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An efficient protocol for genomic DNA extraction from formalin-fixed paraffin-embedded tissues

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ABSTRACT

Formalin-fixed paraffin-embedded tissues (FFPET) represent the largest source of archival biological material available for genomic studies. In this work we present an advanced protocol for extraction of high quality DNA from FFPET that can be applied in several molecular studies. Although cat mammary tumours (CMT) are the third most frequent tumour in cats the recovery of significant number of samples for molecular studies are in some way restricted to FFPET samples. We were able to obtain high quality DNA from FFPET of thirty six CMT that were subjected to pre-fixation and fixation processes routinely used in the veterinary hospitals. The quality of DNA obtained was tested by PCR amplification using six sets of primers that amplify single-copy fragments. The DNA fragments obtained were further sequenced. This protocol was able to provide FFPET gDNA that can be amplified and sequenced for larger fragments up to 1182 bp.

1. Introduction

With growing interest in the genetic basis of diseases the amount of genomic DNA available from biological samples may limit the practicality of genomic analysis (Aviel-Ronen et al., 2006; Gilbert et al., 2007b). Formalin-fixed paraffin-embedded tissues (FFPET) have been used for decades and represent the largest source of archival biological material available for genomic studies (Aviel-Ronen et al., 2006). In some cases, like rare diseases, the FFPET represents the ultimate DNA resource available for genetic analysis that otherwise would be unfeasible (Gilbert et al., 2007a).

Previous studies using FFPET of cat mammary tumours (CMT) described molecular changes in oncogenes such as Her2/neu (De Maria et al., 2005; Winston et al., 2005; Morris et al., 2008) or tumour suppressor genes such as *p53* (Murakami et al., 2000; Nasir et al., 2000; Morris et al., 2008) by immunohistochemical studies. The detection of mutations in the *p53* tumour suppressor gene was previously reported in CMT but in fresh tissues (Mayr et al., 1998). In fact, the nuclear DNA extraction and analysis of cat FFPET has only been reported regarding the amplification of a 108 bp single copy fragment from *p53* gene as a positive control for gDNA extraction with a commercial protocol (Kidney et al., 2002). An interesting opportunity for comparative oncology has been recognized in naturally occurring tumours in domestic animals (MacEwen, 1990; Vail and MacEwen, 2000) and, more specifically, CMT may be considered a good model for their human counterpart (MacEwen, 1990; Hansen and Khanna, 2004; Zappulli et al., 2005).

The use of nucleic acids extracted from FFPET in procedures of molecular genetics is highly dependent on its quality and quantity. Different authors state that a suitable DNA extraction method from FFPET is essential and needs to be chosen in relation to specific endpoints of a precise investigation; for instance, the increased length of amplicon or the increase of effective amplifiable copy number (Wang et al., 1996; Poljak et al., 2000; Gilbert et al., 2007b). Therefore diverse nucleic acid extraction methods already described from FFPET, enable the use of DNA extracted for specific approaches: nested PCR–SSCP assay (Wang et al., 1996), RAPD–PCR (Jacobs et al., 2007), real-time quantitative PCR (Gjerdrum et al., 2004), Southern blot hybridisation (e.g. Dubeau et al., 1986; Jackson et al., 1990; Rogers et al., 1990), flow cytometry (Leers et al., 1999), microarray comparative genomic hybridization (Vékony et al., 2007) and SNP BeadArrays (Oosting et al., 2007).

It is well known that the quality and quantity of nucleic acid extracted are highly dependent on a large number of parameters concerning all the procedures that involved the FFPET handling (before and after the DNA extraction itself) (Greer et al., 1991; Romero et al., 1997; Benavides et al., 2006; Gilbert et al., 2007a). Sample

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manipulation (i.e. type and amount of tissue, and its preservation before fixation), fixation methodology (i.e. which fixative was chosen, fixative pH, and temperature and time of fixation) as well as post-fixation features (i.e. temperature and period of storage) are considered the most important nucleic acid pre-extraction parameters (Greer et al., 1991; Miething et al., 2006; Wang et al., 1996; Poljak et al., 2000). In order to optimize the quality and quantity of the nucleic acid extraction, pre-extraction procedures to remove paraffin from fixed tissues (e.g. use of Xylene and ethanol washes) is believed to be important; otherwise it leads to poor quality of the samples and inhibition of subsequent PCR reactions. However, a growing number of authors do not employ specific protocols to remove the paraffin (e.g. Stanta and Schneider, 1991; Wu et al., 2002; Shi et al., 2004).

In the wide variety of methods for DNA extraction from FFPET, the majority was applied to collections of human samples, fixed with formalin (the fixative generally used in histopathological practice) (Gjerdrum et al., 2004). In veterinary clinics the procedures used are essentially the same. In fact, the fixative formalin is one of the main problems with respect to the quality of DNA extracted from FFPET, namely the increased formalin pH (Poljak et al., 2000). Moreover, formaldehyde (HCHO) is the main constituent of formalin that is responsible for the cross-linkage between proteins and DNA or RNA, which in turn could be limiting for further use of the nucleic acids (e.g. Zsikla et al., 2004; Moller et al., 1977). Finally, one of the recent methods described to remove formaldehyde from the specimens is the application of graded ethanol washes followed by a drying critical point (Fang et al., 2002). This method is presented as increasing both DNA yield, and maximum size of PCR amplifiable fragment. Unfortunately, this study involved only eight FFPET samples and there was a lack of subsequent interest in this method (Gilbert et al., 2007a). In DNA extraction methods itself it is important to consider some factors as the composition of the extraction buffer (with or without incorporation of proteinase-K), and time and temperature of the digestion step (Gilbert et al., 2007a).

In this work we present an advanced protocol for extraction of high quality DNA from FFPET that can be applied in several molecular studies. Although cat mammary tumours are the third most frequent tumour in cats (Zappulli et al., 2005; Seixas et al., 2008) the recovery of significant number of samples for molecular studies are in some way restricted to FFPET collected during a long period of time. Here, we were able to obtain high quality DNA from FFPET of thirty six cat mammary tumours that were subjected to pre-fixation and fixation processes routinely used in the veterinary hospitals. The quality of DNA obtained was tested by PCR amplification using six sets of primers that amplify single-copy fragments. The DNA fragments obtained were further sequenced. The protocol described here was able to provide FFPET gDNA that can be amplified and sequenced for larger fragments up to 1182 bp.

2. Materials and methods

Thirty six cat mammary tumours FFPE samples were obtained from two histopathology laboratories from the University of Porto: Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP) and Instituto de Ciências Biomédicas de Abel Salazar (ICBAS). All samples were collected and formalin fixed in various veterinary hospitals and the following routine paraffin wax embedding procedures were undertaken in the histopathology laboratories. Frozen samples ($n = 30$) from cat mammary tumours and normal muscle and skin tissues were used as controls and were kindly provide by the Trás-os-Montes Veterinary Hospital and ICBAS. All tissues were evaluated by pathology procedures; the cat mammary tumour biopsies represent histological heterogeneous samples. All biopsies and necropsies were carried out between the years of 2005 to 2007.

2.1. DNA Extraction from FFPE tissues

The DNA extraction from FFPE tissues were performed using the Automatic Nucleic Acid Isolation System (QuickGene 800, Fujifilm Life Science). The system uses a porous ultra thin membrane and an automatically pressurizing unit that promote binding, washing and elution steps at low pressure. In order to apply this system to the isolation of genomic DNA from FFPET, several modifications were carried out: melting and removal of the paraffin; time and temperature of the lyses procedure; and the elution time. All other steps of the procedure in the DNA extraction protocol were executed according to the one described by the manufacturer "QuickGene DNA Tissue Kit S" (Fujifilm Life Science).

Tissue sections were observed by light microscopy to determine the location of tumour cells. After slide observation, the paraffin wax blocks were sliced with a blade in the area of interest. DNA was extracted from approximately 5 to 20 mg fragments of paraffin-embedded tissue samples, cut in small sections and collected in a 0.5 ml microcentrifuge tube.

The paraffin of each sample was melted in 50 μl of a Tween 20 solution (0.5% Tween 20, 1 mM EDTA, 50 mM Tris–HCl pH 8.5) at 95 °C for 10 min, followed by a second round of incubation at 65 °C for 10 min. The tissue digestion was done with proteinase K (MDT with EDT reagents in the original protocol from QuickGene DNA Tissue Kit S, Fujifilm Life Science) at 65 °C in an overnight water bath with agitation as described by manufacture. The samples were then centrifuged at 16000 rcf for 5 min, at 0 °C. At this time it is possible to observe a paraffin layer in the top of the digestion solution. In order to eliminate all the paraffin it is advisable to remove the paraffin layer using a glass Pasteur pipette closed in the tip. An additional proteinase K digestion at 65 °C, during 1 h, was performed. Finally, an extra step for the elimination of the residual paraffin was added and this specific procedure was evaluated in the general performance of the protocol regarding integrity, quantity and quality of the gDNA obtained. Therefore, all samples were centrifuged at 15000 rcf for 35 min at 4 °C and the supernatant was collected avoiding the paraffin residues.

As described by the manufacturer, cell lyses was performed by incubating the samples with the cell lyses buffer (LDT in the protocol from QuickGene DNA Tissue Kit S, Fujifilm Life Science) during 10 min at 70 °C and DNA was precipitated by adding 240 μl of ethanol.

At this time, all the lysates were transferred to the purification columns of the automatic nucleic-acid isolation system QuickGene-800. The program "DNA Tissue" was used with the time of elution set to its maximum value. The protocol was optimized for the standardized final volume of 100 μl (the elution buffer used was the one furnished by the manufacturer).

The use of DNA samples obtained from FFPE tissues on subsequent procedures is depends on the quality of the DNA and its storage. The DNA samples should be stored at −20 °C for longer storage periods, however better results were achieved when extracts were not frozen.

2.2. DNA Extraction from frozen tissue samples

The isolation of genomic DNA from frozen tissues was performed with the QuickGene DNA Tissue Kit S (Fujifilm Life Science) following the procedures described by the manufacturer.

2.3. Evaluation of gDNA from paraffin-embedded tissue

In order to analyse the integrity of the genomic DNA extracted all samples were subjected to a 1% agarose gel electrophoresis. The quantity and quality of the DNA extracted was measured in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies,