# DEVELOPMENT OF TYPING METHODS BASED ON PYROSEQUENCING TECHNOLOGY FOR THE ANALYSIS OF SIX BOVINE GENES RELATED TO MARBLING

Ripoli M.V., Rogberg-Muñoz A., Liron J.P., Giovambattista G.

Instituto de Genética Veterinaria Ing. Fernando N. Dulout (IGEVET ex CIGEBA), Facultad de Ciencias Veterinarias, UNLP-CONICET, CCT La Plata. CC 296. CP B1900AVW, La Plata, Argentina.

Corresponding author: Ripoli M.V. 60 y 118 s/nº La Plata. CC 296. CP B1900AVW, La Plata, Argentina.
Tel/fax: 54 221 421 1799,

mvripoli@fcv.unlp.edu.ar

## ABSTRACT

Several methods such as PCR-RFLP, OLA, DNA sequencing, PCR-SSCP and ARMS-PCR have been developed to detect the allelic variation at substitutions K232A of *DGAT1*, GH6.1 of *GH*, F279Y of *GHR*, R4C of *LEP*, I74V of *FABP4*, and at the transition in the *TG* 5´ leader sequence. Most of these methods are manual processes and therefore increase the time spent on the assay and limit the number of animals analyzed. Herein, we describe the development of pyrosequencing-based methods for the bovine *DGAT1*, *GH*, *GHR*, *LEP*, *FABP4* and *TG* genes, whose polymorphisms have been associated with variation in carcass composition. This method was validated by analyzing DNA samples belonging to the Aberdeen Angus and Hereford breeds previously typed by PCR-SSCP, PCR-RFLP and/or DNA sequencing. The results obtained showed that, after sequencing or whole-genome association studies (discovery step), the pyrosequencing-based technique seems to be useful to validate (validation step) a particular single nucleotide polymorphism (SNP) in a candidate gene in a previously mapped region in independent populations (with different genotypes and/or production systems). We conclude that pyrosequencing may be useful in high-throughput SNP genotyping of candidate genes in breeds of cattle and other animal species, making it a fast and interesting screening method for population or association studies.

**Key words:** bovine molecular markers, polymorphism, pyrosequencing, marbling

## RESUMEN

Diversos métodos como PCR-RFLP, OLA, secuenciación de ADN, PCR-SSCP y ARMS-PCR han sido desarrollados para detectar las variaciones alélicas presentes en las sustituciones K232A del gen *DGAT1*, GH6.1 del gen *GH*, F279Y de *GHR*, R4C de *LEP*, I74V de *FABP4*, y en la transición detectada en la secuencia 5´ líder del gen *TG*. La mayoría de estos métodos son procesos manuales que consumen mucho tiempo para realizar el ensayo y limitan el número de animales analizados. En el presente trabajo se describe el desarrollo de métodos de pirosecuenciación aplicables a los genes bovinos *DGAT1*, *GH*, *GHR*, *LEP*, *FABP4* y *TG*, cuyos polimorfismos han sido asociados a variaciones en la composición de la carcasa. Este método se validó mediante el análisis de muestras de ADN pertenecientes a las razas Aberdeen Angus y Hereford previamente tipificadas por PCR-SSCP, PCR-RFLP y/o secuenciación de ADN. Los resultados obtenidos evidenciaron que, después de estudios de secuenciación o asociación genómica (etapa de descubrimiento), la pirosecuenciación sería de gran utilidad para validar (etapa de validación) un polimorfismo de nucleótido único (SNP) particular en un gen candidato localizado en una región previamente mapeada en poblaciones independientes (con diferentes genotipos y/o sistemas de producción). Concluimos que los métodos basados en pirosecuenciación pueden ser de gran utilidad en la genotipificación de alto rendimiento de SNPs de genes candidatos en razas bovinas y en otras especies animales, representando un rápido e interesante método de validación para estudios poblacionales o de asociación.

**Palabras clave:** marcadores moleculares bovinos, polimorfismos, pirosecuenciación, marmoleo

## INTRODUCCIÓN

Several single nucleotide polymorphisms (SNPs) have been associated with fat traits in cattle (*Bos taurus*) (de Koning, 2006). Intramuscular fat deposition, or marbling, is an important trait for meat quality since it confers juiciness, flavor and tenderness to beef, hence it contributes directly to the price of beef in international markets.

AcylCoA-diacylglycerol-acyltransferase 1 (DGAT1) is a microsomal enzyme that catalyzes the final step of triglyceride synthesis. A lysine/alanine (K232A) substitution in the protein encoded by the bovine *DGAT1* gene has been shown to be associated with milk fat content (Grisart *et al.*, 2002; Spelman *et al.*, 2002; Winter *et al.*, 2002) and fat deposition in different bovine breeds (Sorensen *et al.*, 2006; Thaller *et al.*, 2003).

Growth Hormone (GH) plays a major role in tissue growth, fat metabolism and homeorhesis (Shingu *et al.*, 2004; Beauchemin *et al.*, 2006; Thomas *et al.*, 2007). The bovine GH gene shows different polymorphisms (Lucy *et al.*, 1991; Zhang *et al.*, 1993; Kirkpatrick *et al.*, 1993), most of which have been associated with differences in carcass composition, marbling and milk production (Lee *et al.*, 1996; Yao *et al.*, 1996; Lechniak *et al.*, 2002, Di Stasio *et al.*, 2005; Curi *et al.*, 2005; Barendse *et al.*, 2006; Thomas *et al.*, 2007). In particular, the GH6.1 polymorphism, also known as AluI RFLP (Yao *et al.*, 1996). is caused by a C to G nucleotide change in exon 5 of the gene, which gives rise to two alleles that are responsible for alternative forms of bovine GH with a Leucine or Valine amino acid residue at position 127.

Growth Hormone Receptor (GHR) has a major role in the regulation of GH action in most tissues. The F279Y polymorphism of the *GHR* gene has been associated with milk traits and carcass quality, especially milk fat content and fat deposition (Blott *et al.*, 2003; Viitala *et al.*, 2006; White *et al.*, 2007). This polymorphism is caused by a T to A replacement in exon 8 and results in a substitution of a Phenylalanine to a Tyrosine residue at position 279 in the mature polypeptide.

Thyroglobulin (*TG*) is the precursor of T3 and T4 thyroid hormones, which have an important role in metabolic regulation and, among other functions, affect lipid metabolism. Barendse *et al.* (2001) reported that the C to T transition in the TG 5´ leader sequence is highly associated with intramuscular fat deposition in long-fed cattle and defines the `2´ (C) and `3´ (T) alleles. Barendse *et al.* (1999, 2004) also found that the TG `3´ allele is more frequent in animals with higher marbling scores.

Leptin (*LEP*) is a protein hormone that plays a major role in whole-body energy metabolism. *LEP* is one of the best physiological markers of body weight, food intake, energy expenditure (Houseknecht *et al.*, 1998; Woods *et al.*, 1998), reproduction (Cunningham *et al.*, 1999; Garcia *et al.*, 2002), and certain immune system functions (Lord *et al.*, 1998). Polymorphisms in the coding regions of the LEP gene in cattle have been associated with serum LEP concentration (Liefers *et al.*, 2003), feed intake (Liefers *et al.*, 2002; Oprzadek *et al.*, 2003), milk yield (Liefers *et al.*, 2002; Buchanan *et al.*, 2003), body fatness (Buchanan *et al.*, 2002; Nkrumah *et al.*, 2004 a, b) and marbling scores (http://ca.igenity.com/igenity_beef1.html). In particular, the C/T polymorphism situated in exon 2 of *LEP* (Liefers *et al.*, 2002), which leads to an Arginine (R) to Cysteine (C) substitution at amino acid 4 (R4C) in the LEP molecule.

The fatty acid binding protein 4 (FABP4) plays a major role in the regulation of lipid and glucose homeostasis through interaction with peroxisome proliferator activated receptors (PPARs), which act as transcription factors in adipocyte differentiation (Mandrup and Lane, 1997). Michal *et al.* (2006) and Lee *et al.* (2010) identified several SNPs in the *FABP4* gene, which have been associated with economically relevant characteristics such as marbling and subcutaneous fat deposition in cattle. In particular, the I74V polymorphism (A to G transition) situated in exon 2, which results in a substitution of an Isoleucine to Valine amino acid residue in the mature polypeptide.

Several methods, such as PCR-RFLP, OLA, DNA sequencing, PCR-SSCP and ARMS-PCR, have been developed to detect the allelic variation at substitutions K232A of *DGAT1*, GH6.1 of *GH*, F279Y of *GHR*, R4C of *LEP*, I74V of *FABP4*, and at the transition in the TG 5´ leader sequence in different breeds (Blott *et al.*, 2003; Buchanan *et al.*, 2002; Cho *et al.*, 2007; Corva *et al.*, 2004; Hoashi *et al.*, 2008; Ripoli *et al.*, 2006; Viitala *et al.*, 2006; Winter *et al.*, 2002; Yao *et al.*, 1996). Most of these methods are manual processes and therefore limit the number of animals analyzed. Improvement of high-throughput methods based on sequencing, pyrosequencing, real-time PCR, TaqMan assay, and microarrays have been developed in recent years. The availability of high-throughput genotyping methods is a valuable tool to

rapidly discover, screen and validate polymorphisms in animal genotyping for association or population studies in animal production. Pyrosequencing™ (Ronaghi *et al.*, 1996, 1998) is a real-time DNA sequencing technique based on the detection of released pyrophosphate (PPi) during DNA synthesis. This technique has been successful for both confirmatory sequencing and *de novo* sequencing (Ahmadian *et al.*, 2000; Ronaghi *et al.*, 1999). After an oligonucleotide is hybridized to a single-stranded DNA template, a cascade of enzymatic reactions starts with the nucleic acid polymerization reaction primed by an internal primer. Each of the four deoxyribonucleotide triphosphates (dNTPs) is then individually added to the reaction mixture, and inorganic PPi is released as a result of nucleotide incorporation by polymerase. Visible light is generated proportionally to the number of incorporated nucleotides (Ronaghi, 2001) detected by a CCD camera and seen as peaks in a pyrogram™.

The aim of this study was to develop a pyrosequencing-based typing method applicable to high- and medium-throughput genotyping of the K232A, GH6.1, F279Y, R4C, and I74V substitutions in the *DGAT1*, *GH*, *GHR*, *LEP*, and *FABP4* genes respectively and at the transition in the TG 5´ leader sequence in different cattle breeds. We applied this method to screen these polymorphisms in a sample of individuals belonging to the Hereford and Aberdeen Angus cattle breeds**,** previously genotyped by direct sequencing, PCR-RFLP and/or PCR-SSCP.

## MATERIALS AND METHODS

*Sample collection and DNA extraction*

Blood samples were collected from 25 Hereford and 25 Aberdeen Angus cattle. Total DNA was extracted from blood samples using the DNAzolâ reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

*Polymorphism detection assays*

*DGAT1 analysis by PCR-SSCP:* The K232A polymorphism was analyzed by PCR-SSCP and DNA sequencing as described in Ripoli *et al.* (2006).

*GH analysis by PCR-RFLP:* The GH6.1 polymorphism was analyzed by PCR-RFLP as described in Yao *et al.* (1996).

*GHR analysis by PCR-sequencing:* The F279Y polymorphism was analyzed by PCR-DNA sequencing using the primers described in Blott *et al.* (2003) for the PCR (see below), and then sequenced in a MegaBACE 1000 automatic sequencer (GE Healthcare, USA).

*TG analysis by PCR-RFLP:* The C to T transition in the TG 5´ leader sequence was analyzed by PCR-RFLP as described in Barendse *et al.* (2001).

*LEP analysis by PCR-RFLP:* The R4C polymorphism was analyzed by PCR-RFLP according to Liefers *et al.* (2002).

*FABP4 analysis by PCR-sequencing:* The I74V polymorphism was analyzed by PCR-DNA sequencing using primers specially designed for the PCR (see below), and then sequenced in a MegaBACE 1000 automatic sequencer (GE Healthcare).

*Pyrosequencing analysis of polymorphisms*

*a) Preparation of PCR products*

*DGAT1:* A 176 bp fragment of from the DGAT1 gene spanning the K232A substitution (exon 8) was amplified as in Ripoli *et al.* (2006). The forward primer was biotinylated for the subsequent purification step. The internal sequencing primer, complementary to the forward strand, was designed using Pyrosequencing Primer SNP Design 1.01 software (http://www.pyrosequencing.com) (Table 1). This primer is located upstream the SNP selected to differentiate the A and K alleles. The PCR was performed separately with 2 µl DNA in a 25 µl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 µM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research, Boston, MA, USA - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94°C for 45s, 63°C for 45s and 72°C for 45s, plus a final extension at 72°C for 10 min.

*GH:* A 259 bp fragment of exon 5, including the GH6.1 polymorphism, was amplified by an adaptation of the method undertaken by Schlee *et al.* (1994). The reward primer was biotinylated for the subsequent purification step and the internal sequencing primer was designed complementary to the reward strand (Table 1). This primer is located upstream the SNP selected to differentiate the C and G alleles. The PCR was performed separately with 2 µl DNA in a 25 µl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 µM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94°C for 45s, 63°C for 45s and

72ºC for 45s, plus a final extension at 72ºC for 10 min.

*GHR:* A 342-bp fragment of the GHR gene of exon 8, including the F279Y substitution, was also amplified according to Blott *et al.* (2003). The forward primer was biotinylated for the subsequent purification step. The internal sequencing primer was designed complementary to the forward strand (Table 1). This primer is located upstream the SNPs selected to differentiate the A and T alleles. The PCR was performed separately with 2 μl DNA in a 25 μl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 μM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94°C for 45s, 63°C for 45s and 72ºC for 45s, plus a final extension at 72ºC for 10 min.

***TG:*** A 266 bp fragment spanning part of the TG 5´ leader sequence that included the C/T transition was amplified with specially designed primers. The reward primer was biotinylated for the subsequent purification step, and the internal sequencing primer was designed complementary to the reward strand (Table 1). This primer is located upstream the SNP selected to differentiate the "2" and "3" alleles. The PCR was performed separately with 2 μl DNA in a 25 μl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 μM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94°C for 45s, 58°C for 45s and 72ºC for 45s, plus a final extension at 72ºC for 10 min.

***LEP:*** A 269-bp fragment of exon 2, including the R4C mutation, was amplified with primers specially designed. The forward primer was biotinylated for the subsequent purification step and the internal sequencing primer was designed complementary to the forward strand (Table 1). This primer is located upstream the SNP selected to differentiate the C and T alleles. The PCR was performed separately with 2 μl DNA in a 25 μl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 μM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94ºC for 45s, 60ºC for 45s and 72ºC for 45s, plus a final extension at 72ºC for 10 min.

*FABP4:* A 295-bp fragment of exon 2 including the I74V polymorphism was amplified using primers specially designed. The forward primer was biotinylated for the subsequent purification step and the internal sequencing primer was designed complementary to the forward strand (Table 1). This primer is located upstream the SNP selected to differentiate the A and G alleles. The PCR was performed separately with 2 μl DNA in a 25 μl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 μM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94°C for 45s, 63°C for 45s and 72ºC for 45s, plus a final extension at 72ºC for 10 min.

*b) Pyrosequencing*

After each PCR template generation, the products were purified by capturing the biotinylated strands with streptavidin-coated Sepharose beads (Streptvidin SepharoseTM High Performance, GE Healthcare). This immobilized biotinylated strands were used as pyrosequencing template (Ronaghi *et al.* 1998; Ronaghi, 2001). Pyrosequencing was carried out with the internal sequencing primer diluted to 0.3 μM in the annealing buffer provided by the supplier, using the Pyro Gold Reagent Kit (Biotage, AB, Uppsala, Sweden). A PyroMark Prep Workstation (Biotage AB) was used for all steps other than bead addition and transfer. Samples were run on a PSQTM96 System instrument, and outgoing results were analyzed using pyrosequencing software (Biotage AB).

*Calculation of allele frequencies*

The ARLEQUIN 2.0 software package (Schneider *et al.* 2000) was used to calculate the allele frequencies for each locus in each population studied.

Table 1. Oligonucleotide primers used in this study.

| Gene | Primer type | Sequence (5´to 3´) | Region |
|---|---|---|---|
| **DGAT1** | Forward | CTTGCTCGTAGCTTTGGCAGG | Exon 8 |
| | Reverse | CGAAGAGGAAGTAGTAGAGATC | |
| | Internal | AGCTCCCCCGTTG | |
| **GH** | Forward | TAGGGGAGGGTGGAAAATGGA | Exon5 |
| | Reverse | GGCACTTCATGACCCTCAGGT | |
| | Internal | CCCTTGGCAGGAG | |
| **GHR** | Forward | GTGGCTATCAAGTGAAATCATTGAC | Exon 8 |
| | Reverse | ACTGGGTTGATGAAACACTTCACTC | |
| | Internal | TAGAAAATATGAGTAAA | |
| **LEP** | Forward | TAACGGAGCACGTGGGTGT | Exon 2 |
| | Reverse | CGGTTCTACCTCGTCTCCCA | |
| | Internal | TGTCATCCTGGACCTTGC | |
| **TG** | Forward | CTTTGGCCTTTACCCCTGAAG | UTR 5´ |
| | Reverse | CCAGGGACGAATGTGTGTGA | |
| | Internal | CTGGGTTGGGAAGAT | |
| **FABP4** | Forward | TCATCAGTTTGAATGGGGGT | Exon 2 |

## RESULTS

Pyrosequencing allowed us to quickly detect all the SNPs analyzed. This method allowed the detection of homozygous and heterozygous genotypes, and the pyrograms obtained for each genotype were coincident with those predicted by the software (PSQ 96 MA 2.1.1.) The method was validated by genotyping 33 DNA samples from Hereford and Aberdeen Angus cattle previously analyzed by direct sequencing, PCR-RFLP and/or PCR-SSCP.

The allele frequencies for the breeds studied are shown in Table 2. Alleles FABP4 A, LEP T, TG 2, DGAT1 A and GH C were the most common variants in the cattle breeds analyzed. Noteworthy, DGAT1 A variants were fixed in Hereford. The exception was *GHR*, where allele GHR A was the most abundant variant in Aberdeen Angus and the least abundant in Hereford. These results are in agreement with data previously reported (Ripoli *et al.*, 2006, 2011).

Table 2. Estimated gene frequencies (in percentage) for six SNPs analyzed in Aberdeen Angus (AA) and Hereford (HE) cattle breeds.

| Breed | DGTA1 | | FABP4 | | LEP | | TG | | GH | | GHR | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | K | A | G | C | T | 2 | 3 | C | G | A | T |
| **AA** | 77.08 | 22.92 | 60.00 | 40.00 | 22.92 | 77.08 | 78.00 | 22.00 | 80.00 | 20.00 | 86.00 | 14.00 |
| **HE** | 100.00 | - | 72.00 | 28.00 | 25.00 | 75.00 | 98.70 | 1.30 | 85.00 | 15.00 | 12.50 | 87.50 |

## DISCUSSION

Multiple SNPs, such as K232A, GH6.1, F279Y, R4C, I74V and TG 5´, have been associated with economically important traits. It is thus necessary to have rapid and efficient SNP evaluation techniques in order to validate these SNPs in independent populations. As a first approach to screen polymorphisms simultaneously in a large number of individuals, SSCP typing is a very useful tool. However, since it is highly temperature- and ion concentration-dependent, electrophoresis reproducibility is a relevant point to be considered. On the other hand, PCR-RFLP is also a useful tool for genotyping, but it is complicated when one should genotype a large number of animals; also, this technique could generate results of false heterozygotes as a consequence of partial digestion. In the case of direct sequencing, the process can also become complex and time-consuming when working with a large number of individuals. In contrast, pyrosequencing, as an automatic sequencing method, is easy to standardize, and furthermore, its throughput is 96 samples in approximately 20 minutes (Ronaghi, 2001; Wittwer *et al.*, 1997). In addition, this technique sequences the flanking regions of the mutation analyzed, confirming the genotyped region and avoiding false heterozygotes.

During the last years, high-throughput and new-generation technologies (microarrays, whole-genome sequences, etc.) have grown exponentially. These techniques allow analyzing several polymorphisms simultaneously, with a very low cost. The pyrosequencing-based method developed in the present work is more expensive than the methods mentioned above (about 1 U$S each SNP). However, this method is useful and the total cost per experiment is more accessible when, after sequencing or whole genome association studies (discovery step), it is necessary to validate a particular SNP in a candidate gene in a previously mapped region in independent (validation step) populations. Also, it is a useful and less expensive when it is necessary to validate a SNP previously associated with a characteristic of production in different genotypes and production systems.

Pyrosequencing is more efficient and faster than direct sequencing, RFLP or SSCP analysis. SNP analysis in large population studies is highly improved due to the reduction in the amount of reagents used, the automation in outcome acquisition and result interpretation. This could aid in the rapid and efficient analysis of SNPs in many genes associated with economic traits in cattle.

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