WORLD ANTI-DOPING AGENCY
play true

Laboratory Guidelines

Gene Doping Detection based on Polymerase Chain Reaction (PCR)

Version 1.0
January 2021

WORLD ANTI-DOPING AGENCY
play true

TABLE OF CONTENTS

[For the purpose of this these Laboratory Guidelines, *Code* definitions are in *Italics*. *International Standard* definitions are Underlined.]

1.0 Objective

These Laboratory Guidelines have been developed to ensure a harmonized approach in the application of Analytical *Testing* using polymerase chain reaction (PCR) for detection of Gene Doping in sport [1-4]. These Laboratory Guidelines provide direction on the *Sample* pre-analytical preparation, performance of the test and interpretation of test results.

2.0 Scope

These Laboratory Guidelines follow the rules established in the *World Anti-Doping Agency's* (*WADA*) *International Standard* for Laboratories (ISL) [5] and relevant *Technical Documents* (*TD*s) regarding the Analytical *Testing* of blood *Samples*. These requirements are fully applicable and shall be respected. These Laboratory Guidelines contain additional recommendations to facilitate the implementation of the Analytical Methods specific to Gene Doping based on PCR.

3.0 Introduction

These Laboratory Guidelines present information and technical requirements on the application of direct PCR-based Analytical Methods for the identification of Gene Doping agents. Direct Analytical Methods to detect Gene Doping target differences between sequences of a doping gene (also referred to as a transgene) and an endogenous gene in human genomic deoxyribonucleic acid (hgDNA).

The most likely form of a doping gene is based on complementary DNA (cDNA) derived from the gene's messenger RNA (mRNA) sequence. Unlike the endogenous genes in human genomic DNA, in which protein-coding exons are separated by non-coding introns, there are no introns in a cDNA-based transgene. Therefore, DNA sequences that correspond to junctions between the adjacent exons (exon/exon junctions) are unique to the cDNA and are not present in the relevant endogenous gene in the human genome. Therefore, the Analytical *Testing* Procedure described herein to detect Gene Doping is based on PCR assays specifically designed to detect these exon/exon junction target sequences.

4.0 Test Requirements

4.1. Test Method Validation Requirements

Prior to implementation of a Gene Doping Test in routine Analytical *Testing*, the Laboratory shall validate the test's performance, including:

- DNA extraction efficiency [using an appropriate Positive Extraction Control (PEC)];

  *[Comment: This can be done by demonstrating the ability to detect as positive DNA extracted from 1 mL of PEC spiked with 1500 or fewer copies of DNA Reference Material (RM). Alternatively, the Laboratory shall demonstrate that the PCR cycle threshold (Ct) * values for the PEC are indicative of high extraction recovery.]*

* Ct, in real-time PCR, is defined as the cycle at which the fluorescence from the reaction crosses a specified threshold level that distinguishes the signal from background levels [SOURCE: ISO 16577:2016 (en), 3,32]

- PCR specificity [using appropriate no template control (NTC) and positive template control (PTC)];
- PCR efficiency and linearity (using an appropriate PTC);
- PCR sensitivity (assays shall be able to reliably detect 10 or less copies of DNA RM in the PTC);
- Correct identification of specific amplicons by DNA Melt Curve Analysis (MCA) [for the Initial *Testing* Procedure (ITP)] or Fragment Size Analysis (FSA) [for the Confirmation Procedures (CPs)];
- Robustness (with regard to different batches of reagents and, if applicable, different equipment and analysts).

  [Comment: For more guidance on method validation, including the preparation and use of extraction and template controls, refer to the WADA-approved Gene Doping Test Protocol. In addition, the use of these controls shall be specified in the Laboratory's Standard Operating Procedure (SOP) for the Analytical Method.]

4.2. Test Method Accreditation Requirements

- Successfully participate in at least one round of *WADA*-approved educational External Quality Assessment Scheme (EQAS) or in inter-Laboratory collaborative study. In cases of identified deficiencies, proper corrective action(s) shall be documented and implemented;
- Obtain ISO/IEC 17025 accreditation for the Gene Doping Test from an Accreditation Body that is a full member of the International Laboratory Accreditation Cooperation (ILAC) and a signatory to the ILAC Mutual Recognition Agreement (ILAC MRA).

4.3. Pre-analytical Procedure

Upon reception of the "A" and "B" *Samples* in the Laboratory, the following steps should be followed:

- Check that the blood *Samples* have been collected in tubes containing K_2EDTA (dipotassium ethylenediaminetetra-acetic acid) as anticoagulant (such as those used for the collection of *Athlete Biological Passport* (*ABP*) blood *Samples*) [6]. Such blood *Samples* shall be kept in a refrigerated state (not frozen) following collection and during transportation to the Laboratory.

  To avoid any potential cross-contamination that may lead to false positive results, *Samples* collected for the purpose of *ABP* blood analysis shall **not** be used for the application of this PCR-based Gene Doping test. "A" and "B" *Sample* K_2EDTA tubes shall be collected for Gene Doping detection; any other tests that may be performed on these *Samples* (for example, for homologous blood transfusion (HBT) on whole blood, or for erythropoietin receptor agonists (ERAs) on plasma obtained after centrifugation) shall be done on Aliquots taken following the completion of the Gene Doping test;
- Check the status of the *Sample(s)* (*e.g.* evidence of hemolysis) and the integrity of the collection tubes. The Laboratory shall note and record any unusual condition of the *Sample* and include it in the Test Report to the *Testing* Authority (TA);

- The test is to be performed in whole blood:
  - Process the "A" *Sample* as soon as possible as whole blood. If a blood *Sample* starts separating into plasma and cells, the content shall be homogenized by gentle inversion of the tube 8-10 times;
  - If the *Samples* are not to be processed immediately, they can be stored in the *Sample* collection tube refrigerated at approximately 4°C for a maximum of thirty (30) days. Alternatively, the *Samples* can be centrifuged in a swing-out rotor at 1300 g for 10 min at room temperature and stored frozen at -20°C or -80°C in the *Sample* collection tube. *Sample* freezing should follow the following procedure: *Samples* should be refrigerated for 2 hours and gradually frozen at -20°C (for example, in a foam box) before being transferred for storage at -20°C (up to 3 months) or- 80°C (more than 3 months).The content of the *Samples* shall be homogenized after thawing by gentle inversion of the tube 8-10 times before analysis.

4.4. Analytical *Testing* Procedure

For the conduct of the analytical procedures for DNA extraction and PCR, refer to the *WADA*-approved Gene Doping Test Protocol and the Laboratory's SOP.

To minimize chances of cross-contamination during the analytical procedure, best laboratory practice for performing molecular biology techniques shall be followed. Review the recommendations in the *WADA*-approved Gene Doping Test Protocol prior to commencing the test.

In case of contradiction between the *WADA*-approved Gene Doping Test Protocol, the Laboratory SOP and these Laboratory Guidelines, the latter document shall prevail.

4.4.1. Initial *Testing* Procedure

- DNA shall be extracted from a single Aliquot of the "A" *Sample*. Extracted DNA shall be analyzed by PCR assay in duplicate;
- The PCR assay used for the ITP shall target a specific exon/exon junction sequence of the transgene. The PCR assay to be used for the ITP is specified in the Gene Doping Test Protocol;
- Post-PCR MCA shall be conducted to determine the melting temperature of the amplified DNA product;
- The ITP shall include appropriate negative and positive quality controls to check for:
  - the efficiency of the DNA extraction procedure (including testing for potential contamination during extraction);
  - the correct functioning of the PCR assay (including the testing for potential contamination and matrix interferences in PCR); and
  - the correct identification of the melting temperature of specific DNA products (from the RM and from the transgene, if applicable) by MCA analysis and, if necessary, for additional verification by FSA.

The Laboratory shall verify the performance of the employed assays over time through the use of QC-charts for PCR Ct values of positive controls (PEC for DNA extraction and PTC for PCR amplification). The criteria for acceptance of the controls shall be specified in the method's SOP.

[Comment: For more guidance on the preparation, use and acceptance criteria for test quality controls, refer to the WADA-approved Gene Doping Test Protocol.]

4.4.2. Confirmation Procedures

4.2.2.1 "A" CP

In the case where the ITP returns a Presumptive *Adverse Analytical Finding* (PAAF) as per Table 1 below, an assay which targets a different exon/exon junction sequence of the target gene (confirmation assay) shall be used for the "A" CP. The PCR assay to be used for the CP is specified in the Gene Doping Test Protocol.

DNA shall be extracted from a new Aliquot of the original "A" *Sample* (or split "B" *Sample*, if applicable). Extracted DNA shall be analyzed in the confirmation PCR assay in duplicate.

4.2.2.2 "B" CP

For the "B" CP, the same PCR assay used in the "A" *Sample* CP shall be applied to DNA extracted from an Aliquot taken from the "B" *Sample.* Extracted DNA shall be analyzed in PCR in duplicate.

The following requirements shall be applied to the CPs:

- In accordance with the ISL, the Laboratory shall have a policy to define those circumstances where the CP of "A" or "B" *Sample* should be repeated;
- In cases where amplification in the PCR assay is observed in one or both replicates, FSA shall be conducted on the PCR-positive replicate(s) to determine the size of amplified DNA product;
- The CP shall include appropriate negative and positive quality controls (including controls prepared in whole blood) to check for:
  - the efficiency of the DNA extraction procedure (including testing for potential contamination during extraction),
  - the correct functioning of the PCR assay (including the testing for potential contamination and matrix interferences in PCR), and
  - the correct identification of the size of specific DNA products (from the RM and from the transgene, if applicable) by FSA analysis.
- The Laboratory shall verify the performance of the employed assays over time through the use of QC-charts for PCR Ct values of positive controls (PEC(s) for DNA extraction and

PTC for PCR amplification). The criteria for acceptance of the controls shall be specified in the method's SOP.

5.0 Interpretation and Reporting of Results

Prior to reporting *Sample* Test Results, results for quality controls shall be evaluated and accepted according to validated criteria, which shall be specified in the method's SOP.

For further guidance on interpretation of Test Results, refer to the *WADA*-approved Gene Doping Test Protocol.

5.1. Initial *Testing* Procedure (ITP)

5.1.1. *Negative* Finding

The ITP shall produce a Negative Finding if:

- No amplification by PCR is observed in either of the duplicates, or
- One or both replicates are amplified in PCR, but neither of them demonstrates the presence of a peak consistent with the amplicon from the transgene when analyzed by MCA (Table 1).

5.1.2. Presumptive *Adverse Analytical Finding* (P*AAF*)

The ITP shall produce a PAAF if at least one (1) replicate amplified in PCR demonstrates the presence of a peak, which is consistent with the amplicon from the transgene when analyzed by MCA (Table 1).

5.2. Confirmation Procedures (CPs)

5.2.1. Negative Finding

The CP shall produce a Negative Finding if:

- No amplification by PCR is observed in either of the duplicates, or
- One or both replicates are amplified in PCR, but neither of them demonstrates the presence of an amplicon that is consistent in length with the amplicon from the transgene when analyzed by FSA (Table 1).

5.2.2. *Atypical Finding* (*ATF*)

The result of the CP shall be reported as an *ATF* if the FSA establishes the presence of a PCR amplicon consistent with the amplicon from the transgene in one (1) out of the two (2) replicates analyzed (Table 1).

5.2.3. *Adverse Analytical Finding* (*AAF*)

The result of the CP shall be reported as an *AAF* if the FSA of the two (2) positive PCR replicates from the CP establishes the presence of an amplicon consistent in length with the amplicon from the transgene (Table 1).

Table 1. Interpretation and reporting of test results

Initial *Testing* Procedure (ITP)			**Confirmation Procedure (CP)**		
PCR	**MCA**	**Interpretation / Reporting**	**PCR**	**FSA**	**Interpretation / Reporting**
−/−	-/-	Negative Finding			
−/+	−/−	Negative Finding			
	−/+ or **+/−** or **+/+**	***PAAF***	−/−	NA/NA	Negative Finding
			−/+	NA/−	Negative Finding
				NA/+	***ATF***
			+/+	−/−	Negative Finding
				−/+	***ATF***
				+/+	***AAF***
+/+	−/−	Negative Finding			
	−/+ or **+/−** or **+/+**	***PAAF***	−/−	NA/NA	Negative Finding
			−/+	NA/−	Negative Finding
				NA/+	***ATF***
			+/+	−/−	Negative Finding
				−/+	***ATF***
				+/+	***AAF***

NA (not analyzed): No FSA is performed on a replicate that is not amplified in PCR.

PCR: Negative (-) and Positive (+) refer to the absence or the presence of amplification.

MCA: Positive (+) refers to a melt temperature (Tm), which is consistent with that for the amplicon from the transgene (doping gene). Negative (-) refers to the absence of a peak on the melt curve consistent with that for the amplicon from the transgene.

FSA: Positive (+) refers to an amplicon which is consistent in length with the amplicon from the transgene. Negative (-) refers to an amplicon which is either not detected or not consistent in length with the amplicon from the transgene.

6.0 Bibliography

[1] Saiki RK *et al*. "Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia" *Science* **230**: 1350–54 (1985)

[2] Baoutina A *et al*. Gene doping detection: evaluation of approach for direct detection of gene transfer using erythropoietin as a model system. *Gene Therapy* **17**, 1022–1032 (2010)

[3] Baoutina A *et al*. Improved detection of transgene and nonviral vectors in blood. *Hum Gene Ther Methods* **24**(6): 345-354 (2013)

[4] Baoutina A *et al*. Synthetic certified DNA reference material for analysis of human erythropoietin transgene and transcript in gene doping and gene therapy. *Gene Therapy* **23**: 708–717 (2016)

[5] The World Anti-Doping Code International Standard for Laboratories. https://www.wada-ama.org/en/what-we-do/science-medical/laboratories

[6] *WADA* Guidelines on Blood *Sample* Collection. https://www.wada-ama.org/en/resources/world-anti-doping-program/guidelines-blood-sample-collection