

# High sensitive detection of follistatin by Imperacer®: An initial study on the way to analytical gene-doping tests

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### Abstract:

**For the understanding of interactions in the muscle-growth directing myostatin-pathway and therefore also for the establishment of high sensitive detection strategies for gene-doping, detailed studies of protein concentration levels in biological matrices and appropriate highly sensitive analytic techniques for quantitative monitoring of these biomarkers are required. In this work, the development of a high sensitive assay for follistatin based on the Imperacer® technology is described. The quantification limit in human serum was found at 60 pg/ml in only 6 µl sample volume. With this initial assay we demonstrate the first application in a set of analytical tests for a high sensitive myostatin pathway related profiling.**

### Introduction:

Great progress has been achieved over the past years by means of innovative molecular techniques which have led to the discovery of new growth factors involved in the regulation of muscle development. These findings provide new starting points to understand the mechanisms involved in the adaptation of skeletal muscle to exercise training. One of these newly identified growth factors is myostatin, a member of the transforming growth factor- $\beta$  family of proteins that has been demonstrated to play a fundamental role in the regulation of skeletal muscle growth during embryogenesis.[1] Blocking of the myostatin signalling transduction pathway by specific inhibitors and genetic manipulations has been shown to result in a dramatic increase of skeletal muscle mass (figure 1). New understanding of the role of myostatin gene expression in growth and development, along with research into the structural and functional characteristics of the myostatin protein, has offered researchers several potential methods to manipulate the pathway. So far, several proteins (e.g., follistatin, mutant activin type II receptors, and myostatin propeptide) demonstrated to act effectively as myostatin signalling blockers both in vitro, in cell culture and also in animals. These proteins are interconnected in a number of biological pathways: as typical example, the reproduction-correlated protein follistatin, initially identified as follicle-stimulating hormone inactivating regulator, revealed multifunctional regulatory properties e.g. by neutralization of activin.

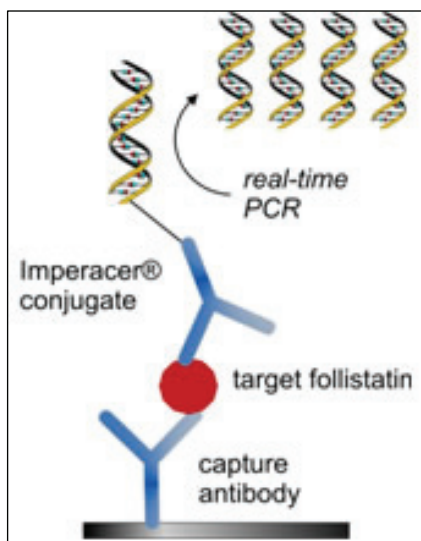


**Figure 1:** Belgian Blue Cattle: Deletions in the myostatin gene of these animals have resulted in a non-functional gene which is responsible for the excessive muscle hypertrophic phenotype.

Knocking out of the follistatin receptor for example resulted in a mouse phenotype very similar to myostatin knockout mice.[2] Drugs or genetic manipulations with the ability to modulate myostatin signalling may have the potential to enhance physical performance in athletes and therefore probably represent a new class of doping substances.

To identify manipulations of myostatin signaling, a promising strategy is the analysis of ratios of factors and molecules associated with the myostatin signal transduction pathway, a so-called molecular fingerprint. Manipulations either by application of a factor or by inhibition of its signaling will change these ratios, resulting in a different fingerprint. A methodological strategy to simultaneously analyze the expression of a variety of these biomarkers and relevant signal transduction molecules with high sensitivity is the detection by Imperacer® Immuno-PCR (figure 2).[3]

We here report on the development of a high sensitive Imperacer® test for follistatin in human serum as initial study to establish a myostatin gene-doping test. The general scheme of this assay is based on a two-sided (sandwich) immunoassay; the read-out is based on real-time PCR (figure 2). The development of an Imperacer® assay for this myostatin pathway protein is part of a World Anti Doping Agency (WADA) funded project and demonstrates the initial milestone for a set of multiplex and polyplex assays[4] to establish a high sensitive analytical tool for (i) studying of the relationship and biological response of the biomarkers involved in muscle growth and (ii) the detection of myostatin pathway related doping. The outcome of this project, including similar assays for further biomarkers and studies of their interactions will be published elsewhere shortly.



**Figure 2:** Schematic description of the Imperacer® technology. The protein follistatin is used to bridge a capture antibody with a target-specific Imperacer® conjugate. The conjugate contains an antibody and a DNA moiety. The DNA marker is finally amplified and visualized by real-time PCR.

### Experimental Section:

Described buffers and reagents are part of the Imperacer® development Kit (Chimera Biotec), the antibody-DNA conjugate was synthesized by Chimera Biotec. A detailed step-by-step protocol for Imperacer® assays was recently published and is available online from Nature Protocols.[3] The following paragraph summarizes briefly the adaptation of this biomarker standard protocol to the follistatin detection: The follistatin-specific detection antibody was linked with a DNA marker sequence for the preparation of the follistatin-specific Imperacer® antibody-DNA conjugate CHI-FOL. All washing steps were carried out automatically, using a Tecan Columbus Pro microplate washer. TopYield® modules (Nunc™) were incubated overnight at 4°C with 1 µg/ml anti human follistatin) in coating capture-antibody (polyclonal goat buffer). The modules were washed with wash buffer A and subsequently blocked for 30 sec at RT Direct Block solution. After plate coating and blocking, an antigen dilution series was

prepared by spiking target-negative human serum samples with follistatin. A total assay volume of 30  $\mu\text{l}$  was added to each well, consisting of 6  $\mu\text{l}$  spiked serum sample and a 1:1000 dilution of CHI-FOL conjugate in 24  $\mu\text{l}$  SDB+. All samples were tested in duplicates and the plate was incubated for 30 min at RT.

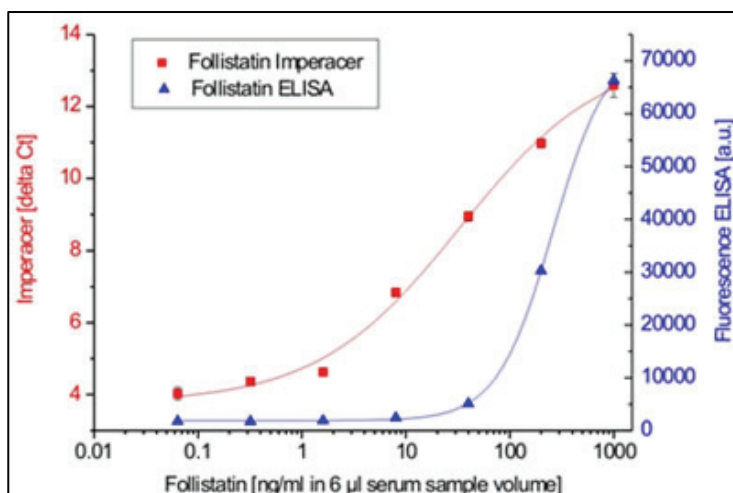
Subsequently a final washing step with buffer B and buffer A, real-time PCR-mastermix for Imperacer<sup>®</sup> was added and real-time PCR carried out using a Stratagene MX3005P device according to standard procedure.[3] The real-time PCR-cycler records  $R_n$  for each cycle  $\Delta$  the increase of the normalized fluorescence signal ( during DNA amplification. The software calculates the threshold cycle (Ct), which represents the first PCR cycle at which the reporter signal exceeds the signal of the baseline (“Threshold”), set it in the phase Ct values were calculated by  $\Delta$  where signal increases linearly. The subtracting the Ct values obtained for each signal from the total number of cycles carried out in the experiment.

### Results and Discussion:

As schematically depicted in figure 2, the Imperacer<sup>®</sup> technology was used to establish a sandwich-based assay for the high sensitive analyses of follistatin. An ELISA assay was established in parallel using the same antibodies and proteins.

In a first set of experiments, shown in figure 3, the detection limits obtained with Imperacer<sup>®</sup> and ELISA (red and blue line in figure 3) are directly compared. An increase in sensitivity of two orders of magnitude was observed for Imperacer<sup>®</sup>. The limit of detection (LOD) of the Imperacer<sup>®</sup> assay is 30 pg/ml (180 fg/well) follistatin in human serum. LOD was determined by significant difference ( $3 \times \text{SNC}$ ) between the measured average signal of the negative control without antigen and the signal thereby calculated for the lowest concentration (DIN32645). The limit of quantification (LOQ) was calculated at 60 pg/ml follistatin in human serum. The LOQ was set at the lowest concentration actually detected in the calibration curve with a recovery  $\geq 90\%$ . Average recovery based on a back-calculation of measured data points compared to the calculated calibration curve showed an average recovery of  $112\% \pm 20\%$ . The average precision of the calculated concentrations was  $9.8\% \pm 6\%$ .

The results from this initial evaluation demonstrate the functionality of the follistatin Imperacer<sup>®</sup> assay allowing to detect biological relevant concentrations of the follistatin, which are typically  $\geq 1$  ng/ml in human sample material. The increase in sensitivity and - even more important - the minimization of sample volume to only 6  $\mu\text{l}$  per test compared to 100  $\mu\text{l}$  per test for typical ELISA systems enabling to focus on other biological fluids, like saliva, which can nowadays not used for significant testing.



**Figure 3:** Imperacer<sup>®</sup> and ELISA for the detection of follistatin in human serum. 6  $\mu\text{l}$  of spiked human serum were used to generate a calibration curve and to determine the general assay parameters. The Imperacer<sup>®</sup> revealed an approx. 100fold increase in sensitivity compared to the ELISA.

**Conclusions:**

Here the successful establishment of an Imperacer® assay for the detection of myostatin pathway related protein follistatin was demonstrated successfully. The follistatin assay is thereby a template for the development of a number of similar assays for the interconnected biomarkers. The next steps will be the detection of myostatin itself, myostatin propeptide, the follistatin related FSTL and Activin in serum, plasma, saliva, and skeletal muscle tissue homogenates. Additional milestones will be the application of these Imperacer® assays to investigate the physiology of protein expression under different physiological conditions and to determine ratios of selected members of the myostatin signal transduction pathway in different biological matrices in order to investigate the inter individual variability and the effect of training on such ratios. In a future studies we will try to analyse if application of myostatin inhibitors will change such fingerprints.

**Acknowledgments:**

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