

Detection of neuronal tissue in meat using tissue specific DNA modifications

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A method has been developed to differentiate between non-muscle tissues such as liver, kidney and heart and that of muscle in meat samples using tissue specific DNA detection. Only muscle tissue is considered meat from the point of view of labelling (Food Labelling [Amendment] (England) Regulations 2003) and Quantitative Ingredient Declaration (QUID), and also certain parts of the carcass are prohibited to be used in raw meat products (Meat Products [England] Regulations 2003). Included in the prohibited offal are brain and spinal cord. The described methodology has therefore been developed primarily to enforce labelling rules but also to contribute to the enforcement of BSE legislation on the detection of Central Nervous System (CNS) tissue. The latter requires the removal of Specified Risk Material (SRM), such as bovine and ovine brain and spinal cord, from the food chain. Current methodologies for detection of CNS tissue include histological examination, analysis of cholesterol content and immunodetection. These can potentially be time consuming, less applicable to processed samples and may not be readily adapted to high throughput sample analysis. The objective of this work was therefore to develop a DNAbased detection assay that exploits the sensitivity and specificity of PCR and is potentially applicable to more highly processed food samples. For neuronal tissue, the DNA target selected was the promoter for Glial Fibrillary Acidic Protein (GFAP), a gene whose expression is restricted to astroglial cells within CNS tissue. The promoter fragments from both cattle and sheep have been isolated and key differences in the methylation patterns of certain CpG dinucleotides in the sequences from bovine and sheep brain and spinal cord and the corresponding skeletal muscle identified. These have been used to design a PCR assay exploiting Methylation Specific PCR (MSP) to specifically amplify the neuronal tissue derived sequence and therefore identify the presence of CNS tissue in an assay sample.

Keywords. Bovine, ovine, Glial Fibrillary Acidic Protein, GFAP, meat, neuronal, central nervous system.

1. INTRODUCTION

Methods are required that enable differentiation between muscle and non-muscle tissues such as liver, kidney and heart in meat samples. This is because only muscle tissue is considered meat from the point of view of labelling (Food Labelling (Amendment) (England) Regulations 2003) and Quantitative Ingredient Declaration (QUID), and also certain parts of the carcass are prohibited to be used in raw meat products (Meat Products (England) Regulations 2003). Included in the prohibited offal is brain and spinal cord. The described methodology has therefore been developed primarily to enforce labelling rules and also to contribute to the enforcement of BSE legislation requiring the detection of Central Nervous System (CNS) tissue. This requires the removal of central nervous system tissue from carcasses prior to processing (Government UK, 1989). To permit effective enforcement there is a need for the development of robust CNS specific detection methods that are applicable to a wide range of processed meat products. Methods for the detection of CNS tissue in meat

include direct tissue examination (Bauer *et al.*, 1996), histological preparation and microscopic examination (FSIS/USDA, 1998) and the analysis of cholesterol content (Lücker *et al.*, 1999; Schmidt *et al.*, 1999). The latter method has used antibodies directed against CNS specific proteins to enable the immunogenic detection of CNS tissue. One of these (Schmidt *et al.*, 1999) utilises an ELISA method based upon the detection of Glial Fibrillary Acidic Protein (GFAP), a protein whose expression is restricted to the CNS (Kaneko, Sueoka, 1993). Our studies have also utilised detection of GFAP but at the DNA rather than the protein level. Such an assay may be more suitable for processed products where DNAdegradation can be overcome by designing a PCR based assay that generates small amplicons and also has the potential to be utilised on a range of assay formats.

Methylation of cytosine occurring in CpG dinucleotides in the promoter of a gene is one of several ways of controlling tissue specific regulation (Condorelli *et al.*, 1994; Condorelli *et al.*, 1997; Teter *et al.*, 1994). Several tissue specific genes have been shown to possess unmethylated promoter sequences in

expressing tissue but be heavily methylated in those where little or no expression occurs (Yeivin *et al.*, 1993). In several cases, methylation of specific CpG dinucleotides within the promoter has been shown to correlate with transcriptional repression and hence control gene expression (Li *et al.*, 1993; Yoder *et al.*, 1997).

One technique that can be used to study promoter methylation is the chemical modification of cytosine to uracil by bisulphite treatment. In this reaction all cytosines are converted to uracil but those that are methylated (5-methylcytosine) are resistant to modification (Wang *et al.*, 1980). Following amplification and sequencing, the converted bases will appear as thymine compared to cytosine in the native sequence. The sequence differences resulting from bisulphite modification of specific bases can also be used to design PCR primers specific to the methylation status of a target template, so called Methylation Specific PCR (MSP) (Herman *et al.*, 1996), allowing discrimination of methylated and non-methylated sequences. By utilising differences in the methylation patterns of the GFAP promoter sequence in bovine and ovine brain and muscle tissue, we have been able to use MSP to design an assay that enables the specific detection of brain tissue in both raw and processed mixed meat samples.

2. DETERMINATION OF METHYLATION STATUS

Using the high degree of conservation that exists in the published promoter sequences for human (Besnard *et al.*, 1991) and rat GFAP (Condorelli *et al.*, 1994), it was possible to design PCR primers to conserved regions to amplify ca. 1700 bp of the bovine and sheep forms of the promoter. Each target was then sequenced to obtain the native form of the promoter sequence prior to methylation analysis (GenBank Accession numbers AF251845 and AF251846).

Genomic DNA was isolated from samples of bovine and ovine brain and spinal cord. Bisulphite treatment of the DNA was then carried out essentially as described by Frommer *et al.* (1992) with some modifications (Paulin *et al.*, 1998; Woodcock *et al.*, 1997). This procedure chemically converts unmethylated cytosine residues to uracil and leaves methylated cytosine residues unconverted giving a permanent record of the methylation status of the gene in each tissue type. Subsequent PCR amplification then records the uracil residues as thymine. PCR primers were designed to amplify only from bisulphite modified DNAs and not any native (unmodified) strands. Care was taken to exclude any CpG residues from the primers where possible. For each tissue type

the target gene was amplified and cloned into a plasmid vector and replicate plasmids were sequenced to give a representation of the percentage methylation in each tissue. Each tissue specific set of sequences was then compared to that of the native sequence to determine any methylation differences (as the presence of thymine or cytosine at a CpG target site) between sequences resulting from muscle or neuronal derived amplicons. Three cytosine residues contained between 90 and 100% methylation in muscle derived DNA and 10% or less in neuronal tissue (based upon replicate analysis), making them suitable for assay design.

3. ASSAY DESIGN

PCR primers were designed to discriminate between methylated (unmodified by bisulphite treatment) and unmethylated (modified by bisulphite treatment) sequences to enable discrimination between skeletal muscle and neuronal tissue derived DNA. Primers were designed to encompass a terminal nucleotide at the 3' end corresponding to the unmethylated form (i.e. that derived from neuronal tissue) of cytosine in a discriminatory CpG site identified within the promoter sequence. This would be converted to uracil by bisulphite modification and hybridise effectively with the terminal adenosine of the primer. This ensures that Taq Polymerase cannot efficiently amplify a promoter sequence containing the methylated form of the gene (i.e. that derived from muscle tissue) because the cytosine residue is unchanged, leading to a 3' mismatch (**Figure 1**).

Figure 2 illustrates an example of the tissue specificity of the neuronal tissue detection assay for ovine neuronal tissue. An amplicon is visible for DNA derived from brain tissue but absent for that from muscle, heart and liver.

4. TRANSFER OF ASSAYS TO OTHER FORMATS

4.1. Use of a reference gene

In order to detect tissue specific differences in samples due to methylation status and not as a result of differing amounts of input target material, it is important to have some way of normalising the target specific data before comparison. To this end a reference gene system was developed. Primers and probes were designed to amplify part of the target promoter region that contained no CpG sites in order to ensure that amplification was not methylation dependent. This gave the total signal from all the gene copies present in the reaction (regardless of methylation status) and an indication of whether the

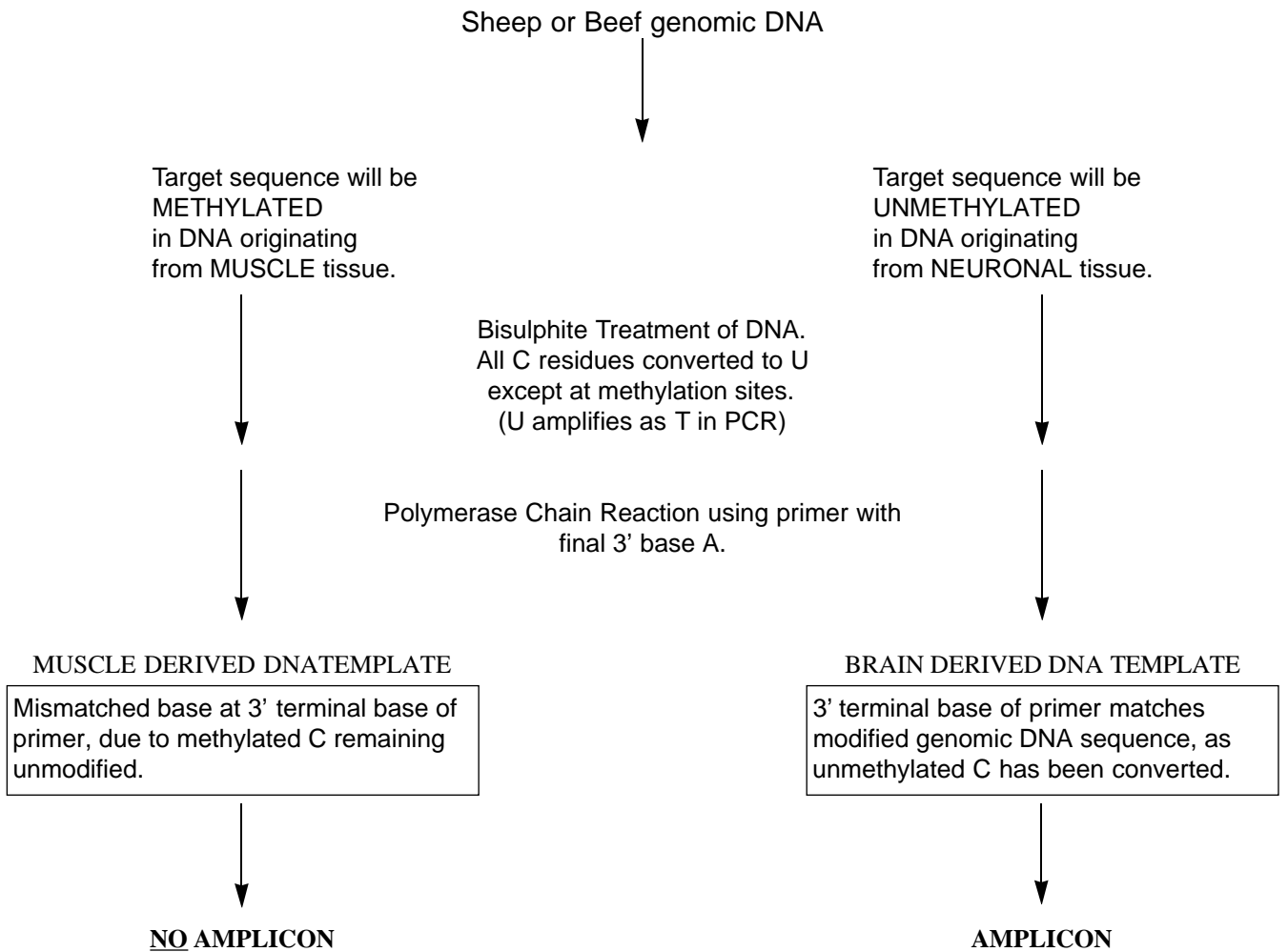


Figure 1. Diagram illustrating how it is possible to use the methylation sites identified in target promoters to design a PCR assay using Methylation Specific PCR (MSP) for the detection of neuronal tissue.

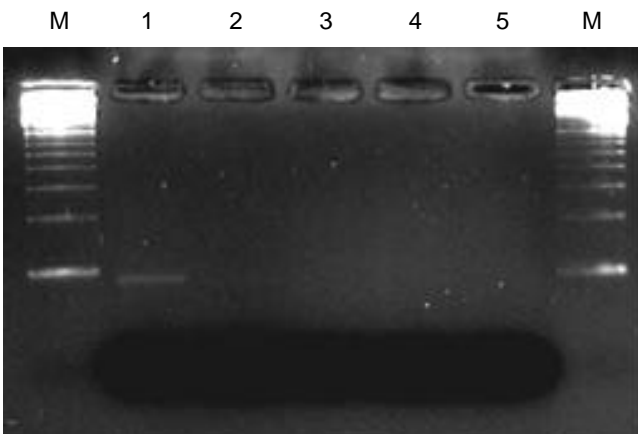


Figure 2. Demonstration of the tissue specificity of the ovine neuronal detection assay. Amplification was carried out on bisulphite treated DNA from brain (lane 1), muscle (lane 2), heart (lane 3) and liver (lane 4). Lane 5 contains a minus DNA control. Lane M contains molecular weight markers.

levels of target gene in each reaction were the same. It also enabled the ratio of the amount of target tissue specific signal to that of the total promoter signal (methylated plus unmethylated) to be calculated to normalise for any differences.

4.2. Real time PCR detection of neuronal tissue

One technique that has been applied for sensitive detection of methylated alleles using real time PCR detection is the MethyLight assay (Eads *et al.*, 2000). This utilises methylation specific amplification primers as with MSP but also allows the use of methylation state specific TaqMan probes to add an extra level of discrimination. It also enables the accumulation of fluorescence resulting from the increase in amplicon concentration during amplification to be measured, potentially enabling quantitative PCR measurement. Using this principal, the assays were further refined to incorporate a

fluorescent TaqMan probe containing a methylation site in the GFAP promoter to enable the transfer of the method to the ABI 7700 SDS Prism system. This enables discrimination of DNA derived from bovine spinal cord, brain or muscle, by measuring the cycle at which the accumulated fluorescence signal for each amplicon crosses a given threshold (**Figure 3**).

This reaction can be run in duplex with the reference gene to enable the ratio of the neuronal specific form to that of the total gene concentration to be measured as the difference in threshold cycle (Ct). Using a series of admixtures of neuronal tissue in muscle we obtain a linear relationship between Ct and target tissue concentration demonstrating the quantitative potential of the assay (**Figure 4**).

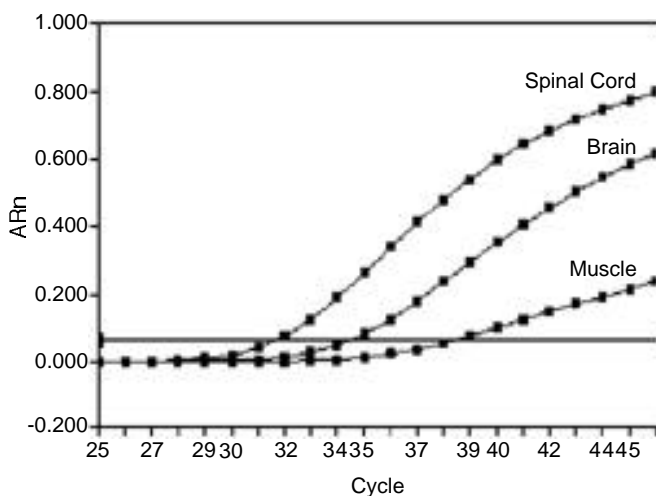


Figure 3. TaqMan based differentiation of bovine brain, spinal cord and muscle using the ABI SDS Prism 7700 platform.

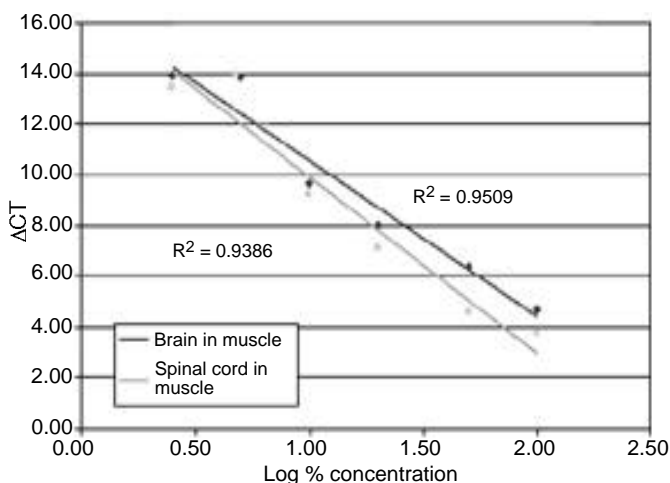


Figure 4. Linear response between Ct and target tissue concentration when a series of admixtures of decreasing amounts of neuronal tissue in a background of muscle are detected using a TaqMan system on the ABI SDS Prism 7700.

4.3. Capillary electrophoresis (CE) based detection of neuronal tissue

The Agilent CE based detection system relies on the use of conventional PCR followed by CE based separation of amplicons in a polyacrylamide matrix using a 12 well Labchip. This gives a high level of product separation and the potential for quantitative measurement by being able to measure the net fluorescent signal obtained for each PCR product and is a relatively low cost alternative to that of real time PCR. Reactions were carried out as a duplex utilising the ratio of the total GFAP amplicon signal obtained compared to that of the neuronal specific amplicon signal, to normalise and attempt to quantitate the level of non-muscle specific amplicon. This method has been proven to work in a similar fashion for the quantitative detection of Roundup Ready soya (Burns *et al.*, 2003) using the ratio of the lectin gene signal (detecting GM plus non-GM soya) to that of the Roundup Ready soya specific amplicon (GM specific). It enables a cut off limit to be set, that is designated as the background signal resulting from any residual non-methylated GFAP sequence normally present in muscle tissue which will be linked to the possible presence of GFAP protein in peripheral nervous system tissue. Any signal designated as resulting from neuronal tissue would therefore have to be above this as compared to a 100% muscle control run on the chip.

By using admixtures of neuronal tissue in muscle, a linear relationship was demonstrated between target concentration and signal obtained and we were able to set a threshold for any background non-methylation present in the muscle sample, giving the basis for quantitative measurement (**Figure 5**). This demonstrates the clear potential of the CE system as an alternative platform for this type of assay.

5. SUMMARY

Currently, visual or histological examination, cholesterol content or more recently developed immunoassays can be used to detect neuronal tissue in meat products. Some of these methods are subjective and time consuming and may not be applicable to highly processed samples. We report here a new technique for the detection of CNS tissue in meat products that exploits the robustness of DNA, a molecule that is relatively resistant to the rigours of food processing and may give greater applicability to both raw, processed and cooked meat products.

To enable the use of DNA as the target molecule we focused on GFAP, a gene that has been characterized as a marker for CNS tissue and has previously been utilised at the protein level for the

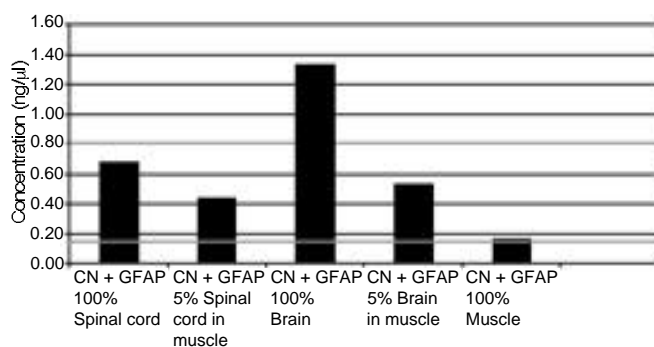


Figure 5. Capillary Electrophoresis (CE) based detection of bovine neuronal tissue in a background of muscle. This shows the relationship between target concentration (% brain or spinal cord in muscle) and signal obtained when the target specific PCR is run on an Agilent CE system and concentration results obtained are normalised to an internal reference gene. The grey horizontal line shows the threshold set in this instance, to show the background non-methylation present in samples (determined by the 100% muscle sample). It can be seen that the 100% and 5% brain and spinal cord in muscle mixtures give a signal above this threshold and in proportion to the target tissue concentration.

immunogenic detection of CNS tissue in meat (Schmidt *et al.*, 1999). Tissue specific methylation patterns for the promoter of this gene have been reported in several species (Condorelli *et al.*, 1994, 1997; Teter *et al.*, 1994) and this has enabled us to design a PCR based assay to discriminate between differentially methylated forms of the same sequence and hence identify the tissue from which the template was derived. By utilising the technique of methylation sensitive PCR (MSP) it was possible to design a PCR based assay specific to the methylation status of the GFAP promoter in the target tissue and hence be able to detect bovine and sheep brain and spinal cord tissue in a background of non-target muscle tissue.

In conclusion, we have demonstrated the first use of a PCR based assay for the specific detection of DNA derived from neuronal tissue in muscle admixtures. This has utilised methylation specific PCR (MSP), a technique previously only used to distinguish between tumour and non-tumour tissue in carcinogenesis. Further work is now underway on the use of one non-gel based detection platform, the Agilent CE system, in order to fully validate the method for the detection of neuronal and other non-muscle targets in a muscle background.

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Bibliography

- Bauer NE., Garland T., Edwards JF. (1996). Brain emboli in slaughtered cattle. *Vet. Pathol.* **33**, p. 600.
- Besnard F., Brenner M., Nakatani Y., Chao R., Purohit HJ., Freese E. (1991) Multiple interacting sites regulate astrocyte-specific transcription of the human gene for glial fibrillary acidic protein. *J. Biol. Chem.* **266** (28), p. 18877–18883.
- Burns M., Shanahan D., Valdivia H., Harris N. (2003). Quantitative event-specific multiplex PCR detection of Roundup Ready soya using LabChip™ technology. *Eur. Food Res. Technol.* **216** (5), p. 428–433.
- Condorelli DF., Nicoletti VG., Barresi V., Caruso A., Conticello S., de Vellis J., Giuffrida Stella AM. (1994). Tissue-specific DNA methylation patterns of the rat glial fibrillary acidic protein gene. *J. Neurosci. Res.* **39**, p. 694–707.
- Condorelli DF., Dell'Albni P., Conticello SG., Barresi V., Nicoletti VG., Caruso A., Kahn M., Vacanti M., Albanese V., de Vellis J., Giuffrida Stella AM. (1997). A neural-specific hypomethylated domain in the 5' flanking region of the glial fibrillary acidic protein gene. *Dev. Neurosci.* **19**, p. 446–456.
- Eads CA., Danenberg KD., Kawakami K., Saltz LB., Blake C., Shibata D., Danenberg PV., Laird PW. (2000). Methylight: a high-throughput assay to measure DNA methylation. *Nucl. Acids Res.* **28** (8), E32.
- FSIS/USDA. (1998). Proposed rules: meat produced by advanced meat/bone separation machinery and recovery systems. *Fed. Reg.* **63**, p. 17959–17965.
- Frommer M., McDonald LE., Millar DS., Collis CM., Watt F., Grigg GW., Molloy PL., Paul CL. (1992). A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA* **89**, p. 1827–1831.
- Government UK. (1989). *The bovine offal (prohibition) regulations*. United Kingdom SI No. 2061.
- Herman JG., Graff JR., Myohanen S., Nelkin BD., Baylin S. (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA* **93**, p. 9821–9826.
- Kaneko R., Sueoka N. (1993). Tissue-specific versus cell type-specific expression of the glial fibrillary acidic protein. *Proc. Natl. Acad. Sci. USA* **90**, p. 4698–4702.
- Li E., Beard C., Jaenisch R. (1993). Role for DNA methylation in genomic imprinting. *Nature* **366**, p. 362–365.
- Lücker E., Eigenbrodt E., Wenisch S., Failing K., Leiser R., Bulte M. (1999). Development of an integrated procedure for the detection of central nervous tissue in meat products using cholesterol and neuron-specific enolase as markers. *J. Food. Prot.* **62**, p. 268–276.
- Paulin R., Grigg GW., Davey MW., Piper AA. (1998). Urea improves efficiency of bisulphite-mediated sequencing of 5'-methylcytosine in genomic DNA. *Nucl. Acids Res.*

- 26, p. 5009–5010.
- Schmidt GR., Hossner KL., Yemm RS., Gould DH., O'Callaghan JP. (1999). An enzyme-linked immunosorbent assay for Glial Fibrillary Acidic Protein as an indicator of the presence of brain or spinal cord in meat. *J. Food Prot.* **62**, p. 394–397.
- Teter B., Finch CE., Condorelli DF. (1994). DNA methylation in the Glial Fibrillary Acidic Protein gene: map of CpG methylation sites and summary of analysis by restriction enzymes and by LMPCR. *J. Neurosci. Res.* **39**, p. 708–709.
- Wang RY.-H., Gerke CW., Ehrlich M. (1980). Comparison of bisulphite modification of 5-methyldeoxycytidine and deoxycytidine residues. *Nucl. Acids Res.* **8**, p. 4777–4790.
- Woodcock DM., Lawler CB., Linsenmeyer ME., Doherty JP., Warren WD. (1997). Asymmetric methylation in the hypermethylated CpG promoter region of the human L1 retrotransposon. *J. Biol. Chem.* **272**, p. 7810–7816.
- Yeivin A., Razin A. (1993). Gene methylation patterns and expression. In Jost JP., Saluz HP. (Eds). *DNA methylation: Molecular biology and biological significance*, 8 ed. Basel: Birkhauser, p. 523–568.
- Yoder JA., Walsh CP., Bestor TH. (1997). Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* **13**, p. 335–340.

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