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Establishment and quality evaluation of a glioma biobank in Beijing Tiantan Hospital

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Background: We established a glioma biobank at Beijing Tiantan Hospital in November, 2010. Specialized staffs have been trained to collect, store and manage the biobank in accordance with standard operating procedures.

Methods: One hundred samples were selected to evaluate the quality of glioma samples stored in the liquid nitrogen tank during different periods (from 2011 to 2015) by morphological examination, RNA integrity determination, DNA integrity determination, housekeeping gene expression and protein integrity determination.

Results: The majority of samples (95%) remain high RNA quality for further analysis with $RIN \geq 6$. All samples remain high DNA and protein quality without significant degradation.

Conclusion: Storage conditions of our biobank are suitable for long-term (at least 5 years) sample preservation with high molecular quality.

1 Establishment and quality evaluation of a glioma biobank in 2 Beijing Tiantan Hospital

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17 Abstract :

18 **Background:** We established a glioma biobank at Beijing Tiantan Hospital in November,
19 2010. Specialized staffs have been trained to collect, store and manage the biobank in accordance
20 with standard operating procedures.

21 **Methods:** One hundred samples were selected to evaluate the quality of glioma samples stored
22 in the liquid nitrogen tank during different periods (from 2011 to 2015) by morphological
23 examination, RNA integrity determination, DNA integrity determination, housekeeping gene
24 expression and protein integrity determination.

25 **Results:** The majority of samples (95%) had high RNA quality for further analysis with
26 $RIN \geq 6$. Quality of DNA and protein of all samples remained stable without significant
27 degradation.

28 **Conclusion:** Storage conditions of our biobank are suitable for long-term (at least 5 years)
29 sample preservation with high molecular quality.

30 **Introduction**

31 Gliomas refer to a group of tumors that arise from the glial tissue of the central nervous
32 system. Basically, they can occur anywhere in the central nervous system, both brain and spine.
33 Gliomas are the most commonly occurring tumors of the central nervous system which make up
34 about 27% of all brain tumors and 80% of all malignant primary brain tumors (Agnihotri et
35 al.,2013; Wilson, Karajannis&Harter,2014; Aldape et al.,2015). The incidence rate of glioma is
36 approximately 4.67-5.73 per 100000 persons (Ostrom et al.,2014). Glioblastoma (GBM) is the
37 most malignant type of gliomas. The median survival time of patients with GBM is 12–15
38 months (Bryukhovetskiy et al.,2016).

39 Tumor biobanks aim to systematically collect, store, manage and utilize tumor tissues from
40 surgical removal , blood samples and also the corresponding participant data for further use of
41 scientific projects and more specifically, for clinical and translational research (Watson,
42 Kay&Smith,2010; Kang et al.,2013). Relying on the neurosurgery discipline, a well-managed and
43 normative glioma biobank was established in our hospital in November, 2010. Using
44 morphological examination, RNA integrity determination, DNA integrity determination,
45 housekeeping gene expression and protein integrity determination, the quality of the glioma
46 tissues stored in the liquid nitrogen tank for 1-6 years (collected from 2011 to 2015) was
47 evaluated in order to provide high quality samples for medical research.

48 **Materials and methods**

49 *Laboratory equipment*

50 The lab is equipped with -80°C freezers, -150°C freezers and liquid nitrogen tanks. Each freezer
51 has a specialized temperature monitoring system to detect real-time temperature. When the
52 temperature inside freezers is abnormal, alarm messages will be sent to biobank managers.
53 Central air-conditioning system keeps the room temperature around 20°C.

54 ***Collection and storage of tumor tissues***

55 Excised glioma tissues from patients were collected immediately by trained and experienced
56 staffs as soon as tumor samples were removed from the body during neurosurgery. Consent to use
57 resected tissues was obtained from all cancer patients prior to surgery. This study was approved
58 by the Ethics Committee of Beijing Tiantan Hospital (KY2014-021-02). On the basis of
59 pathological diagnosis, the remaining tissue samples were gathered. Blood and necrotic tissue on
60 the surface of the samples were washed off with pre-cooling PBS. Then the tumor samples were
61 cut into small pieces (about 0.5 cm x 0.5 cm, thickness < 0.5 cm) under aseptic conditions,
62 repackaged in cryotubes and quickly frozen in liquid nitrogen tank in the operating room for
63 temporary storage. All samples were frozen within 30 min after resection (Tonje et al.,2016). On
64 the same day, they were transferred to liquid nitrogen tanks in the biobank for long-term
65 preservation. Registration of samples were done at the same time. Clinical information of patients
66 including name, gender, age, pathological diagnosis, clinical diagnosis and treatment were all
67 recorded and stored in the database.

68 ***Morphology characteristics***

69 One hundred samples were selected to conduct the following experiments from 2011 to 2015.
70 For each year, twenty samples were selected randomly. Firstly, the tissue fragments were fixed
71 in 10% formalin before thawed for 24 hours then embedded in paraffin. Next, five-micrometer
72 tissue sections were cut, dewaxed and stained with Hematoxylin–Eosin (HE) in accordance with
73 standard procedures (Iigen et al.,2014).The slides were observed by an experienced pathologist.
74 Tumor samples which were comprised of $\geq 80\%$ tumor nuclei and $\leq 20\%$ necrosis ($\leq 50\%$
75 necrosis for GBM) were considered fit for other research.

76 ***RNA isolation***

77 Total RNA was isolated using TRIZOL reagent (Invitrogen, Stockholm, Sweden). 1ml
78 TRIZOL reagent was added into an RNase-free tube. 100mg frozen tissue of each sample was
79 then removed into the tube and homogenized using RNase-free pestles. The homogenized

80 tissue was incubated in TRIZOL reagent for 10 minutes at room temperature. After those, 200ul
81 chloroform was added into each tube, and the tubes were manually shaken for 15 seconds before
82 placed at room temperature for 10 minutes. The tubes were centrifuged at 12,000rpm for 15
83 minutes at 4°C, after which the aqueous phase was transferred to a new RNase-free tube.
84 Afterwards, 500ul isopropyl alcohol was added into each tube and incubated at -20°C for 20
85 minutes. The tubes were centrifuged at 12,000rpm for 10 minutes at 4°C. The supernatant was
86 discarded, and 1ml 75% ethanol was added into each tube to wash RNA pellet. Finally, the tubes
87 were centrifuged at 7500g for 10 minutes. The supernatant was discarded, and the RNA pellet
88 was air-dried and subsequently dissolved into 30ul RNase-free water.

89 ***RNA yield and integrity determination***

90 A Nanodrop spectrophotometer (Thermo Fisher Scientific) was used to determine
91 concentrations (ng/ul) and purity of RNA samples. The A260/A280 ratio was measured to
92 indicate RNA purity. The ratio of samples with high purity is 1.8~2.1 (Sanabria et al.,2014). An
93 Agilent 2100 bioanalyzer in conjunction with the RNA 6000 Nano and the RNA 6000 Pico
94 LabChip kits (Agilent Biotechnologies, Palo Alto, CA) was used to evaluate RNA integrity,
95 from which RNA integrity number (RIN) was calculated. A scoring system between 1 and 10
96 were used in the RIN software, with 1 representing degraded RNA and 10 representing very high-
97 quality, intact RNA (Griffin et al.,2012). In literature, samples were divided into four quality
98 groups according to RIN: RIN<5, not reliable for demanding downstream analysis; 5≤RIN<6,
99 suitable for quantitative reverse transcription-PCR (RT-qPCR); 6≤RIN<8, suitable for gene array
100 analysis; and RIN>8, suitable for all downstream techniques (Kap et al.,2014). The assays were
101 performed according to the manufacturer's instructions.

102 ***cDNA synthesis and real-time quantitative PCR (RT-qPCR) for housekeeping genes***

103 Extracted RNA (1ug) was reverse-transcribed to first-strand cDNA using the PrimeScript RT
104 reagent Kit With gDNA Eraser (TaKaRa). Afterwards, 2ul cDNA solution was used for a 40-
105 cycle SYBR Green PCR assay with the SYBR Premix EX Taq reagent (TaKaRa). The same

106 sample was run three different times in the same experiment to remove any outliers. Primer sets
107 were as follows: human ACTB: forward primer 5'-TTAGTTGCGTTACACCCTTTCTTG -3';
108 reverse primer 5'- GTCACCTTCACCGTTCCAGTTTT-3'; human GAPDH: forward primer 5'-
109 CTATAAATTGAGCCCGCAGCC-3'; reverse primer 5'-GCGCCCAATACGACCAAATC-3',
110 The Thermal Cycler DiceReal Time System (TaKaRa) was used for qRT-PCR under the
111 following conditions: 95°C for 30s, 95°C for 5s , 58°C for 30 s , 72°C for 30 s.

112 ***DNA isolation***

113 100mg frozen tissue of each sample was pulverized to isolate genome DNA. DNA isolation
114 was accomplished using the EasyPure Genomic DNA kit (Transgen Biotech) according to the
115 manufacturer's instructions. In the end, the DNA pallet was dissolved into 50ul ddwater(PH>8.0).

116 ***DNA yield and PCR for housekeeping genes***

117 A Nanodrop spectrophotometer (Thermo Fisher Scientific) was used to determine concentrations
118 (ng/ul) and the A260/A280 of DNA samples. After that, 50ng genomic DNA was used for a 40-
119 cycle SYBR Green PCR assay with the SYBR Premix EX TaqII reagent (TaKaRa). Each
120 measurement was performed in triplicate to remove any outliers. Primer sets were as follows:
121 human ACTB: forward primer 5'-AAGACCTGTACGCCAACACA-3'; reverse primer 5'-
122 CTGGATGTGACAGCTCCCC-3'; the primer set of human GAPDH was the same as mentioned
123 before.

124 ***β-Globin PCR amplification***

125 Four different length fragments of the housekeeping gene β-globin were amplified to evaluate
126 DNA quality. The maximum amplicon size positively correlates with DNA quality. PCR
127 amplification was performed with 50 ng of tissue DNA using DNA Polymerase High Fidelity
128 (TransTaq). The primers are shown in Table 1. PCR was performed using the following
129 conditions: primerI : initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturing
130 at 94°C for 30s, annealing at 56°C for 30s, extension at 72°C for 30s, and final extension at 72°C
131 for 5 min ; primerII : initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturing
132 at 94°C for 30s, annealing at 52°C for 30s, extension at 72°C for 30s, and final extension at 72°C

133 for 5 min ; primer III = initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturing
134 at 94°C for 30s, annealing at 52°C for 30s, extension at 72°C for 1 min, and final extension at
135 72°C for 5 min ; primer IV = initial denaturation at 94°C for 5 min, followed by 35 cycles of
136 denaturing at 94°C for 30s, annealing at 54°C for 30s, extension at 72°C for 1min, and final
137 extension at 72°C for 5 min. PCR products were analyzed on agarose gels.

138 ***Protein isolation and western blot***

139 100mg frozen tissue of each sample was pulverized to isolate tissue protein. First, the
140 powdered tissue was incubated in 500ul RIPA (Applygen) containing 50x protease inhibitor
141 (Applygen) on the ice for at least 15 minutes. Afterward, the solution was centrifuged at
142 12000rpm for 15 minutes at 4°C. Then, the supernatant was decanted to a new fresh tube, which
143 was the final protein solution we needed. The protein concentration was measured by BCA assay
144 (Applygen) according to the manufacturer's instructions. Electrophoresis was performed using
145 12% acrylamide gel, 20ug protein was run from each sample on the gel and proteins were
146 transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) .The membranes were
147 blocked with 5% non-fatty milk in PBS containing 0.1% of Tween-20 for one hour and incubated
148 with monoclonal anti-ACTB antibody (Tansgen) overnight. Then the membranes were washed
149 thrice for 5 minutes before addition of HRP conjugated anti-mouse antibody for 1 hour.
150 Membranes were then washed and treated with ECL Western Blotting Substrate (Fisher
151 Scientific) according to manufacturer's instructions.

152 ***Statistical analysis***

153 Statistical differences were analyzed using one-way ANOVA test. The criterion for significance
154 was set at P value < 0.05 .

155 **Results**

156 ***Composition of the biobank***

157 The total number of cancer cases reached 3686 from 2010 to 2016. 3,686 patients were
158 consented and tissue was collected from each patient. Fig.1 showed the constituent ratio of

159 glioma subtypes. The vast majority subtypes of the biobank were oligo-astrocytoma, astrocytoma
160 and glioblastoma.

161 ***Morphology characteristics***

162 According to the HE staining results, all tumor samples were comprised of $\geq 80\%$ tumor
163 nuclei and $\leq 20\%$ necrosis ($\leq 50\%$ necrosis for GBM). Fig.2 shows the representative images
164 from the 100 samples. No significant tissue decomposition was detected in all selected samples.

165 ***RNA integrity of different storage duration***

166 The majority of RNA samples had a A260/A280 ratio ranging between 1.8 and 2.1 which
167 indicate high purity (Fig.3). The RIN results of the selected 100 samples showed that 95%
168 samples in the biobank were suitable for RT-qPCR and gene array analysis with $RIN \geq 6$ (Fig.4).
169 Besides, no significant change in the RNA quality was found among different storage periods
170 (from 2011 to 2015).

171 ***RT -qPCR for Housekeeping genes***

172 RT -qPCR for housekeeping genes (GAPDH , ACTB) was performed to verify the accuracy of
173 RIN determination. Except for the 5% samples with $RIN < 6$, of which the ct values were
174 relatively higher, gene expression levels of all the other 95% samples remained stable under
175 different storage periods (Table 2 and Fig.5). No significant difference was found.

176 ***DNA integrity of different storage durations***

177 The A260/A280 and PCR for genomic housekeeping genes (GAPDH , ACTB) were
178 performed to evaluate DNA degradation level. DNA samples had a A260/A280 ratio ranging
179 between 1.8 and 2.0 with the exception of two samples showed ratios of 2.05 and 2.07 (Fig.6),
180 suggesting contamination during the phenol extraction. According to the PCR results, all the ct
181 values were stable of different storage durations, no significant difference was found (Fig.7).
182 DNA quality was assessed also by PCR amplification of β -globin gene fragments. The integrity
183 of DNA was scored according to the number of amplified bands of increasing size from 1 to 4.
184 According to our results, 100% of extracted DNA samples were of good quality with at least

185 three amplified β -globin bands of increasing size. No significant difference was found in DNA
186 quality of different storage durations.

187 *Western blot*

188 Western blot of ACTB was performed to predict tissue protein quality. Western blot analyses
189 revealed that all the samples were enriched for ACTB (Fig.8). No significant protein degradation
190 was found.

191 **Discussion**

192 Tumor biobanks aim to collect and store sufficient number of tumor samples with high quality
193 for basic cancer research .Clinical and basic researchers use these samples to carry out molecular
194 biology, cell biology, genetics, transcriptomics, genomics and proteomics research in order to
195 explore new standard of tumor classification, diagnosis, treatment and prognosis. Prolonged
196 storage periods of tumor samples allows researchers to design studies to identify biomarkers of
197 aggressiveness and responses to different drug treatments which increases their value
198 (Hewitt,2011; Olivieri et al.,2014). Thus, establishment of tumor biobanks with high quality
199 samples plays an important role in personal medicine and translational research.

200 Compared with other moluculers, the RNA molecule is less stable and more likely to degrade
201 by RNases because its ribonucleotides contain a free hydroxyl group in the pentose ring (Zhang
202 et al.,2016). However, in our study, we didn't find significant RNA degradation in samples
203 preserved in the liquid nitrogen tank (-196°C) for 1-6 years (from 2011 to 2015). Except for few
204 samples (5%) with $RIN < 6$, the majority of them (95%) remain high quality for further analysis
205 with $RIN \geq 6$. We verified the accuracy of RIN determination by RT-qPCR of housekeeping genes
206 (GAPDH , ACTB). The number of cycles required to reach a detectable threshold level for
207 fluorescence is defined as ct. Ct is inversely correlated with the amount of template RNA (Riemer
208 et al.,2012). Thus, RNA samples with high quality have a relatively lower ct value whereas RNA
209 samples with degradation have a relatively higher ct value of housekeeping genes. Our results of
210 RT-qPCR were in accordance with RIN determination. All the 95% samples with $RIN \geq 6$ have

211 stable ct values of housekeeping genes (ACTB: $14.32 \pm 0.47 \sim 14.70 \pm 0.65$; GAPDH: 21.11 ± 0.75
212 $\sim 22.17 \pm 1.01$). Our findings were consistent with those of Andreasson et al. (2013), who
213 assessed RIN values in endocrine tissues stored at -80°C for approximately 30 years and found
214 that long-term storage in -80°C did not adversely affect the quality of the RNA extracted from
215 the tissues. In our study, we found that glioma tissues preserved in liquid nitrogen (-196°C) could
216 well maintain RNA quality for at least 5 years. There are also other studies showed similar results
217 such as Hebel et al.(2013)detected RIN values in blood samples stored at -80°C for 4–19 years
218 and at -196°C for 11–19 years and found no adversely correlation between RNA quality and
219 storage duration.DNA and protein are more stable than RNA. QPCR reactions of genomic
220 housekeeping genes (GAPDH , ACTB) showed no signs of DNA degradation as the ct values
221 were stable (ACTB: $22.63 \pm 0.63 \sim 23.06 \pm 0.57$; GAPDH: $32.77 \pm 0.63 \sim 33.32 \pm 1.50$). β -Globin
222 PCR amplification showed that 100% of extracted DNA samples were of good quality with at
223 least three amplified β -globin bands of increasing size. Since there is not a standard method to
224 evaluate tissue protein quality, we just evaluated one housekeeping protein through western blot
225 to roughly predict protein quality. Protein degradation was not found from ACTB western blot
226 analysis.

227 According to our research, storage conditions of our biobank are suitable for long-term (at least
228 5 years) sample preservation with high molecular quality. However, there are also limitations of
229 our research. Heterogeneity has been found in various human tumors and glioblastoma (GBM) is
230 a highly heterogeneous tumor (Diaz-Cano,2012; Furnari et al.,2015). In our research, we only
231 studied the tumor nuclei and necrosis proportion, but we did not study whether the pathological
232 type of samples preserved in our biobank is consistent with the previous pathological diagnosis.
233 About protein quality, we only studied one protein, but it is far from suggesting all proteins are of
234 good quality. We only studied gliomas and are not sure whether the other tumors have similar
235 quality results. Moreover, our biobank established only for a short period (presently up to 6
236 years), we should further verify the quality over time to find optimal storage periods.

237 **Conclusion**

238 Storage conditions of our biobank are suitable for long-term (at least 5 years) sample
239 preservation with high molecular quality.

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242 helping with the collection of tissue samples.

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Table 1 (on next page)

Primers used for β -globin gene amplification by PCR

Amplicon	Forward primer	Reverse primer	Size (bp)
I	GAAGAGCCAAGGACAGGTAC	CAACTTCATCCACG TTCACC	268
II	GCTCACTCAGTGTGGCAAAG	GGTTGGCCAATCTACTCCCA GG	536
III	ATTTCCACCCCTTAGGCTG	TGGTAGCTGGATTGTAGCTG	989
IV	GGTTGGCCAATCTACTCCCAGG	TGGTAGCTGGATTGTAGCTG	1327

Table 2 (on next page)

Gene expression levels under different storage durations

Year	gene	Ct values (Average \pm SD) of samples with		
		RIN < 5	5 \leq RIN<6	RIN \geq 6
2011	ACTB	16.17 \pm 0.14		14.50 \pm 0.41
	GAPDH	23.21 \pm 0.28		21.13 \pm 0.85
2012	ACTB	18.26 \pm 0.00		14.39 \pm 0.66
	GAPDH	26.10 \pm 0.00		21.69 \pm 1.00
2013	ACTB	15.79 \pm 0.00	15.11 \pm 0	14.40 \pm 0.58
	GAPDH	23.24 \pm 0.00	22.10 \pm 0	21.11 \pm 0.75
2014	ACTB			14.70 \pm 0.65
	GAPDH			22.17 \pm 1.01
2015	ACTB			14.32 \pm 0.47
	GAPDH			21.62 \pm 0.66

Figure 1

Constituent ratio of glioma subtypes

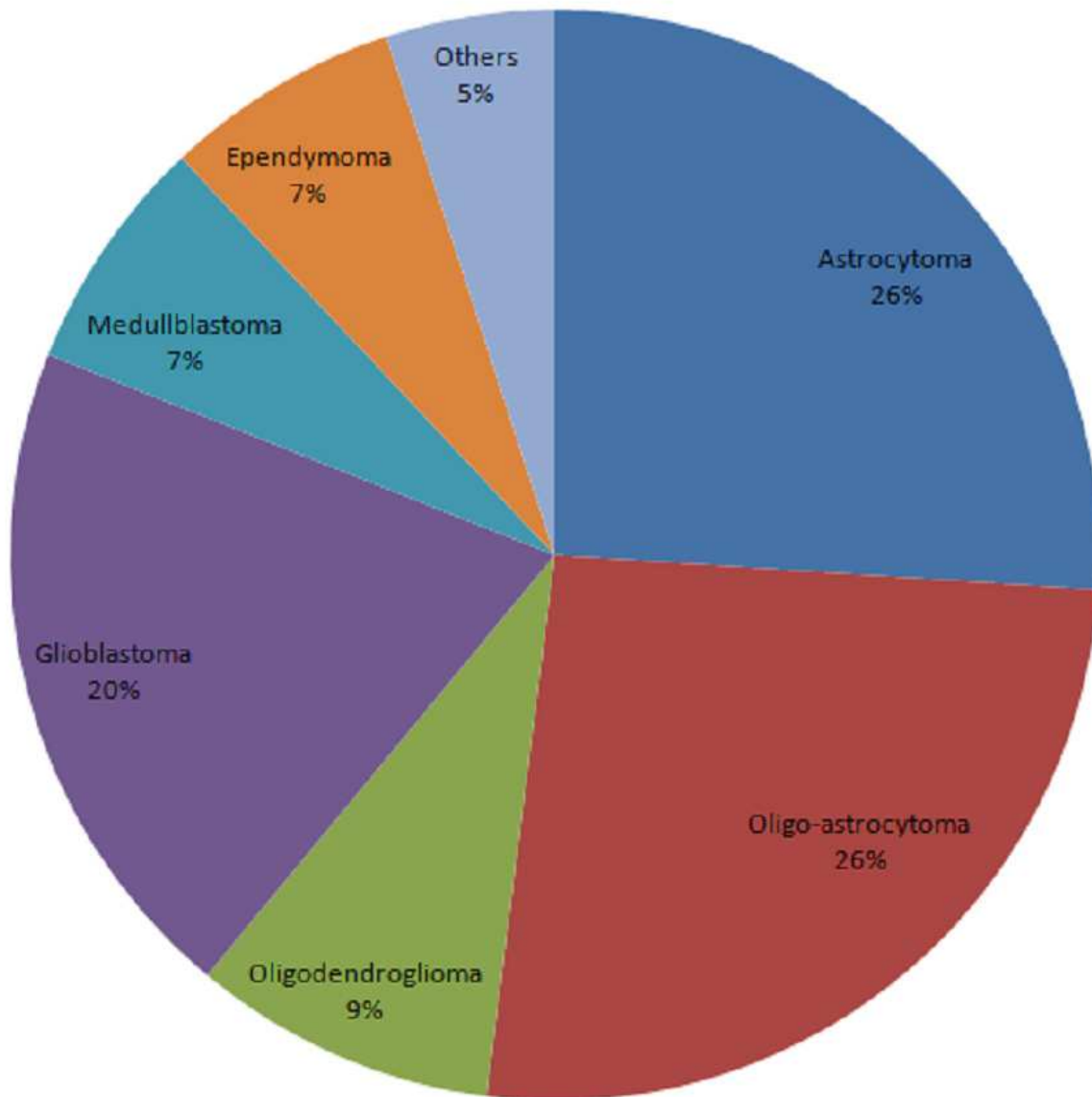


Figure 2

Morphology of glioma tissues preserved in liquid nitrogen of different storage periods (from 2011 to 2015)

(A) Morphology of glioma tissues preserved in liquid nitrogen in 2011. (B) Morphology of glioma tissues preserved in liquid nitrogen in 2012. (C) Morphology of glioma tissues preserved in liquid nitrogen in 2013. (D) Morphology of glioma tissues preserved in liquid nitrogen in 2014. (E) Morphology of glioma tissues preserved in liquid nitrogen in 2015.

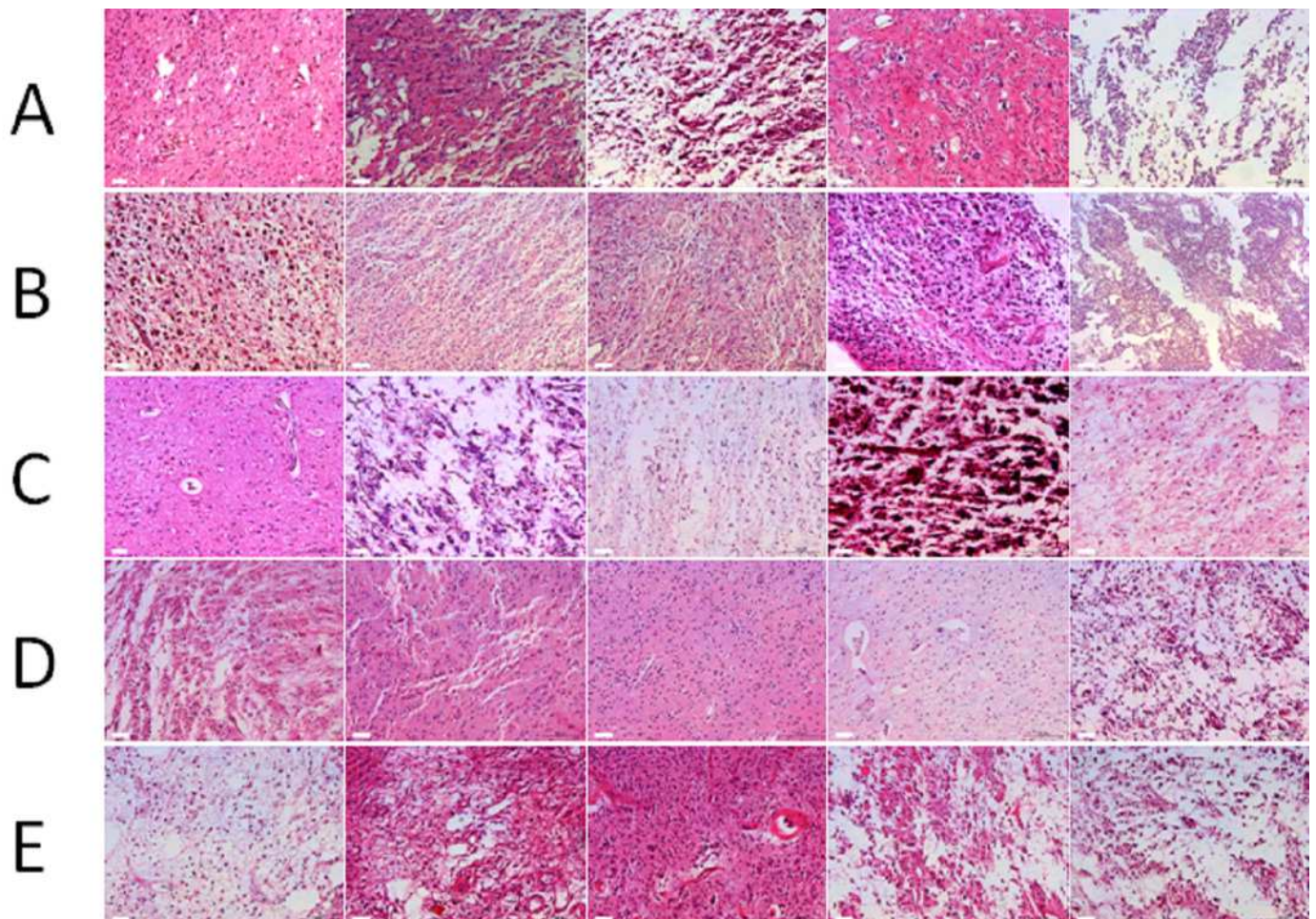


Figure 3

Ratio 260/280 of RNA samples

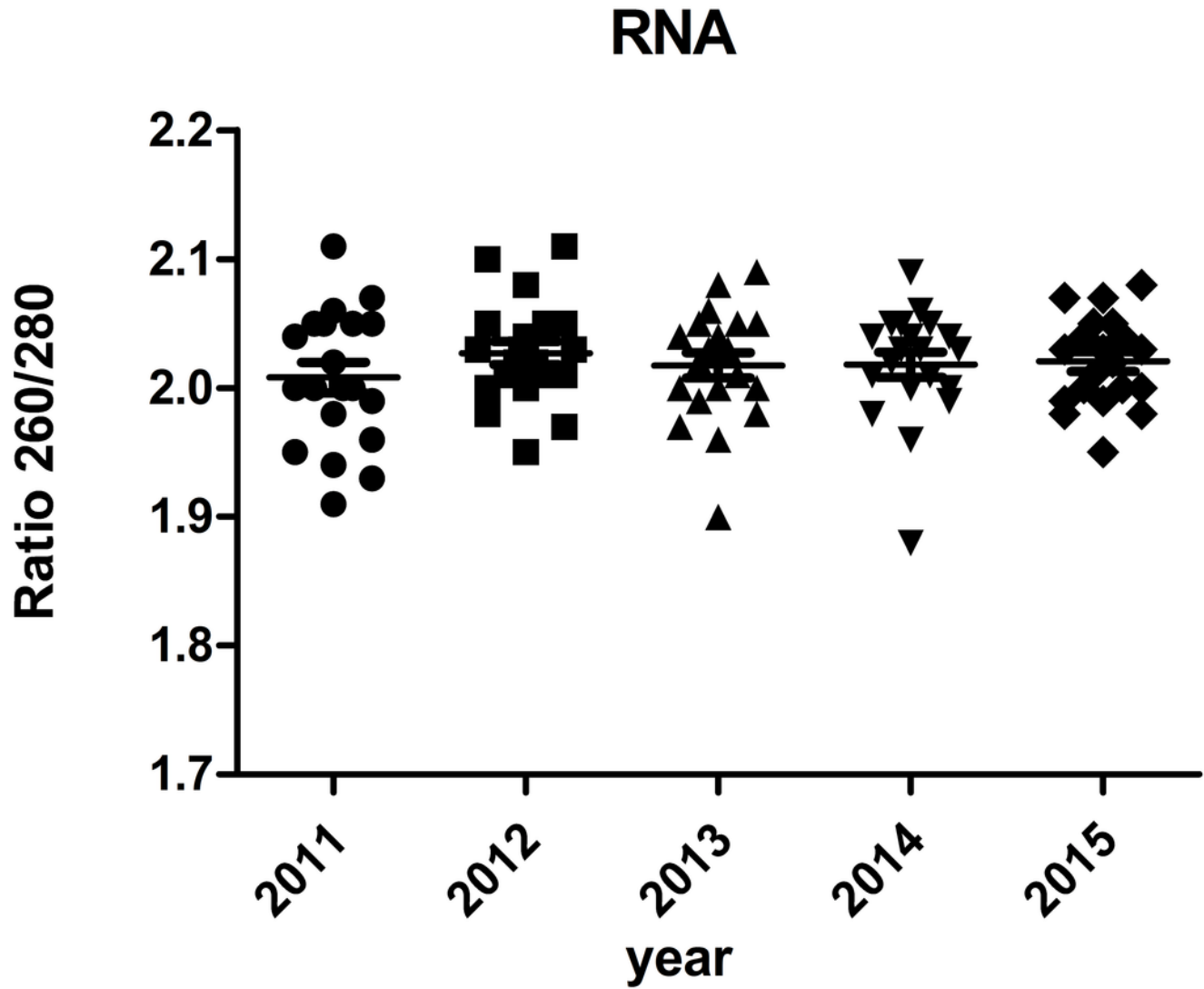


Figure 4

RNA integrity number of selected samples of different storage periods

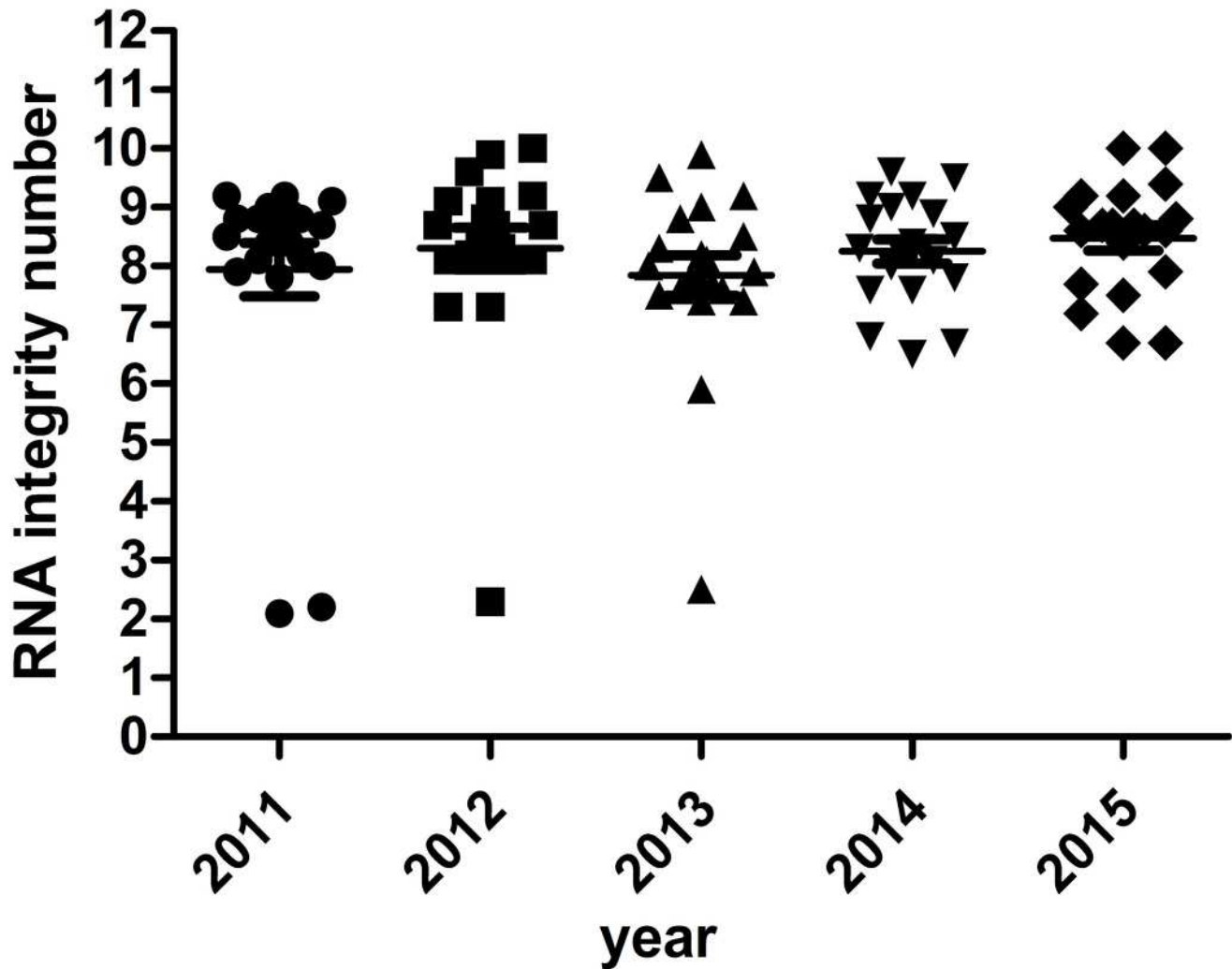


Figure 5

Gene expression levels under different storage durations

(A) Gene expression levels of ACTB; (B) Gene expression levels of GAPDH.

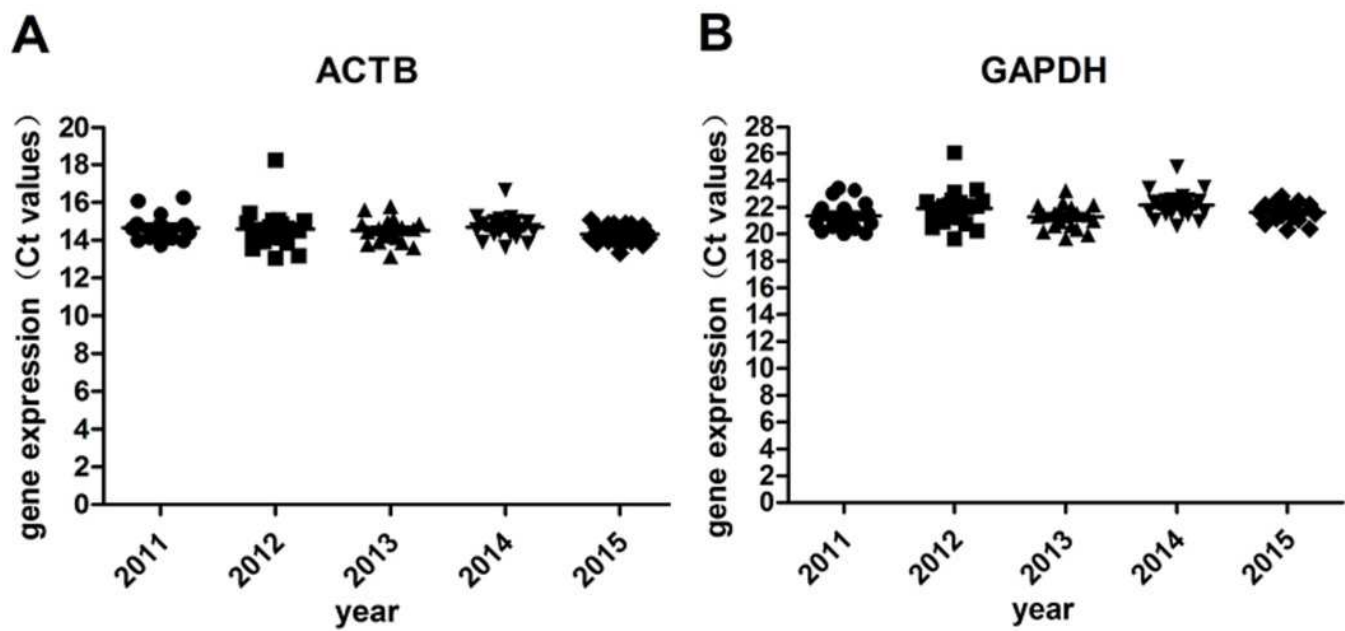


Figure 6

Ratio 260/280 of DNA samples

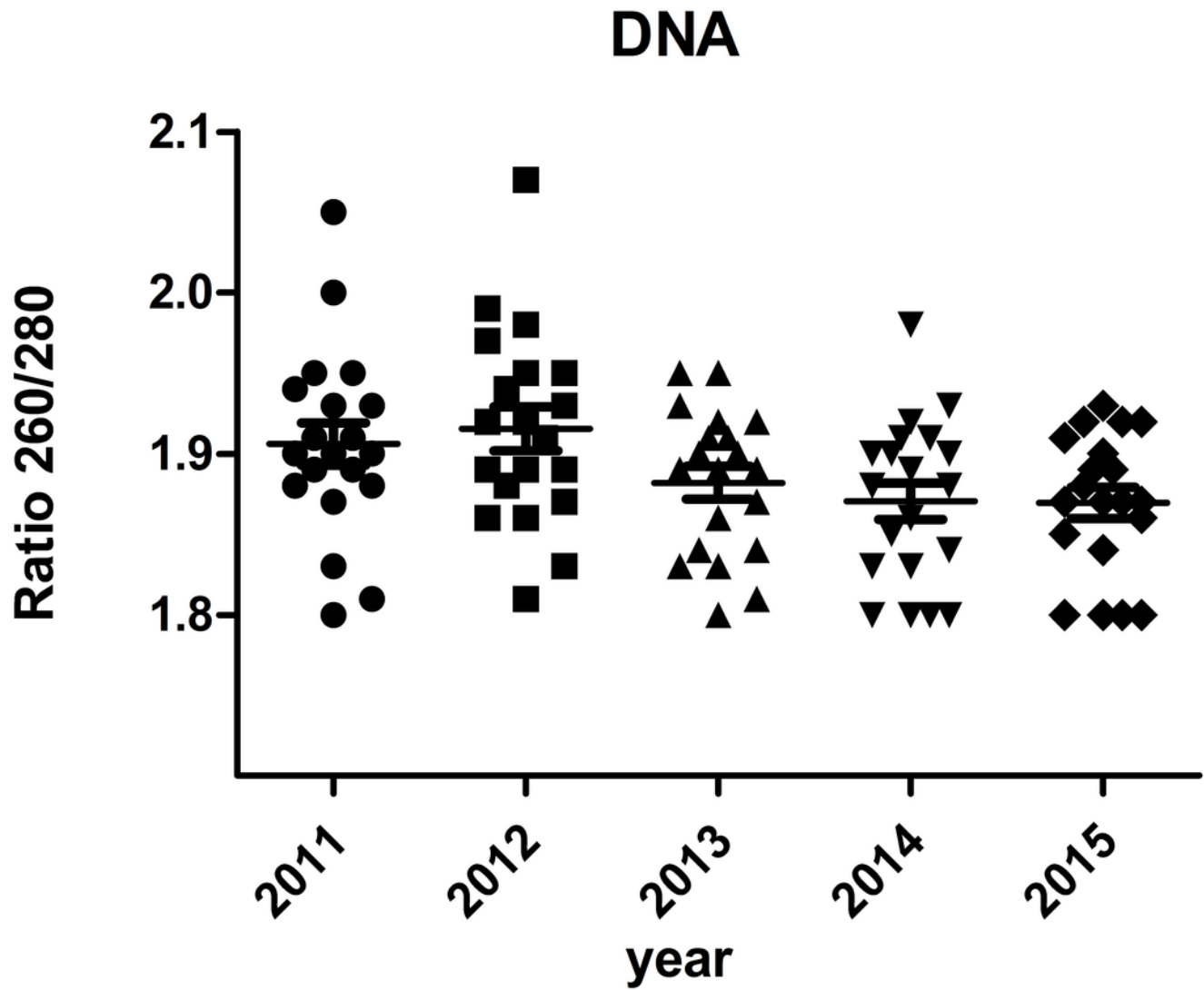


Figure 7

Ct values of genomic housekeeping genes under different storage durations

(A) Ct values of ACTB. (B) Ct values of GAPDH.

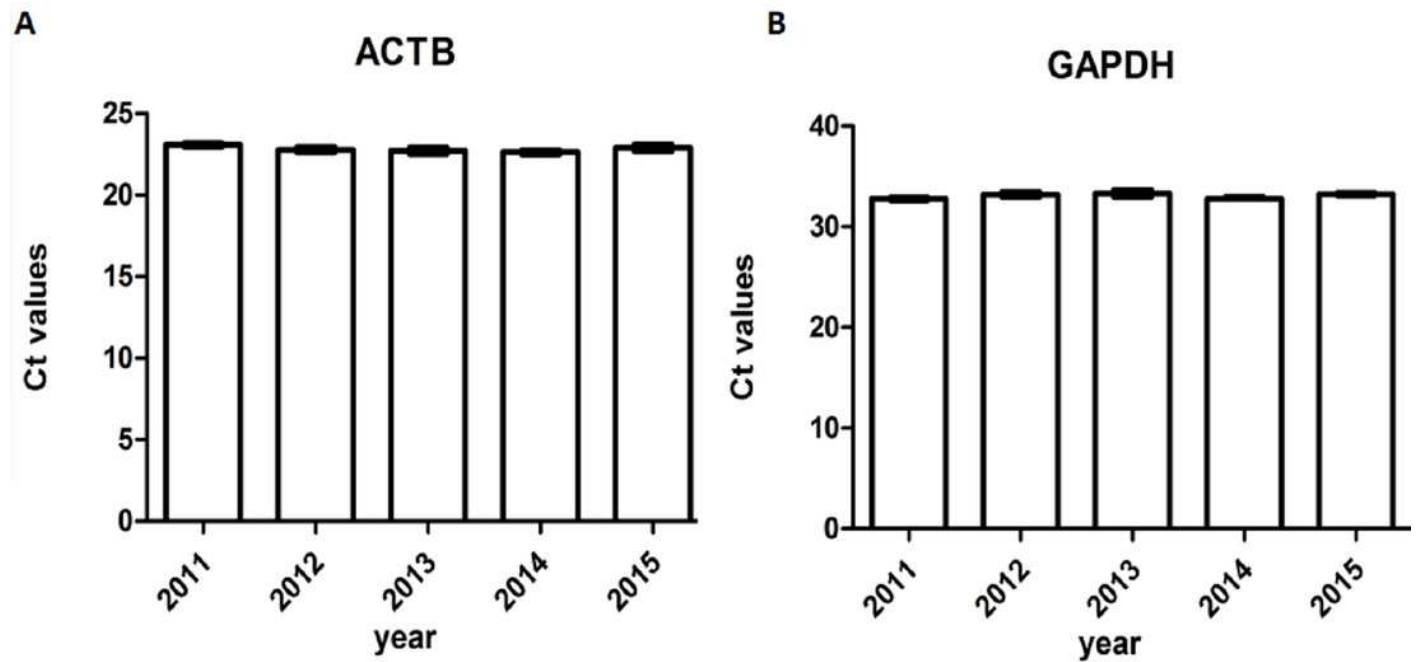


Figure 8

Western blot analysis of ACTB of selected samples under different storage periods

(A) Western blot analysis of ACTB of selected samples in 2011. (B) Western blot analysis of ACTB of selected samples in 2012. (C) Western blot analysis of ACTB of selected samples in 2013. (D) Western blot analysis of ACTB of selected samples in 2014. (E) Western blot analysis of ACTB of selected samples in 2015.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

