

A Universal Primer Set for PCR amplification of Nuclear Histone H4 Genes from all Animal Species

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Abstract

In order to control the quality of genomic DNA of samples from a wide variety of animals, a hemi-nested PCR assay specifically targeting a nuclear gene has been developed. The histone H4 gene family comprises a small number of genes considered among the most conserved genes in living organisms. Tissue samples from necropsies and from cells belonging to 43 different species were studied, 8 samples from invertebrates and 35 from vertebrates covering all classes. Ancient DNA samples from three siberian woolly mammoths (*Mammuthus primigenius*) dating between 40,000 and 49,000 years before present were also tested for PCR amplification. Performance of HIST2H4 amplification were also compared with those of previously published universal PCRs (28S rRNA, 18S rRNA, cytochrome b). Overall, 95% of species studied yielded an amplification product including some old samples from gorilla and chimpanzees. The data indicate that the HIST2H4 amplimers are thus suitable for both DNA quality testing as well as species identification in the animal kingdom.

Introduction

Since the appearance of PCR, several tests have been described which enable DNA amplification from most vertebrate species. However, these assays aim at conserved segments of the mitochondrial genome or at ribosomal genes both present at several hundred to several thousand copies per cell in metazoan organisms. Such targets are readily detectable but reflect only distantly the status of non ribosomal nuclear genes in tissues (Bataille et al. 1999; Meyer et al. 1994; Naito et al. 1992). In addition, mitochondrial DNA (mtDNA) migrates regularly to chromosomal DNA where fragments may be stabilized as pseudogenes (Bensasson et al. 2001; Wallace et al. 1997). Consequently, mistaking nuclear mtDNA for *bona fide* mitochondrial genomes may lead to erroneous conclusions when performing species identification (Vartanian and Wain-Hobson 2002). The development of additional molecular tools enabling the study of nuclear DNA in phylogenetically distant animals is still needed. To assess more accurately the quality of clinical samples after genomic DNA extraction, a PCR assay targeting a nuclear gene was developed. Nuclear DNA would supply a much higher set of characters for phylogenetic analysis and open up the possibility of studying genetic loci involved in determining genotypic traits in extinct species.

Histones are structural and functional components of chromatin. Five classes of histones were originally characterized (H1, H2A, H2B, H3, H4). The number of histone genes per haploid genome varies substantially from species to species from a few copies in mammals and birds up to ~500 copies organized in tandem clusters in certain sea urchin species. In mammals and birds histones genes are usually mapping at few genomic loci that contain clusters or individual genes. In humans, the largest cluster of histone genes HIST1 is located on chromosome 6 (6p21-p22), and two smaller clusters HIST2 (1q21) and HIST3 (1q41) are located on chromosome 1. In addition to the histone genes on these clusters, a histone H4 gene (HIST4) was identified on chromosome 12.

We focused on histone H4 genes considered as some of the most conserved genes in eukaryotes (DeLange and Smith 1971; Thatcher and Gorovsky 1994; Wells and Brown 1991). Each of the 14 human histone H4 genes encodes the same protein. The H4 genes in HIST2 and HIST4 on chromosome 1 and 12 are

thus very similar to the genes in HIST1 on chromosome 6, indicating that there is a strong selective pressure to preserve a similar nucleotide sequence in the coding region. Even so, histones H3 and H4 evolve on the order of 10 times more slowly than H2A and H2B (Thatcher and Gorovsky 1994). In addition, pseudogenes of histone H4 exist in the human genome sequence.

In the current report, we present a method for amplification of nuclear histone H4 genes that is suitable for DNA quality testing as well as for species identification in the animal kingdom.

Material and Methods

Samples and DNA extraction

Tissues samples from necropsies and from cell lines belonging to 43 different species were studied and include 8 samples from invertebrates and 35 from vertebrates (Table 1). Samples were digested in SDS/proteinase K buffer (0.1 mg/ml) at 37°C for 6 to 12 h, and extracted twice with phenol and once with chloroform. DNA was then ethanol-precipitated and resuspended in TE buffer. Besides liver necropsies, a collection of samples considered as degraded was investigated. A series of 11 faeces from chimpanzees living in the wild were extracted using the MasterPure™ complete DNA and RNA purification kit (Epicentre, Tebu-bio, Le Perray-en-Y., France) according to the manufacturer's procedure. Nine DNA samples were extracted from teeth of chimpanzees (n=7) and gorillas (n=2) dating from between 1883 and 1948. Finally, three DNA samples from siberian woolly mammoths (*Mammuthus primigenius*) dating from between 40,000 and 49,000 years before present were also analyzed (Table 2). Tooth and mammoth DNA was prepared according to protocols previously described (Debruyne et al. 2003; Hassanin et al. 1998).

Polymerase chain reaction

Approximately, 50 ng of genomic DNA were subjected to 35 cycles of amplification according to a "step-down" protocol in a Techne Genius thermal cycler (OSI, Saint-Quentin-en-Yvelines, France) (Hecker and Roux 1996). The "step-down" procedure enhances the specificity of PCR by avoiding mispriming during the initial cycles. Reactions were performed in 25 µl of a mixture containing 1X PCR buffer [50 mM KCl, 10 mM Tris (pH 8.9), 0.1 %

Tween 20, 1.5 mM MgCl₂, 200 μM dATP, dGTP, dTTP, dCTP], 50 pmol of each primer, 1.25 unit of Taq polymerase (Life Technologies, Cergy-Pontoise, France). Degenerate primers were designed in a conserved region of the human HIST2H4 gene (1q21) and its orthologs published in databases. Amplified segments were mapped between nucleotides 96 and 307 (211 bp) of HIST2H4 gene (accession # BC019846) for the first round amplification using H4F2s, 5'TSCGIGAYAACATYCAGGGIATCAC and H4F2er, 5' CKYTTIAGIGCRTAIACCACRTCCAT, primers and nucleotides 96 and 277 (181 bp) for the internal one (I: inosine, K: G/T; R:A/G; S: C/G; Y: C/T). For the hemi-nested protocol, primer H4F2s was combined with H4F2ir 5' GTIACIGTCTTSCKYTTGGCGTGCTC on 2 μl of the primary amplification and subjected to 35 additional PCR cycles according to the same procedure except that reactions were performed in the presence of 7% DMSO. Following PCR, amplification products were loaded on a 2% agarose gel (Eurobio, Les Ulis, France), stained with ethidium bromide and visualized on a UV transilluminator. Appropriate precautions to avoid cross contamination were taken while performing the PCR and negative amplification controls remained negative. Primers used to amplify 28S rRNA, 18S rRNA and cytochrome b PCR have been published elsewhere (Kocher et al. 1989; Meyer et al. 1994; Naito et al. 1992).

Sequences analysis

For all 43 species amplified fragments were purified from agarose gels (Qiaex II kit, Qiagen, France) and ligated in the TOPO TA cloning vector (InVitrogen, France). Sequencing was performed using Thermosequenase (USB, Amersham) on an ABI373A sequencer (Applied Biosystems). Five clones were sequenced for each of these 43 species. In addition, between 3 and 5 clones from each mammoth specimens were sequenced. Sequences were aligned by using the CLUSTAL W program (Thompson et al. 1994). Nucleotide distances were determined with DNAdist of the Phylip package version 3.5 (Felsenstein 1993). The tree was derived by neighbor-joining (NJ) analysis applied to pairwise distances calculated using the Kimura two-parameter method to generate unrooted trees. Robustness of the NJ phylogenetic tree was tested by bootstrap analysis. Horizontal branch lengths are drawn to scale with the bar indicating 0.1 nucleotides replacements per site. The final output was

generated with Treeview (Page 1996). The number at each node represents the percentages of bootstrap replicates (out of 100). Only bootstrap values of > 60 are given. The sequences can be found in GenBank under accession numbers: AY675260-AY675296.

Results

Target gene and samples studied

Our aim was to develop a robust PCR method to assess the quality of genomic DNA from all animal species. We performed PCR amplification on 43 genomic DNA samples from animals belonging to non-vertebrate (n=8) as well as vertebrate (n=35) branches. Among vertebrates, all traditional classes (fishes, amphibians, reptiles, birds, mammals) were represented (Table 1). A subset of DNAs studied (n=8) were extracted from liver tissues obtained from animals suffering from acute hepatitis whereas remaining samples were obtained from established cell lines (e.g. COS7 from *Cercopithecus aethiops*), laboratory model specimens (e.g. zebra fish or chicken), routine practice in veterinary medicine (ovarian or testicular ablation from dog and cat respectively). In addition, DNA was extracted from three groups of different samples, ancient and extinct species, 11 faeces from chimpanzees collected in the wild, 9 teeth of gorilla and chimpanzees collected between 1883 and 1948 and finally 3 samples from Siberian woolly mammoths (*Mammuthus primigenius*) conserved in permafrost since the Pleistocene (Debruyne et al. 2003).

Amplification from vertebrates, non-vertebrates and clinical samples

All vertebrate samples (n=35) yielded amplification products either after a single round or a hemi-nested amplification procedure (Table 1). Those of the vertebrates samples yielding amplimers only after the second round of amplification originated in most cases (9/11 cases) from degraded tissues specimens obtained from animals suffering from severe acute hepatitis or from inappropriately stored samples (Figure 1A and 1B). In the remaining cases (n=25/36, 67%), a single round of PCR cycles was sufficient to obtain a detectable amplimer. As expected, after 35 cycles, amplification of the smaller

internal (181 bp) fragment was overall more efficient than that of the larger one (211 bp, 25 vs. 20 positive samples). For invertebrates, the hemi-nested procedure was often necessary (n=3/9) to obtain a sufficient amplification product (Table 1).

Comparison with previously published wide-spectrum PCR methods

To ascertain the usefulness of the method, we compared the performance of HIST2H4 amplification with those of previously published universal PCR on the same DNA samples. 18S/28S rRNA or cytochrome b amplifications were reviewed. The performance of HIST2H4 amplification was similar to that of 28S rRNA amplification, with 43 (95%) and 41 (91%) positive samples, respectively. Both techniques were performed according to a nested and hemi-nested procedure, thus explaining the high fraction of successful assays. The performances of 18S rRNA and cytochrome b PCRs were poorer, with 25 (55%) and 17 (38%) positive samples, respectively (Table 1). This difference is most likely attributable to the lack of a nested procedure. The sequenced DNA from HIST2H4 gene were occupying in the phylogenic tree, a position consistent with their place in evolution indicating the robustness of the tree. However, with the primers used in the current report amplification precludes the detection of HIST1H4 B, C, D, E, G and HIST4H4, out of the 14 human histone H4 genes due to the presence of disparate mismatches in the third base of the primer sequences. Hence, the alignment of HIST2H4 sequences gave a major form of 80% (4/5), however, in some animal species, 1 or 2 HISTH4 sequences were also amplified from another histone H4 locus (see supplementary information).

Relative sensitivity of the method

We next examined the amount of DNA required for HIST2H4 assay. An absolute comparison on different genomes is not possible because the exact genome sizes and composition with regard to the number of histone H4 genes are not known for most of the animal species. Broad differences in amplification efficiency attributable to the number of histone H4 genes per genome (sometimes in accordance with genome size) or to particular homology with degenerate primers may be detected by comparing different high molecular weight DNA samples (Bensasson et al. 2001; Ishaq et al. 1993).

Six different samples were tested by serial ten-fold dilutions ranging from 100 ng to 0.1 fg. Species were chosen in each of the vertebrate classes (*Bufo bufo*, *Mabuya quinquetanea*, *Melopsittacus undulatus* and *Cercopithecus aethiops*) and a non-vertebrate was also added (*Locusta migratoria*). First round of amplification yielded clear amplicones within a range of 1 ng (*Bufo bufo*, *Mabuya quinquetanea*, *Melopsittacus undulatus*, *Cercopithecus aethiops*) to 10 pg (*Locusta migratoria*) whereas the second round of PCR extended the detection range from 2 pg (*Mabuya quinquetanea*, *Cercopithecus aethiops*) to 20 fg (*Locusta migratoria*, *Bufo bufo*, *Melopsittacus undulatus*) (data not shown).

Degraded, ancient and extinct DNA

Some samples, considered as difficult to explore *i.e.* chimpanzee faeces, ape teeth and mammoth tissues were then subjected to PCR for the HIST2H4 target gene. Amplification was successful in 6 out of 11 DNA samples extracted from chimpanzee faeces (55%) whereas ape teeth yielded a product in 7 out of 9 cases (77%). The oldest DNA extracted from a tooth was dating from 1885 (*G. gorilla*). Finally, we were able to amplify 3/3 Siberian mammoth samples dating from 40,000 to 49,000 years before present (Table 2). These data indicate that HIST2H4 amplification is robust enough to be performed on modern and old remains.

Discussion

Nucleic acid samples from animal livers with acute hepatitis often represent degraded material. The poor quality of DNA may then be the cause of a lack of virus detection in such samples. A reliable method of assessing the quality of DNA specimens is consequently needed to analyse samples taken from different animal species and to avoid false negative results. Recent developments of comparative genomics have produced a substantial amount of sequences from various living organisms thus permitting significant interspecies DNA alignments. This prompted us to develop a novel, robust and simple method of wide-spectrum PCR to test the quality of nuclear DNA in clinical samples and more generally in animals.

The strong conservation of histone H4 across evolution from slime molds and plants to mammals is known since early studies were performed on proteins

(DeLange and Smith 1971). In contrast, protists represent the only group displaying substantial variation within H4 sequences (Bernhard and Schlegel 1998; Van Den Bussche et al. 2000). Our data demonstrate the usefulness of HIST2H4 amplification in virtually all animal species (Table 1). The assessment of nuclear DNA amplifiability, i.e. quality in samples in which DNA is partially degraded is possible for any species when the nested procedure is used. With regard to quantitative aspects, HIST2H4 amplification may rival previously described methods aiming at rRNA or mtDNA. Ribosomal 18S/28S RNA (rRNA) genes are tandemly-organized gene clusters on acrocentric arms of chromosomes (Zardoya and Meyer 1996). The number of rRNA genes per haploid genome varies greatly (50-800 copies) among species. A similar method is based on cytochrome b detection. This gene is encoded by mitochondrial genomes which varies widely in number per cell ($10 - 10^5$) with tissue type, age and pathology (Jansen 2000), thus making them unsatisfactory for an accurate nuclear DNA quality assessment. The method developed in the current report is however, also not strictly aimed at a single copy nuclear gene. Indeed, in sea urchin (e.g. *P. miliaris*, *S. Purpuratus*) or in *D. melanogaster*, histones genes are organized into a single type of tandemly repeated (~100-500 copies) cluster. The situation differs substantially in vertebrates species. In that case, the relatively low repetition (e.g. 14 H4 genes in man, 4 in mouse, 7 in chicken) has no consistent organization. In mammals and birds, clusters of histone genes may be composed of different repeating units and sometimes no basic repeated entity is found in the cluster. Consequently several non allelic genes may be detected with primers used primarily to detect HIST2H4 orthologs (Marzluff et al. 2002). Nevertheless, the relatively small number of paralogs of HIST2H4 makes this assay a rather faithful reflection of the status of a regular nuclear gene. HIST2H4 detection may, thus, represent an alternative or an additional target for characterisation of samples from phylogenetically distant organisms (Figure 2). Similarly to previously published assays, the present procedure can be employed with a wide taxonomic utility (Bataille et al. 1999; Meyer et al. 1994). Since the mitochondrial genome is inherited maternally in most animals, the use of a nuclear DNA amplification method is essential when molecular data based on biparentally inherited sequences are needed (Palumbi and Cipriano 1998; Slade et al. 1998). In addition, recent analyses

showed that phylogenetic results based on mitochondrial genomes often need to be confirmed with data from nuclear genes (Cotton and Page 2002). Similarly, several questions could not be resolved by rRNA sequence comparisons. Histone H4 may thus represent a truly independent marker for testing the robustness of rRNA or mtDNA phylogenies (Bernhard and Schlegel 1998). One peculiarity of histone H4 should, however, be kept in mind. Histone H4 is considered as virtually invariant at the protein level among vertebrates. Fortunately, the extent of overall nucleotide sequence divergence is higher than that of proteins (Piontkivska et al. 2002). Most of the nucleotide sequence variation takes the form of synonymous substitutions reaching apparently the saturation level in many species. It has been shown formerly that this situation may explain that distant species appear closely related on the phylogenetic tree (Piontkivska et al. 2002).

The application spectrum of a novel universal PCR method is wide and can be extended beyond basic research. For examples, in forensic entomology, the molecular characterisation of dipters *larvae* may provide some indications for postmortem interval determination (Sperling et al. 1994). In addition, DNA sequence analysis has become a powerful tool to detect food adulteration and/or to provide forensic evidence for illegal wildlife trade (Bellagamba et al. 2003; Wan and Fang 2003).

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Table 1 : Animal species for which genomic DNA has been subjected to PCR analysis.

Branch	Class	Order	Species		Hepatitis	DNA m.w.	Hist2H4			28S			18S	Cyto.b	
							ext	int	nested	ext	int	nested			
1	Vertebrates	Fishes	Chondrycties	Marble electric ray		<i>Torpedo marmorata</i>	H	+	+	nd	-	+	nd	+	-
2			Cypriniforms	Goldfish		<i>Carassius auratus</i>	H	+	+	nd	-	+	nd	+	+
3				Zebra fish		<i>Brachydanio reiro</i>	H	+	+	nd	-	+	nd	+	-
4		Amphibians	Anoures	Frog		<i>Rana esculenta</i>	H	-	-	+	-	-	-	-	-
5				Toad		<i>Bufo bufo</i>	H	+	+	nd	-	+	nd	+	-
6				African clawed frog		<i>Xenopus laevis</i>	H	-	-	+	-	-	+	-	-
7		Reptiles	Squamates	Agama		<i>Agama agama</i>	L	-	-	+	-	-	-	-	-
8				Mabuya		<i>Mabuya quinquetanea</i>	H	+	+	nd	+	+	nd	+	-
9				Common lizard		<i>Lacerta vivipara</i>	H	+	+	nd	+	+	nd	+	+
10				Tegu		<i>Tupinambis rufescens</i>	L	-	+	nd	-	-	+	+	-
11			Ophidians	House snake		<i>Lamprophis fuliginosus</i>	L	-	-	+	-	-	+	-	-
12		Birds	Galliforms	Chicken		<i>Gallus gallus</i>	H	+	+	nd	+	+	nd	+	+
13				Japanese Quail		<i>Coturnix coturnix japonica</i>	H	+	+	nd	+	+	nd	+	+
14			Psittaciform7s	Budgerigar		<i>Melopsittacus undulatus</i>	H	+	+	nd	-	+	nd	+	+
15			Passeriforms	Bengalese finch		<i>Lonchura domestica</i>	H	-	+	nd	-	+	nd	+	+
16		Mammals	Rodents	Mouse		<i>Mus domesticus</i>	H	+	+	nd	+	+	nd	+	+
17				Woodchuck		<i>Marmota monax</i>	H	+	+	nd	+	+	nd	+	+
18				Arctic squirrel		<i>Spermophilus parryi</i>	H	+	+	nd	+	+	nd	+	+
19				Gerbil		<i>Meriones unguiculatus</i>	H	+	+	nd	+	+	nd	+	+
20			Artiodactyles	Zebu		<i>Bos indicus</i>	H	+	+	nd	+	+	nd	+	+
21			Chiropteres	Bat		<i>Roussettus aegyptiacus</i>	+	L	-	-	+	-	-	+	-
22			Carnivores	Dog		<i>Canis domesticus</i>	H	+	+	nd	+	+	nd	+	+
23				Fennec fox		<i>Fennecus zerda</i>	+	L	-	-	+	-	-	+	-
24				Suricate		<i>Suricata suricatta</i>	+	L	-	-	+	-	-	+	-
25				Cat		<i>Felis catus</i>	H	+	+	nd	+	+	nd	+	+
26			Primates	Ring-tailed lemur		<i>Lemur catta</i>	+	H	+	+	nd	+	+	nd	+
27				Black and white ruffed lemur		<i>Varecia variegata</i>	+	L	-	-	+	-	-	+	-
28				Tufted-ear marmoset		<i>Callithrix geoffroyi</i>	+	H	-	-	+	-	-	+	-
29				Cottontop tamarin		<i>Saguinus oedipus</i>	+	H	+	+	nd	+	+	nd	+
30				Squirrel Monkey		<i>Saimiri sciurus</i>	+	H	+	+	nd	-	+	nd	-
31				Hamlyn's monkey		<i>Cercopithecus hamlyni</i>	+	L	-	-	+	-	-	+	-
32				Vervet monkey		<i>Cercopithecus aethiops</i>	H	+	+	nd	+	+	nd	+	+
33				Rhesus monkey		<i>Macacca mulatta</i>	H	+	+	nd	+	+	nd	+	-
34				Chimpanzee		<i>Pan troglodytes</i>	L	+	+	nd	+	+	nd	+	+
35				Man		<i>Homo sapiens</i>	H	+	+	nd	+	+	nd	+	+
36	Molluscs	Gastropods	Pulmonates	Pond snail		<i>Planorbis corneus</i>	H	-	+	nd	-	-	+	-	-
37				Reticulated slug		<i>Deroceras reticulatum</i>	L	-	-	+	-	-	+	-	-
38				Small striped slug		<i>Arion hortensis</i>	L	-	-	+	-	-	-	-	-
39		Bivalves	Unionoids	Fresh water mussel		<i>Anodonta complanata</i>	L	-	+	nd	-	-	+	-	-
40	Arthropods	Insects	Lepidopterans	Fall armyworm		<i>Spodoptera frugiperda</i>	H	-	+	nd	-	-	+	+	+
41			Orthopterans	Migratory locust		<i>Locusta migratoria</i>	H	+	+	nd	+	+	nd	+	+
42		Chelicerates	Arachnids	European house spider		<i>Tegenaria domestica</i>	H	+	+	nd	+	+	nd	+	+
43		Crustaceans		Brown shrimp		<i>Atya mollucensis</i>	L	-	-	+	-	-	+	-	-

Taxonomic data as branches, class, order, english vernacular names and zoological names are mentioned in the 5 leftmost columns. Hepatitis indicates, whether it was a necropsy sample from animal that died from hepatitis. DNA molecular weight indicates the presence of high (H) or low molecular weight DNA fragments. In the following columns are reported the PCR data from HIST2H4 and 28S rDNA nested PCR as well as 18S rDNA and cytochrome b after a single round of PCR. + an - indicate a positive or negative PCR results respectively. M.w: molecular weight, nd: PCRs have not been performed.

Table 2 : PCR amplification of HIST2H4 from old material.

DNA Origin	Species		Number of specimens	positive H4F2
skin/bone 49000ybp	Woolly mammoth	<i>Mammuthus primigenius</i>	3	3/3 (100%)
teeth 1885 and 1936	Gorilla	<i>Gorilla gorilla</i>	2	1/2 (50%)
teeth 1883-1948	Chimpanzees	<i>Pan troglodytes</i>	7	6/7 (86%)
faeces	Chimpanzees	<i>Pan troglodytes</i>	11	6/11 (55%)

All PCRs were performed according to the hemi-nested procedure.

Figure Legends

Figure 1: Amplification of HIST2H4 from partially degraded DNA samples.

A) Partially degraded DNAs were controlled on a 0.8% agarose gel. Seven DNA samples were extracted from necropsy specimens, (lanes 1-7, *Agama agama*, *Lamprophis fuliginosus*, *Mus domesticus*, *Gallus gallus*, *Roussettus aegyptiacus*, *Fennecus zerda*, *Suricatta suricatta*), three poor quality DNA samples from non-vertebrates (Lane 8-10, *Spodoptera frugiperda*, *Arion hortensis*, *Attya mollucensis*); lane 11 contains the positive control (COS7 cells from *Cercopithecus aethiops*).

B) Agarose gel analysis of the 181bp amplification products obtained after HIST2H4 hemi-nested PCR performed on each of the degraded DNAs samples.

Figure 2: Unrooted tree of HIST2H4 genes. The twenty-nine most representative HISTH4 sequences were aligned and used to construct the tree (NJ, Kimura2-parameter). Bootstrap values are based on 100 replications and only those above 60% are shown. Asterisks represent sequences taken from the gene bank, respectively *Rattus norvegicus* (accession, NM 022686) and *Bos taurus* (accession, NM 173880)

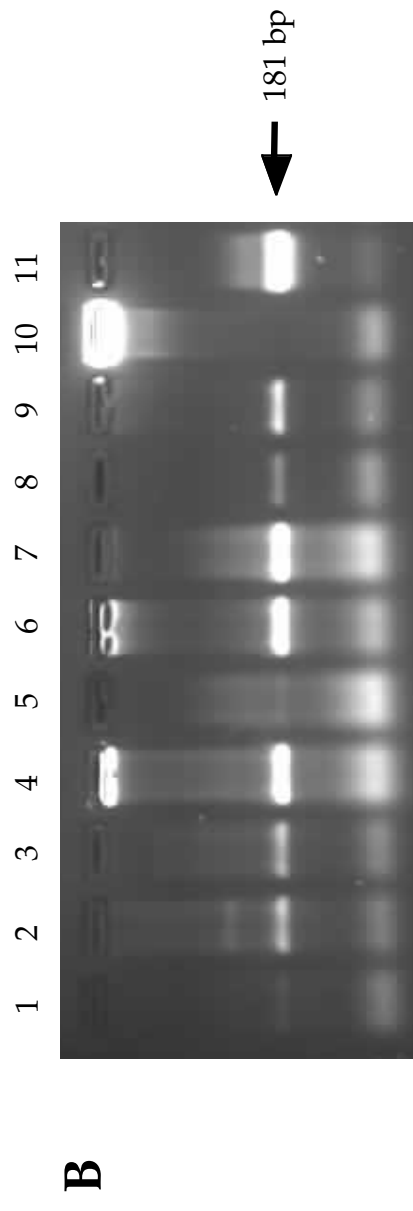
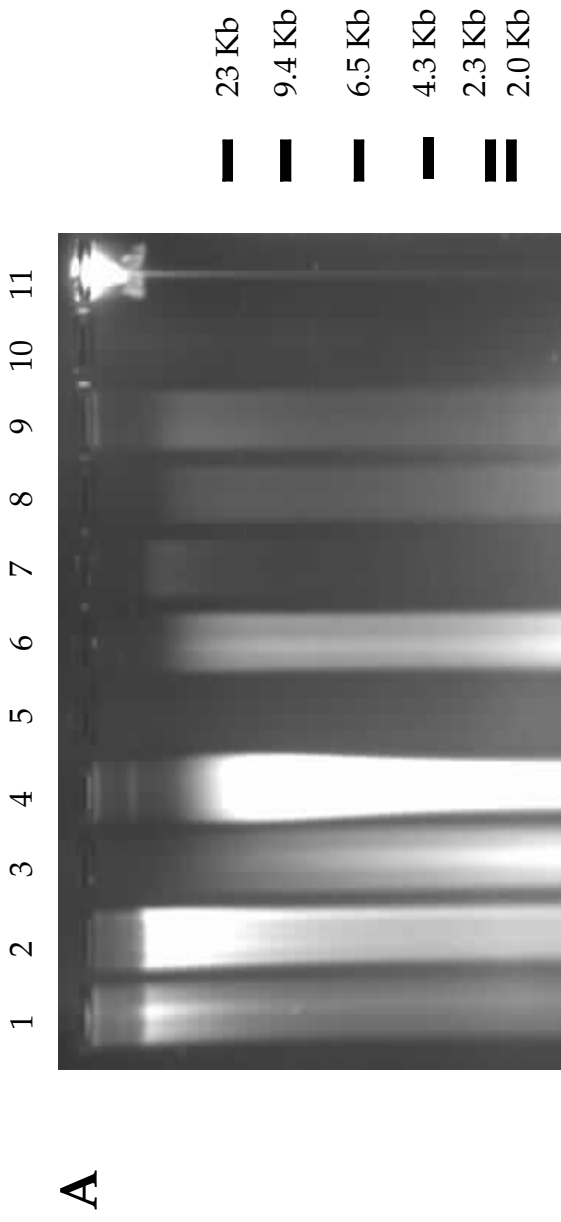


Figure 1

