

1 **Flexible, multi-use, PCR-based nucleic acid integrity assays based on the ubiquitin C gene**

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13 **ABSTRACT**

14 Nucleic acid integrity assessment is an important aspect of quality control for many applications in
15 molecular biology. A number of methods exist (electrophoresis- or PCR-based), but they are not
16 universally applicable. Some of them need huge amounts of input, process certain amount of
17 samples, require expensive equipment, only work specifically on (c)DNA, (m)RNA, certain species
18 or certain tissues, or produce fragments covering a small length range. We investigated if the
19 ubiquitin C gene (UBC) could be used to develop flexible, multi-use PCR-based (deoxy)ribonucleic
20 acid integrity assays. UBC gene analysis (in human, mouse, pig, cow, horse, sheep, dog and cat)
21 shows that UBC is a highly conserved and ubiquitously expressed gene (reference gene in RT-
22 qPCR), that encodes a polyubiquitin precursor (containing tandem repeats of at least 5 ubiquitin
23 monomers of 228 bp) in a single exon. On average, ubiquitin monomers show a nucleic acid
24 sequence identity of 96% at intraspecies level and 93% at interspecies level. Based on a multiple
25 alignment of all monomer ubiquitin sequences of all investigated species, we could design a single
26 degenerated primer pair generating PCR amplicons of 137, 365, 593 and 821 bp on low amounts of
27 high quality DNA of all investigated species (down to 10 pg) and on cDNA reverse transcribed from
28 high quality RNA from different tissues (e.g. heart, liver, brain, kidney). Increasing levels of nucleic
29 acid degradation resulted in a decrease of amplification products starting with the longer amplicons.
30 We conclude that UBC is suited to develop a single, cheap, universal assay to estimate the presence,
31 integrity and amplificability of native mammalian nucleic acids. In addition, we used the same
32 strategy to design a similar assay to check the quality of bisulfite treated mammalian DNA.

34 INTRODUCTION

35 Nucleic acid integrity assessment is an important aspect of quality control faced by every researcher
36 in the decision if a sample is suitable for many of the, sometimes expensive and/or time-consuming,
37 applications in molecular biology. Existing integrity assays rely on electrophoresis or PCR, but they
38 are not universally applicable/available [1-3]. Gel electrophoresis is cheap and easy to perform, but
39 requires high amounts of sample. Chip electrophoresis is more sensitive and needs only a limited
40 amount of sample, but is expensive and requires specialized equipment. In contrast with PCR-based
41 assays, they do not check for PCR amplificability. Traditional PCR assays are cheap, easy to
42 perform, sensitive and require only a limited amount of sample, while qPCR or dPCR assays might
43 be more precise, but more complex, more expensive and require specialized equipment. Because
44 published PCR-based assays are developed application-dependent, they only work specifically on
45 DNA, cDNA, certain species, certain tissues or produce fragments covering a small length range [1-
46 3]. As a result, until now, laboratories that want to check PCR amplificability have to implement a
47 battery of different integrity assays to cover all their experiments.

48 The ubiquitin C gene (UBC) is a highly conserved and ubiquitously expressed gene (frequently
49 used as a reference gene in RT-qPCR), that encodes a polyubiquitin precursor in a single exon [4-5].
50 Here, we want to investigate if the UBC gene could be used to develop a single, cheap, multi-use
51 PCR-based assay to estimate the presence, integrity and amplificability of DNA (isolated from man,
52 mouse, pig, cow, horse, sheep, dog or cat) and cDNA (reverse transcribed from RNA isolated from
53 any tissue type and reflecting RNA integrity).

54 **MATERIALS AND METHODS**

55 **DNA isolation**

56 DNA was isolated from blood by performing a proteinase K digest followed by a phenol/chloroform
57 extraction and an ethanol precipitation. DNA was artificially degraded with 0,1 U RQ1 DNase
58 (Promega). DNA used for bisulfite treatment was isolated with the Quick-DNA Miniprep Plus kit
59 (ZymoResearch). Bisulfite treatment was performed with the EZ DNA Methylation-Lightning Kit
60 (ZymoResearch). Purity and concentration was measured with Nanodrop (Isogen). Integrity was
61 estimated by analysing 1 µg of DNA on a 1% agarose gel.

62 **RNA isolation and cDNA synthesis**

63 Total RNA was isolated using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad),
64 including an on-column DNase treatment to remove genomic DNA. Purity and concentration was
65 measured with Nanodrop (Isogen). Integrity was estimated by analysing 1 µg of RNA on a 1%
66 agarose gel. One µg of total RNA was converted to cDNA with the ImProm-II cDNA synthesis kit
67 (Promega), using both oligo dT and random primers. cDNA was 10 times diluted with water.

68 **PCR**

69 UBC gene sequences were retrieved via NCBI Gene [6]. Pairwise sequence alignment was
70 performed with NCBI Blast [7] and multiple sequence alignment with ClustalW [8]. Primers based
71 on native DNA were designed with Primer-Blast [9] and primers based on bisulfite treated DNA
72 with BiSearch [10]. PCR assays were optimised and performed with TEMPase Hot Start DNA
73 Polymerase (VWR) on a S1000 Thermal Cycler (Bio-Rad). Assay results were validated with
74 primers amplifying other genes. All assay details are listed in Additional file 1.

75 **Agarose gel electrophoresis**

76 DNA, RNA and PCR products were all analysed by 1% agarose gel electrophoresis.

77 **RESULTS AND DISCUSSION**

78 **UBC gene *in silico* analysis**

79 Dot matrix views of intraspecies pairwise nucleic acid sequence alignment of human, murine,
80 porcine, bovine, equine, ovine, canine and feline UBC gene sequences show that all investigated
81 UBC genes encode at least 5 tandem repeats of the UBC monomer of 228 bp (Additional file 2).
82 Intraspecies pairwise nucleic acid sequence alignments of the individual UBC monomer sequences
83 show that they are highly conserved at intraspecies level, with 96% nucleic acid sequence identity
84 averaged over the investigated species (except for the 10th UBC monomer in mouse, that was left
85 out for further analysis). Interspecies pairwise nucleic acid sequence alignments of the individual
86 UBC monomer sequences show that, with an average of 93% nucleic acid sequence identity, they
87 are even highly conserved at interspecies level. Multiple nucleic acid sequence alignment of all
88 individual UBC monomer sequences of the investigated mammals shows positional conservation
89 (Additional file 3).

90 **UBC integrity assay design**

91 The rationale behind the assay design is to choose primers that are able to bind to all UBC
92 monomers of all investigated species in order to amplify a series of fragments of different lengths
93 due to the presence of the tandem repeats (Figure 1). Since these tandem repeats contain
94 uninterrupted coding sequences and UBC is ubiquitously expressed, primers can amplify both DNA
95 and cDNA reverse transcribed from RNA isolated from any tissue (Additional file 4).

96 The amplification rate of the different fragments will be related to the quality of the template. High
97 quality nucleic acids will generate a ladder of amplicons. Decreasing quality will result in a
98 decrease of both the number and the concentration of the amplicons, starting with the longer
99 fragments. By analysing the amplification pattern of this single assay, it is possible to check the

100 presence, integrity and amplificability of the template, being DNA or cDNA from any tissue of any
101 of the investigated mammals.

102 Because of the presence of single ubiquitin monomer sequences in several other genes, primers
103 were chosen not to be able to amplify single monomers in order not to implicate the amplification
104 of the longer UBC fragments. The human UBC nucleic acid sequence containing the first two
105 monomers was used to design primers. The forward primer was forced to be chosen in the last 114
106 bp of the first monomer and the reverse primer in the first 114 bp of the second monomer, in order
107 to allow only amplification of ubiquitin tandem repeats (Figure 1). Potential primers were manually
108 checked for variation based on the multiple nucleic acid sequence alignment of all individual UBC
109 monomer sequences of the investigated mammals (Additional file 3). Optimal primers should
110 contain no or only a few variations, preferably at their 5'-end, and they should not be present in 5
111 consecutive monomers in order not to implicate amplification of fragments up to 1000 bp. Variation
112 outside these 5 consecutive monomers, in species with more than 5 tandem repeats, was ignored.
113 Finally, a primer pair in regions with the least variation was ordered, with degenerate nucleotides
114 where variation occurred, aiming to generate amplicons of 137, 365, 593 and 821 bp (Figure 1;
115 Additional file 1.A).

116 A similar strategy was followed to design the UBC bisulfite integrity assay, aiming to generate
117 amplicons of 105, 333, 561 and 789 bp on the bisulfite treated UBC DNA sense strand (Additional
118 file 1.A).

119 **Validation of the UBC integrity assay**

120 Gradient PCR showed that all desired amplicons were generated on 100 ng high quality porcine
121 DNA using a wide annealing temperature (T_a) range (68°C was chosen as optimal T_a ; Additional
122 File 5.A). The optimized assay was used to check its sensitivity by performing the assay on a 1/10
123 serial dilution series from 100 ng down to 100 fg of high quality porcine DNA. Agarose gel analysis

124 shows that all desired amplicons were generated down to 10 pg of template (equivalent to DNA
125 from 2 cells). One pg input resulted in the amplification of only the smaller bands, while 100 fg
126 resulted in no amplification products (Figure 2.A). Species specificity was confirmed with 100 ng
127 of high quality DNA isolated from all the investigated mammals as a template (Figure 2.B) and
128 tissue specificity was confirmed with cDNA (equivalent to 10 ng RNA) from jejunum, heart,
129 mucosa, liver, brain, kidney, neutrophil and lymph node (Figure 2.D).

130 In order to evaluate if decreasing quality of nucleic acids results in a decrease of both the number
131 and the concentration of the amplicons starting with the longer fragments, the assay was performed
132 on high quality DNA gradually degraded with DNase (Figure 2.C) and on cDNA reverse transcribed
133 from RNA with different degradation levels (Figure 2.D). Surprisingly, for both degraded DNA and
134 RNA, high amplifiability was observed, showing that even heavily degraded nucleic acids will still
135 be useful for a lot of applications.

136 The assay was found to be robust since it was also successfully applied to evaluate DNA from
137 equine vaginal swabs, DNA from faeces from an unknown origin, circulating DNA from equine
138 plasma (cfDNA), contaminating DNA in porcine RNA samples (minus RT control PCR) and cDNA
139 from bovine embryos, and performed by different scientists with different polymerases on different
140 PCR thermal cyclers (data will be published elsewhere).

141 **Validation of the UBC bisulfite integrity assay**

142 DNA was isolated from 8 biological replicates of bovine blood neutrophils. The UBC integrity
143 assay showed that the DNA was of high quality because all desired amplicons were generated
144 (Figure 3.A). After bisulfite treatment, known to damage DNA severely, these samples were used to
145 validate the UBC bisulfite integrity assay. Gradient PCR showed that a Ta range of 52-56°C gave
146 the best results (54°C was chosen as optimal Ta; Additional file 5.B). The optimized assay was
147 performed on the 8 biological replicates. Surprisingly, not only the 2 lower fragments of 105 and

148 333 bp were amplified, but also longer amplicons, although less intense (Figure 3.B). This finding
149 suggest that amplicons longer than the recommended 300 bp could be used for bisulfite sequencing.
150 To verify this, 2 bovine specific assays for bisulfite sequencing, amplifying 442 bp of MPO and 462
151 bp of SOD2, were performed on the bisulfite treated DNA of the 8 biological replicates. Agarose gel
152 analysis showed that both amplicons were successfully amplified on all samples (Figure 3.C-D).

153 **CONCLUSIONS**

154 We conclude that UBC gene features are extremely suited to develop single, cheap and multi-use
155 PCR-based assays to estimate the presence, integrity and amplificability of DNA (isolated from a
156 variety of sample types/qualities from different species) and cDNA (reverse transcribed from RNA
157 isolated from any tissue type, reflecting the RNA integrity and taking into account the efficacy of
158 the reverse transcription). In this paper we describe a single assay useful to investigate native
159 nucleic acids from all tissues of the most investigated mammal species. Surprisingly, the PCR
160 amplificability was a lot higher than expected from electrophoresis-based integrity assay results,
161 what could be of great help for validating degraded samples for (non-)quantification applications.
162 This assay can also be used to check the level of DNA contamination in RNA samples (minus RT
163 control PCR) or to check if an unknown sample contains useful mammalian DNA for further
164 investigation (forensics, species identification). We strongly believe that this assay will prove its
165 usefulness and will contribute to the improvement of quality control strategies. Laboratories that
166 need to check PCR amplificability might directly benefit from this single integrity assay (instead of
167 using a battery of more specific integrity assays) or apply the same strategy to design similar multi-
168 use assays, as we did for estimating the integrity of bisulfite treated DNA from the most
169 investigated mammal species.

170

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174

175 **CONFLICT OF INTEREST**

176 The authors declare no conflict of interest

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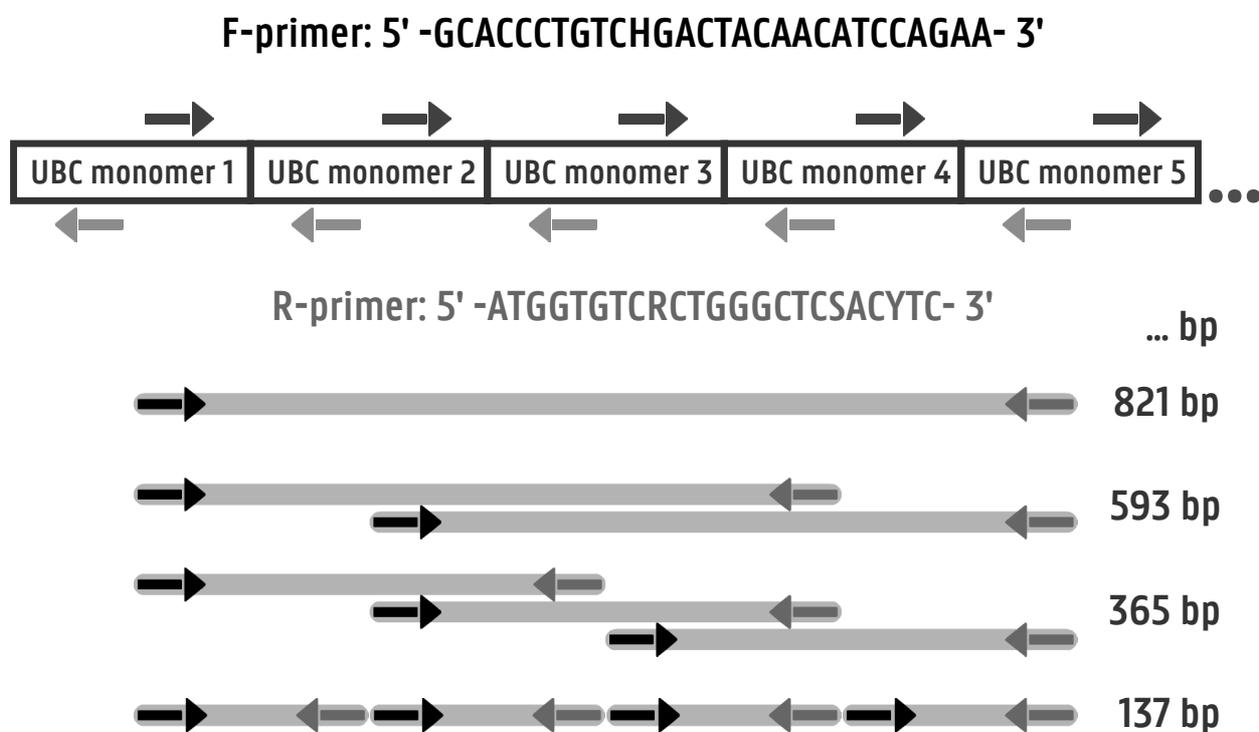
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207 **TITLES AND LEGENDS TO FIGURES**

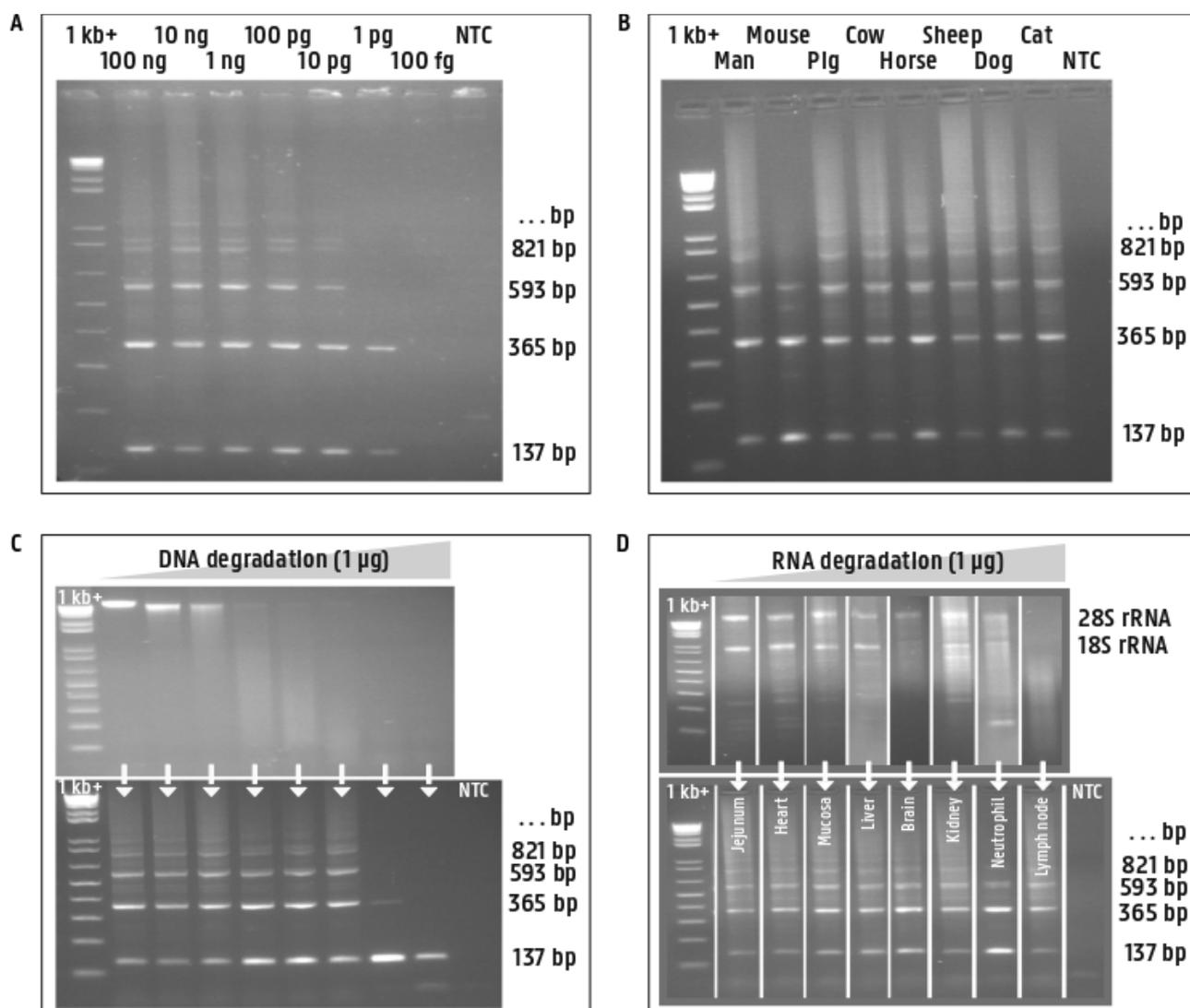
208

209 **Figure 1** UBC integrity assay design strategy, showing primer sequences and amplicon lengths.



211

212 **Figure 2** Agarose gels showing amplicons generated with the UBC integrity assay on (A) a 1/10
213 serial dilution series of porcine DNA, (B) 100 ng DNA isolated from 8 mammal species, (C) 10 ng
214 porcine DNA gradually degraded with DNase (1 μ g gradually degraded DNA is analysed in the
215 upper part, the assay on the corresponding samples in the lower part) and (D) cDNA reverse
216 transcribed from RNA from 8 different tissues with different quality (1 μ g RNA is analysed in the
217 upper part, the assay on cDNA from the corresponding RNA in the lower part).



219

220 **Figure 3** Agarose gels showing amplicons generated with (A) the UBC integrity assay on native
221 DNA isolated from 8 biological replicates of bovine blood neutrophils, and with (B) the UBC
222 bisulfite integrity assay, (C) the *Btau*MPO assay and (D) the *Btau*SOD assay on the corresponding
223 DNA samples after bisulfite treatment.

