, C.; Modlich, U.; Schambach, A.; Candotti, F.; Otsu, M.; Sorrentino, B.; Scobie, L.; Cameron, E.; Blyth, K.; Neil, J.; Abina, S. H.; Cavazzana-Calvo, M.; Fischer, A. *Nature* **2006**, *443*, e5–e6.
(5) Schneider, A. J.; Friedmann, T. *Adv. Genet.* **2006**, *51*, 1–110.
(6) Nicholls, H. *Nat. Biotechnol.* **2008**, *26*, 849.
(7) Wells, D. J. *Br. J. Pharmacol.* **2008**, *154*, 623–631.
(8) Baoutina, A.; Alexander, I. E.; Rasko, J. E.; Emslie, K. R. *J. Gene Med.* **2008**, *10*, 3–20.
(9) Azzazy, H. M. E.; Mansour, M. M. H. *Analyst* **2007**, *132*, 951–957.
(10) Minunni, M.; Scarano, S.; Mascini, M. *Trends Biotechnol.* **2008**, *26*, 236–243.
(11) Teles, F. R. R.; Fonseca, L. P. *Talanta* **2008**, *77*, 606–623.
(12) Jenison, R.; Yang, S.; Haeberli, A.; Polisky, B. *Nat. Biotechnol.* **2001**, *19*, 62–65.
(13) Turner, A. P. F. *Nat. Biotechnol.* **1997**, *15*, 421–421.
(14) Vaughan, R. D.; Guilbault, G. G. In *Piezoelectric Immunosensors*; Springer Series on Chemical Sensors and Biosensors Piezoelectric Sensors; Springer: Berlin-Heidelberg, Germany, 2007; pp 237−280.
(15) Marquette, C. A.; Blum, L. J. *Biosens. Bioelectron.* **2006**, *21*, 1424–1433.
(16) Mariotti, E.; Minunni, M.; Mascini, M. *Anal. Chim. Acta* **2002**, *453*, 165–172.
(17) Minunni, M.; Tombelli, S.; Fonti, J.; Spiriti, M. M.; Mascini, M.; Bogani, P.; Buiatti, M. *J. Am. Chem. Soc.* **2005**, *127* (22), 7966–7967.
(18) Baskar, J. F.; Smith, P. P.; Nilaver, G.; Jupp, R. A.; Hoffmann, S.; Peffer, N. J.; Tenney, D. J.; Colberg-Poley, A. M.; Ghazal, P.; Nelson, J. A. *J. Virol.* **1996**, *70*, 3207–3214.
(19) Hallauer, P. L.; Hastings, K. E. *BMC Genet. Epub* October 4, **2000**, 1:1.

10.1021/ac901445b CCC: $40.75
Published on Web 10/30/2009

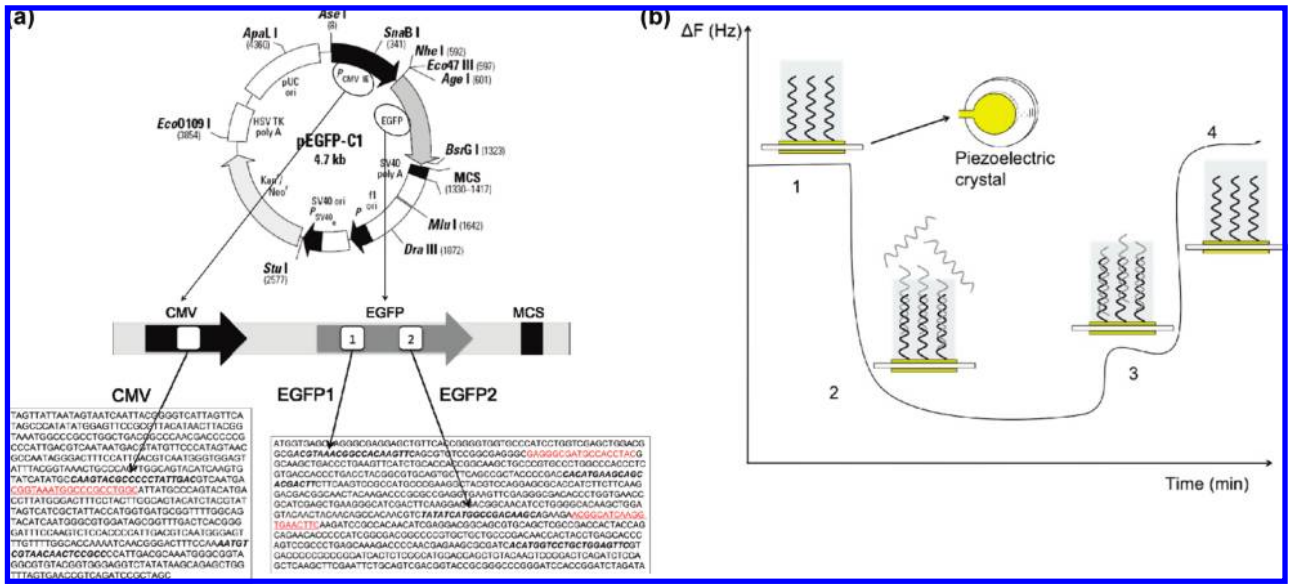

Figure 1. (a) Plasmid pEGFP-C1 map containing the CMV promoter and EGFP gene sequences used in this study as markers of transgenosis. The vector cassette containing the target sequences is evidenced at the bottom of the vector map. At the bottom of the vector map selected marker sequences are evidenced in red. Primer pairs for PCR amplification containing each specific marker sequence are in italic bold. (b) Measuring cycle of DNA-based piezoelectric biosensor (frequency (in hertz) vs time). Step 1, baseline signal in buffer, before target addition (free probe on the surface); step 2, hybridization reaction showed as binding curve (target sequence binds the complementary probe); step 3, washing the surface to remove the unbound target; step 4, regeneration of the surface by dissociating the hybrid (dsDNA) restoring the baseline. The analytical datum is taken as the difference (3 − 1) values in hertz.

For sensor development, we first identified putative vectors used in mammal transgenosis and, within vectors, also related sequences to be referred to as markers of transgenosis. Thus the commercially available plasmid pEGFP-C1 was selected among different vectors (for gene therapy research Riken data bank: http://www.brc.riken.jp/inf/en/index.shtml) as a model system, and two related sequences were then identified as markers of vector presence. In particular, as a target analyte two plasmid regions were chosen: the enhanced green fluorescent protein (EGFP) reporter gene, a mutant of green fluorescent protein, and the *Cytomegalovirus* (CMV) promoter sequences (Figure 1).

For DNA-based sensing development, sensor surfaces were chemically modified for covalent probes binding, by two different immobilization protocols developed for gold surfaces, represented by evaporated electrodes on 9.5 MHz quartz crystals. The approaches used were the thiol/dextran/streptavidin coating with biotinylated probes[20] and the direct linkage of thiolated probes.[21]

The system has been optimized using synthetic oligonucleotides and applied to the detection of the EGFP reporter gene and of CMV promoter sequences first in polymerase chain reaction (PCR) products obtained from pEGFP-C1 purified plasmid and second in the presence of human genomic DNA to evaluate any matrix effect. Finally, detection of these transgenosis markers has been achieved in DNA extracted and amplified from human cell culture (HEK, i.e., human embryonic cells) transformed with the same plasmid, demonstrating the suitability of biosensor approach for transgene detection in samples of human origin.

(20) Löfås, S.; Johnsson, B. *J. Chem. Soc., Chem. Commun.* **1990**, *21*, 1526–1528.
(21) Ulmann, A. *Chem. Rev.* **1996**, *96*, 1533–1554.

EXPERIMENTAL SECTION

Materials and Reagents. 11-Mercapto-1-undecanol (MU), 6-mercapto-1-esanol (MCH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), and streptavidin were purchased from Sigma (Milan, Italy); Dextran 500 from Amersham Biosciences Europe (Milan, Italy); and (±) epichlorohydrin and *N*-hydroxysuccinimide (NHS) from Fluka (Milan, Italy). For thiolated probes, the immobilization buffer was KH_2PO_4 1 M, pH = 3.8; for biotinylated probes, the immobilization buffer was NaCl 150 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4. Hybridization buffer was NaCl 300 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4. Ethanol and all the reagents for the buffers were purchased from Merck (Italy).

PCR primers, ssDNA probes, and targets were purchased from MWG Biotech (Milan, Italy) and are reported in Table 1. dNTPs and *Taq* DNA polymerase were from GE-Healthcare (Milan, Italy). The plasmid pEGFP-C1 was purchased from Clontech, and the commercial genomic human DNA (hDNA) was from Novagen (Wisconsin). The plasmid pEGFP-C1 (Clontech) was transformed in "One Shot TOP10 Chemically Competent" *E. coli* cells (Invitrogen), following the manufacturer specifications. For plasmid DNA extraction, NucleoSpinPlasmid kit (Macherey Nagel, M-Medical, Firenze) was used. Human embryonic kidney cells (HEK), transformed with the same plasmid named HEK-GFP cells as well as the same cell line not transformed (negative control HEK-293), were kindly provided from Dr. Arcangeli (Dipartimento di Patologia e Oncologia Sperimentale, University of Florence, Italy). Genomic DNA was extracted from HEK cells according to the instructions of the Invisorb Spin Tissue Kit (Invitek, Berlin, Germany).

Apparatus. Quartz crystals (9.5 MHz AT-Cut, 14 mm) with gold evaporated (42.6 mm^2 area) on both sides were purchased

Table 1. Oligonucleotide Sequences Used As Surface Bound Probes, Complementary Targets, and Negative Control (Noncomplementary Sequence)[a]

oligonucleotide	DNA sequences
EGFP1 biotinylated probe (18 mer)	5′ biotin-GAG GGC GAT GCC ACC TAC-3′
EGFP1 complementary target (18 mer)	5′-GTA GGT GGC ATC GCC CTC-3′
EGFP2 biotinylated probe (20 mer)	5′ biotin-ACG GCA TCA AGG TGA ACT TC-3′
EGFP2 complementary target (20 mer)	5′-GAA GTT CAC CTT GAT GCC GT-3′
CMV biotinylated probe (20 mer)	5′ biotin-CGG TAA ATG GCC CGC CTG GC-3′
CMV complementary target (20 mer)	5′-GCC AGG CGG GCC ATT TAC CG-3′
noncomplementary sequence (Tnos, 25 mer)	5′-GAT TAG AGT CCC GCA ATT AAT CAT T-3′
forwardegfp1*	5′-ACG TAA ACG GCC ACA AGT TC-3′
reverse egfp1*	5′-CAC ATG AAG CAG CAC GAC TT-3′
forward egfp2*	5′-TAT ATC ATG GCC GAC AAG CA-3′
reverse egfp2*	5′-ACA TGG TCC TGC TGG AGT TC-3′
forward cmv*	5′-CAA GTA CGC CCC CTA TTG AC-3′
reverse cmv*	5′-GGC GGA GTT GTT ACG ACA TT-3′

[a] Primer pairs used in PCR amplifications are also reported.

by International Crystal Manufacturing. The measurements were conducted in methacrylate cells where only one side of the crystal was in contact with the solution. The quartz crystal analyzer used for the measurements was the QCMagic analyzer by Elbatech (Livorno, Italy) and directly recorded by a computer connected to the QCMagic interface.

All PCR experiments were conducted with Thermocycler MJ-Research Ptc-200 (Peltier Thermal Cycler) DNA Engine. Screening of the PCR products was performed by gel electrophoresis (Bio-Rad, CA) and visualized through a UV transilluminator GelDoc System (Bio-Rad). PCR amplicons were quantified using Picogreen dye with a TD-700 fluorometer (Turner Biosystems, Milan, Italy). DNA sequences for probes and primers were purchased from MWG Biotech (Milan, Italy) and are listed in Table 1.

PROCEDURES

Probe Immobilization. The thiolated and biotinylated probes were immobilized on gold surfaces by direct amino-coupling or via biotin-streptavidin binding, after modifying the gold surface via thiol/dextran/streptavidin treatments. Both procedures have been previously reported in Mannelli et al., 2003.[22]

Hybridization Reaction. A total of 60 μL of the DNA solution (synthetic oligonucleotides or PCR amplified samples) in hybridization buffer was added to the measuring cell and the incubation was carried out for 10 and 20 min with oligonucleotides or amplified samples, respectively. For PCR samples, a heat treatment (95 °C for 5 min) followed by cooling in ice for 1 min is required to denature the double strand. The sample is then immediately added to the cell. Then, the surface was washed with hybridization buffer to eliminate the unbound target DNA. The reported frequency shifts (ΔF) are the difference (in hertz) between the final value and the value displayed before the hybridization step, both in the same buffer. The signal is considered positive when $\Delta F > 3$ Hz, which represents 3 times the blank signal, both for oligonucleotides and PCR samples. After each cycle of hybridization, the single stranded probe on the crystal surface was regenerated by two consecutive treatments of 30 s with 1 mM HCl allowing a further use of the sensor. All the experiments are performed at room temperature (20 °C).

(22) Mannelli, I.; Minunni, M.; Tombelli, S.; Mascini, M. *Biosens. Bioelectron.* **2003**, *18*, 129–140.

DNA Template Processing and PCR Conditions. Plasmid DNA was used as a template, carrying both the target analytes: EGFP gene and CMV promoter sequences. The plasmid analyzed was present in three different combinations: (1) present alone, (2) copresent with commercial human genomic DNA (hDNA) to assess its amplificability by PCR, and (3) present in transformed human cells (HEK-GFP). Two different regions of the EGFP gene were considered for the PCR amplification, resulting in 187 bp and 219 bp amplicons, respectively, obtained with egfp1 and egfp2 primer pairs (Table 1 and Figure 1a). For the CMV promoter sequence, a 272 bp region was amplified by using the corresponding primer pair (Table 1 and Figure 1a).

The EGFP amplification protocol in both cases is reported here. Reactions were carried out in 25 μL PCR mixtures containing 1× of the specific reaction buffer (100 mM Tris-HCl, pH 9.0, 15 mM $MgCl_2$, and 500 mM KCl, GE-Healthcare, Milan, Italy), 200 μM each dNTP, 200 nM of each primer, 0.2 U Taq DNA polymerase (GE-Healthcare, Milan, Italy), and 10 ng of plasmid DNA template. The control solution (blank) contained all the PCR reagents with the exception of the DNA template.

The amplification condition was the same for both targets, the EGFP gene and the CMV promoter, except the annealing temperature 54 and 52 °C for 1 min, respectively. Cycler conditions were initial denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 1 min, annealing (54 or 52 °C, respectively) for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 8 min. The amplificability of the target sequences present on the vector was evaluated in the presence of hDNA. A different number copies of pEGFP-C1, i.e., 1, 5, 10, and 50, were added to 100 ng of hDNA. The weight of a pEGFP-C1 single copy was evaluated as follows: pEGFP-C1 $= (4.731 \times 10^3 \times 660)/6.02 \times 10^{23} = 5.19 \times 10^{-6}$ pg. Since the weight of haploid human genomic DNA is 3.75 pg (http://www.ncbi.nlm.nih.gov/projects/genome/guide/human/), the total number of genome copies contained in 100 ng is 26.667.

In the case of HEK cell lines, HEK-GFP and HEK-293, the total DNA was extracted according to the supplying company recommendations. For each PCR reaction tube, 100 ng were used as the template.

The PCR amplification protocols reported above were applied also in these latter two experiments. The amplification products were analyzed by electrophoresis on 2% (w/v) agarose gel

Table 2. Main Analytical Parameters Obtained by Calibrating the Biotinylated Biosensors with the Relative Synthetic Oligonucleotides EGFP1, EGFP2, and CMV

probe	EGFP1	EGFP2	CMV
experimental detection limit (DL) (nM)	50	50	25
frequency shift at DL (Hz)	-7 ± 2	-5 ± 2	-9 ± 1
CVav %	10	6	4
linear range (nM)	0−500	0−500	0−500
linear equation	$y = 0.12x + 0.9892$	$y = 0.21x - 5.83$	$y = 0.25x + 5.69$
R^2	0.998	0.999	0.985

(SeaKem, Cambrex, ME) in TAE buffer (Tris-HCl 2 M, EDTA 100 mM, pH = 8). The profiles obtained were captured as tiff format files by the GelDoc software.

RESULTS AND DISCUSSION

Target Sequence Selection. To identify putative marker sequences of transgenosis suitable for the analysis, the vectors most used in transgenosis for gene therapy protocols have been compared in the Riken data bank (http://www.brc.riken.jp). In particular, two regions present in the plasmid pEGFP-C1 were selected as transgenosis markers: the EGFP reporter gene, a mutant of green fluorescent protein, and the *Cytomegalovirus* promoter sequence. EGFP gene detection was addressed for its wide use as marker of transgenosis event, both in in vitro and in vivo preclinic trials, and thus represents a well-known reporter system. On the other hand, studies on mice have demonstrated the importance of the CMV promoter in high level and constitutive gene expression, i.e., in skeletal muscle.[18,19]

Probe Design for Sensor Development. Probes for EGFP and CMV promoters were designed starting from the selected marker sequences, following different criteria: selecting a region rich in GC (>50%) and minimizing possible secondary structures, i.e., hairpins, etc., to ensure higher binding to the complementary sequence in solution. For EGFP gene sequence, two different probes were chosen with the aim to study the influence of secondary structures on the DNA probe functionality via in silico preliminary evaluation. This has been achieved by free available software (http://mfold.bioinfo.rpi.edu/). For CMV detection, only one probe sequence was designed. All probe sequences are reported in Table 1.

Probe Immobilization and Sensor Calibration with Synthetic Probes. The probes were covalently immobilized on chips (gold electrodes evaporated on quartz crystals) using two immobilization chemistries, respectively: the thiol/dextran/streptavidin coating with biotinylated probes and the direct immobilization of thiolated probes (Mannelli, 2003). Calibration curves were obtained with synthetic complementary oligonucleotides for each DNA probe.

EGFP Gene Sensing. All these sensors resulted specific since negligible signal (<3 Hz in absolute value) were observed when

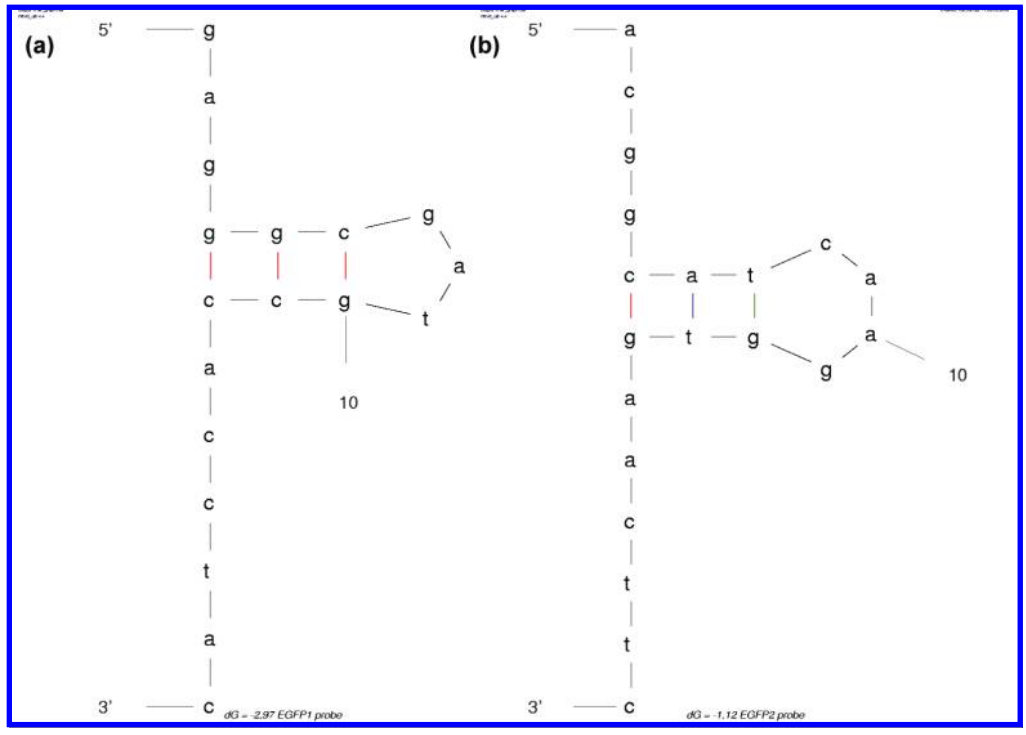

Figure 2. On the left (a) is showed the most thermodynamically favorable (d$G = -2.97$ kcal/mol) secondary structure of the EGFP1 sequence, consisting in a 5-term circular loop with three C−G bonds. On the right (b) is showed the most thermodynamically favorable (d$G = -1.12$ kcal/mol) secondary structure of the EGFP2 sequence, characterized by a lower free energy since it involves a 6-terms weaker loop formed by an A−T match and a T−G mismatch. These data support the experimental finding showing probe EGFP2 biosensor is more sensitive than the EGFP1 probe.

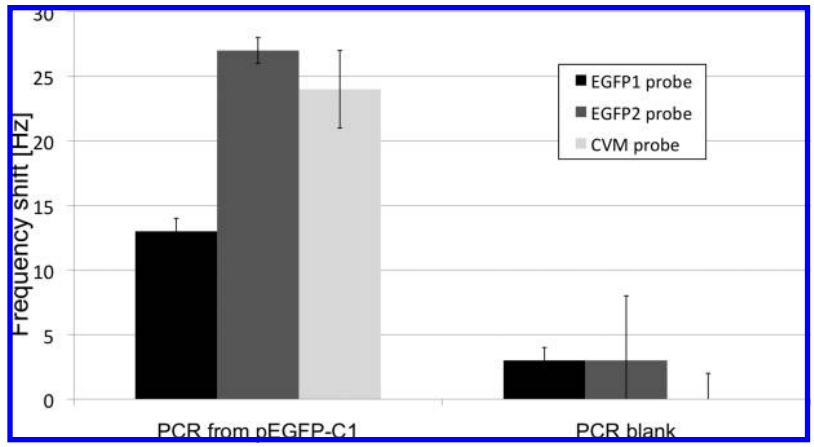

Figure 3. PCR amplified DNA samples, using as a template purified pEGFP-C1 and the relative PCR blanks, were tested on the three different probes EGFP1, EGFP2, and CMV. The concentration of the PCR amplicons was 200 nM for all the samples. Each sample was tested three times ($n = 3$).

they were exposed to the negative control (1 μM). The observed reproducibility was good (5% and 11% CVav % for biotinylated and thiolated probes, respectively). The linearity for the all systems was also good (R^2 from 0.98 to 0.99) with a linear working range of 0−500 nM. Since the best sensitivity and reproducibility were observed using biotinylated probes together with longer sensor lifetime (up to 24 cycles vs 15, using 1 mM HCl for 30 s as dsDNA dissociating agent), further experiments were conducted using thiol/dextran/streptavidin chemistry. Moreover, within biotinylated probes for EGFP, in particular EGFP2 gave the best results, in terms of sensitivity and reproducibility (linear equation $y = 0.21x - 5.83$, CVav % = 6) in comparison with EGFP1 (linear equation $y = 0.12x + 0.9892$, CVav % = 10). The linear range was 0−500 nM for both probes (Table 2). These results on EGFP probes confirm the great influence of secondary structures in the efficacy of hybridization between probes and targets. EGFP1 and EGFP2 relative structures are reported in Figure 2, and they are obtained by inserting into the software's parameters a 20 °C temperature and $[Na^+] = 0.3$ M as the operating conditions.

CMV Promoter Sensing. To detect CMV promoter target sequence, on the basis of EGFP sensor optimization findings, only the biotinylated probe was immobilized on the surface using thiol/dextran/streptavidin chemistry. A linear range of 0−500 nM was also found using synthetic CMV complementary oligonucleotide. The linearity for CMV was also good ($R^2 = 0.984$). The sensor was sensitive ($\Delta F = -9 \pm 1$ Hz for 25 nM target), selective, since negligible (<3 Hz) signal using negative control (1 μM), and reproducible (CVav % = 4). The sensor lifetime was 26 cycles (with HCl 1 mM, 30 s). The relative main analytical parameters of the three biosensors are summarized in Table 2. Thus, the EGFP and CMV optimized sensors, together with EGFP1, were further applied to PCR amplified DNA extracted from bacterial and human transgenic cell culture (HEK-GFP cells) both transformed with plasmid pEGFP-C1.

Detection of Target Sequences in PCR Amplified Samples. Plasmid DNA was first extracted from bacterial cells and amplified by PCR. The samples were thermally denatured (95 °C, 5 and 1 min cooling in ice) to obtain ssDNA able to hybridize the immobilized complementary probe and then applied to the sensor for 20 min. Then the surface was washed with hybridation buffer to remove the unbound target, and the frequency was recorded. The results are shown in Figure 3. The three sensors were able to detect the relative target sequences in amplified DNA, in a selective and reproducible manner, since negligible shifts were found when negative controls were tested and demonstrated the selectivity of the system. The estimated detection limits were 50, 25, and 80 nM for EGFP1, EGFP2, and CMV, respectively. Between biotinylated EGFP1 and EGFP2 probes, better sensitivity and reproducibility was obtained for the latter one. In particular, PCR samples directly tested after amplification at the concentration of 220 nM gave $\Delta F = -13 \pm 1$ for EGFP1 vs $\Delta F = -27 \pm 1$ for EGFP2 with CVav % = 8 and CVav % = 4, respectively. These results are in agreement with those obtained with the corresponding synthetic oligonucleotides.

Table 3. Main Analytical Parameters Obtained with PCR Amplified Samples from Both Purified Plasmid and Transgenic Human Cells[a]

sample type	probe	QCM signal (Δf in Hz)	CVav %	exptl DL (nM)
amplicons from purified vector (pEGFP-C1)	EGFP1	13 ± 1	8	50
	EGFP2	27 ± 1	4	25
	CMV	24 ± 3	12	80
amplicons from HEK-GFP cells transfected with pEGFP-C1	EGFP1	6 ± 1	17	110
	EGFP2	24 ± 1	4	30
	CMV	25 ± 3	12	80

[a] All frequency shifts are referred to samples at the same concentration (220 nM).

Relative to CMV amplified samples, a significant shift was also found for the same concentration ($\Delta F = -24 \pm 3$), with good reproducibility (CVav % = 12). The results are summarized in Table 3.

Reuse of the sensors was possible by use of a denaturing solution (HCl 1 mM for 30 s) to dissociate the dsDNA complex at the sensor surface for a number of cycles comparable to those obtained with synthetic oligonucleotides. These findings have been obtained using purified plasmid as a DNA template and aimed to demonstrate the biosensor applicability to detect transgenosis markers. In a hypothetical gene doping event, the plasmid

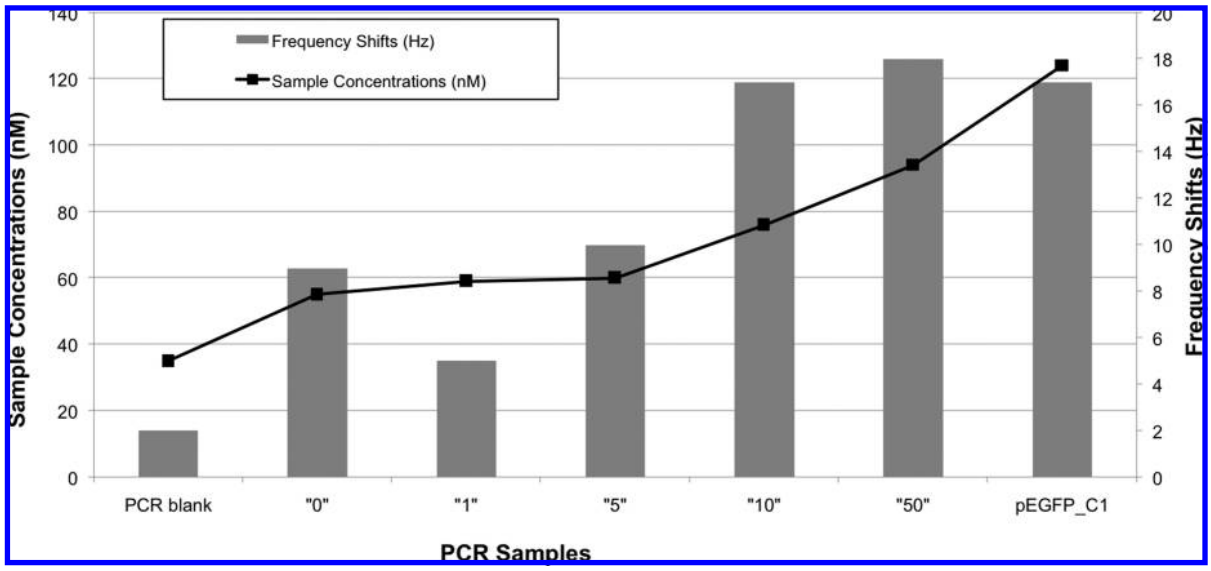

Figure 4. Plasmidic DNA copresent with human DNA to selectively amplify the marker DNA EGFP sequence. pEGFP-C1 template added to human genomic DNA in different copy numbers (0, 1, 5, 10, 50) to 10 ng of bulk hDNA solution. The EGFP marker amplified by PCR using the forward and reverse EGFP2 primers couples to obtain the EGFP2 amplicon. The amplified samples were tested on the EGFP2 biotinylated biosensor, and the results obtained were compared with the relative samples' concentrations.

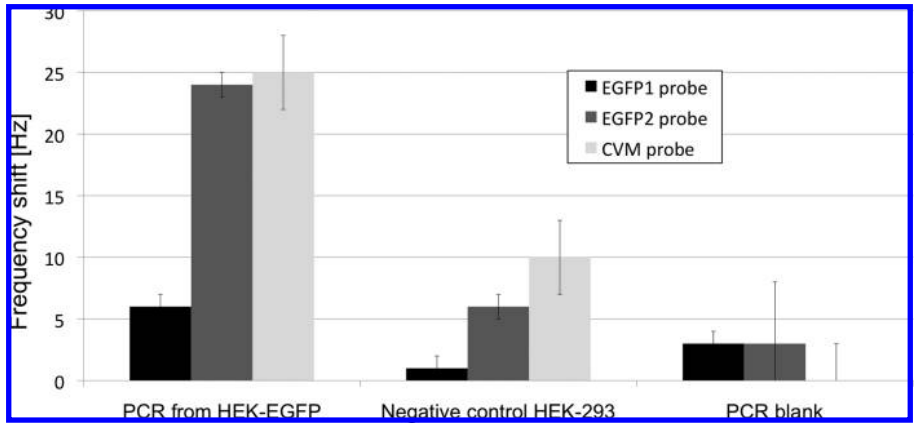

Figure 5. PCR amplified DNA extracted from transgenic human cells (human hembryogenic kidney HEK-EGFP) transformed with plasmid pEGFP-C1 and the relative control (nontransformed cells HEK-293) are tested on EGFP and CMV biosensors carrying the three different probes EGFP1, EGFP2, and CMV. The concentration of the PCR amplicons was 200 nM for all the samples. Each sample was tested three times ($n = 3$).

would be injected into the body, i.e., muscle and thus copresent with human DNA. To mimic this situation, the plasmid template was added to human genomic DNA. Thus different copy numbers of the plasmid (0, 1, 5, 10, 50) were added (100 ng/tube corresponding to 2 ng/μL) to a 10 ng bulk hDNA solution and EGFP2 marker amplified by PCR. The results showed that it was possible to amplify the target sequence in the presence of human genomic DNA and also to detect down to 10 copies by gel electrophoresis (data not shown).

To evaluate the ability of the sensor in detecting target sequences in such complex matrixes, the same PCR samples with hDNA copresent were tested on the sensor, after the denaturing thermal treatment. As shown in Figure 4, the system was able to detect the target sequence down to 10 copies of EGFP amplicons, in agreement with gel electrophoresis results and in a concentration dependent manner. The results obtained here demonstrate that it is possible to selectively detect target sequences on the vector cassette, even when in the presence of bulk human genomic DNA. This achievement allowed us to proceed in testing the sensor with HEK-EGFP (human embryonic kidney) cells transformed with the plasmid pEGFP-C1.

Detection of Target Sequences in Transgenic Human Cells. Transgenic human HEK-GFP cells were obtained by their transformation with plasmid pEGFP-C1 and subsequent in vitro cultivation. The total DNA was then extracted and amplified for further measurements by EGFP (both EGFP1 and EGFP2) and CMV biosensors previously developed. As a negative control, nontransgenic cells consisting in the same cell line but not transformed with pEGFP-C1 (HEK-293) were used.

Both EGFP and CMV sensors gave selective signals with PCR samples relative to the specific markers, since negligible shifts were observed with the negative controls (Figure 5). In particular, probe EGFP1 showed lower hybridization signals ($\Delta F = -6 \pm 1$ Hz) and higher variability (CVav % = 17) with respect to probe EGFP2 ($\Delta F = -24 \pm 1$ Hz, CVav % = 4) using the amplified samples (220 nM). Negligible signals were found with PCR blanks and negative controls (HEK-293) demonstrating the high selectivity of the systems.

For the CMV sensor, tested with corresponding amplicons obtained from transgenic cells, positive responses were found ($\Delta F = -25 \pm 3$ Hz for 220 nM) and the signal was well distinguishable from the one observed with the HEK-293 negative control ($\Delta F = -10 \pm 1$ Hz), demonstrating also in this case the system selectivity. The response showed good reproducibility (CVav % = 12). The results are summarized in Table 3. It was possible to detect transgenic DNA present in human cells transformed with plasmid pEGFP-C1, both in the case of the EGFP gene and CMV promoter sequences, demonstrating the ability of the system to selectively and reproducibly bind target sequences.

CONCLUSIONS

The traceability of transgenosis represents an emerging problem for gene doping analysis. Here it was demonstrated that piezoelectric-based affinity sensing represents an interesting fast and low cost (about $5 U.S./sample) approach for the detection of transgenes in human genome.

For method development, the EGFP gene and CMV promoter sequences were selected as markers of transgenosis to be used for identifying the gene transfer event. In particular, the EGFP gene, widely used for in vitro and in vivo preclinic trials as a useful marker of transgenosis, allowed optimization of the analytical method representing an internal positive control. The biosensor was thus applied to detect the EGFP and also CMV sequences in standard solutions (both of synthetic oligonucleotides and in PCR samples from purified plasmid) and in human transgenic cells genetically modified with the same plasmid. Good reproducibility of the system in marker sequences detection was observed in transgenic human cells with CVav % values of of 4% and 12%, respectively, for the EGFP gene and CMV promoter as target analytes. Target sequences amplificability, down to 10 vector copies per genomic bulk DNA, was achieved demonstrating the presence of genomic DNA does not interfere with marker sequence amplificability, even in the presence of the template's low copy number. Moreover, selective transgene detection was assessed, since significant response was achieved only in the presence of transfected cells while negligible signals were recorded with the negative controls (nontransfected cells).

For further optimization of hybridization stringency, studies should be directed toward probe selection and immobilization conditions, as here we have evidenced that different analytical performances can be achieved using two different probe mappings in different regions of the amplified target analyte. As a further extension of the proposed approach, different probes could be eventually tested to better discriminate among the ones showing better analytical performances for detecting the sequence on the specific transgenic amplified region.

Finally, we would like to conclude with some important issues regarding the preanalytical phase, such as sampling protocols in gene-doping control. For the total analytical approach, sampling issues should be addressed. Unfortunately, no sampling indications for gene doping control are yet available. Sampling issues are strategic for a reliable analysis and at this regard studies on animal models are very useful to explore the vector lifetime and fate after injection for obtaining indications about sampling issues. Some literature[8−10] and on going projects (http://www.wada-ama.org/en/dynamic.ch2?pageCategory.id=347), funded by the World Anti-Doping Agency (WADA), are focused on this issue. It seems very reasonable that muscle exercise might determine the release of muscle fiber DNA into circulation during extreme exercise and also that transgenic material can be found in blood after gene therapy applications.[26,27] We feel that the usual method for biopsies, using a large biopsy needle, is unlikely to be acceptable due to the damage caused. However in contrast, a new method for biopsy using a fine needle aspirate seems to avoid the damage in sampling and seems to be sensitive when coupled to PCR approaches, to pick up evidence of genetic manipulation. It is however limited by the same issues associated with muscle-biopsy samplings. In that the area, samples may be not indicative of the whole muscle, and where gene delivery has been less than 100% efficient, the biopsy may not be sampling the treated area.[28] The approach adopted in this study allows one to work with extracted human DNA from cells and can be eventually transferred to other matrixes of human origin, eventually suggested by animal model studies, which have however to be scaled up to humans.

Further development in label-free, affinity-based sensing for gene doping applications will be in extending this approach to multi-analyte/sequence detection; thus a more exhaustive control can be thought of. With the use of an array approach, a high number of markers of transgenosis can be selected and the relative probes immobilized on the surface, in a multianalyte-based sensing for simultaneous detection of target analytes. One suitable approach will be further based on optical label free sensing such as on surface plasmon resonance imaging (SPR-imaging) transduction,[23−25] allowing for the simultaneous detection of hundreds of interactions. Preliminary work at this regard is in progress, since the World Anti-Doping Association (WADA) has very recently encouraged the affinity biosensors-based approach, by funding (October 2008) a 1 year project entitled "An Integrated Approach with Affinity-Based Biosensing (ABBs) for Gene Doping Detection: A Pilot Study" based on animal model, bioinformatics, and SPR imaging transduction.

ACKNOWLEDGMENT

We thank Prof. Annarosa Arcangeli, Dipartimento di Patologia ed oncologia sperimentale, Università degli Studi di Firenze, for kindly providing human transgenic cells (HEK-EGFP and HEK-293) and the Italian Ministry of Health for financial support, within Programma di Ricerca 2005 sui farmaci, sulle sostanze e pratiche mediche utilizzabili ai fini di doping nelle attività sportive, with the project "Metodi bioanalitici basati sui biosensori a DNA per le nuove frontiere del doping: l'individuazione di geni e proteine esogene", coordinator, M. Minunni.

Received for review July 1, 2009. Accepted October 19, 2009.

AC901445B

(23) Homola, J., Ed. *Surface Plasmon Resonance Based Sensors*; Springer: Berlin-Heidelberg, Germany, 2006.
(24) Lisboa, P.; Valsesia, A.; Mannelli, I.; Mornet, S.; Colpo, P.; Rossi, F. *Adv. Mater.* **2008**, *20*, 2352–2358.
(25) Alastair, W. W.; Lee, H. J.; Corn, R. M. *Anal. Chem.* **2005**, *77*, 3904–3907.
(26) Parker, A. L.; Nicklin, S. A.; Baker, A. H. *Curr. Opin. Mol. Ther.* **2008**, *10*, 439–448.
(27) Baker, A. H.; Mcvey, J. H.; Waddington, S. N.; Di Paolo, N. C.; Shayakhmetov, D. M. *Mol. Ther.* **2007**, *15*, 1410–1416.
(28) Wells, D. J. *Br. J. Pharmacol.* **2008**, *154*, 623–631.