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# Greater than X kb: A quantitative assessment of preservation conditions on genomic DNA quality, and a proposed standard for genome-quality DNA

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Advances in biodiversity genomic sequencing will increasingly depend on the availability of DNA samples—and their guantifiable metadata—preserved in large institutional biorepositories that are discoverable to the scientific community. Improvements in sequencing technology constantly provide longer reads, such that longer fragment length, higher molecular weight, and overall "genome-quality" DNA (gDNA) will be desirable. Ideally, biorepositories should publish numerical scale measurements of DNA quality useful to the user community. However, the most widely used technique to evaluate DNA quality, the classic agarose gel, has yet to be guantified. Here we propose a simple and economical method using open source image analysis software to make gDNA gel images quantifiable, and propose percentage of gDNA "greater than X kb" as a standard of comparison, where X is a band from any widely used DNA ladder with desirably large band sizes. We employ two metadata standards ("DNA Threshold" and "Percent above Threshold") introduced as part of the Global Genome Biodiversity Network (GGBN) Darwin Core extension. We illustrate the method using the traditionally used HindIII ladder and the 9,416 base-pair (bp) band as a standard. We also present data, for two taxa, a vertebrate (fish) and an invertebrate (crab), on how gDNA quality varies with seven tissue preservation methods, time since death, preservation method (i.e. buffers vs. cold temperatures), and storage temperature of various buffers over time. Our results suggest that putting tissue into a buffer prior to freezing may be better than directly into ultra-cold conditions.

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2	conditions on genomic DNA quality, and a proposed standard for
3	genome-quality DNA.
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#### Abstract

16 Advances in biodiversity genomic sequencing will increasingly depend on the availability 17 of DNA samples — and their quantifiable metadata — preserved in large institutional 18 biorepositories that are discoverable to the scientific community. Improvements in 19 sequencing technology constantly provide longer reads, such that longer fragment length, 20 higher molecular weight, and overall "genome-quality" DNA (gDNA) will be desirable. 21 Ideally, biorepositories should publish numerical scale measurements of DNA quality 22 useful to the user community. However, the most widely used technique to evaluate DNA 23 quality, the classic agarose gel, has yet to be quantified. Here we propose a simple and 24 economical method using open source image analysis software to make gDNA gel 25 images quantifiable, and propose percentage of gDNA "greater than X kb" as a standard 26 of comparison, where X is a band from any widely used DNA ladder with desirably large 27 band sizes. We employ two metadata standards ("DNA Threshold" and "Percent above 28 Threshold") introduced as part of the Global Genome Biodiversity Network (GGBN) 29 Darwin Core extension. We illustrate the method using the traditionally used *HindIII* 30 ladder and the 9,416 base-pair (bp) band as a standard. We also present data, for two taxa, 31 a vertebrate (fish) and an invertebrate (crab), on how gDNA quality varies with seven 32 tissue preservation methods, time since death, preservation method (i.e. buffers vs. cold 33 temperatures), and storage temperature of various buffers over time. Our results suggest 34 that putting tissue into a buffer prior to freezing may be better than directly into ultra-cold 35 conditions.

36 Subjects Biodiversity, Bioinformatics, Genomics, Molecular Biology, Zoology

37 Keywords Agarose gels, DNA extractions, Genomic DNA, Tissue preservation

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#### Introduction

39

40	Increasing the length of sequence reads is a core technological challenge in
41	genomic science. Currently, the most widely used bench top technologies can achieve
42	quality reads up to 150–500 bp in length (Glenn, 2011; Loman et al., 2012), but longer-
43	read sequencing technologies are increasingly feasible (Loman and Quinlan, 2014).
44	Therefore, access to high quality, high molecular weight DNA will become increasingly
45	crucial. It is now feasible, practical, and increasingly more common to sequence
46	complete genomes of non-model organisms (e.g. Pisani et al., 2015). As biodiversity
47	genomics expands to rarer, harder-to-access, or vanishing organisms, obtaining "genomic
48	quality" tissues—that provide high molecular weight DNA—becomes a significant
49	challenge.
50	Natural history museums and academic institutions are currently obtaining,
51	curating, and rapidly increasing biodiversity biobank collections (i.e. biorepositories), in
52	order to maintain genomic quality material of non-model organisms, and to make this
53	material available for scientific researchers conducting genomic analyses around the
54	globe (Droege et al., 2014). In turn, making massive tissue and DNA collections
55	discoverable is a priority for data aggregators, such as the Global Genome Biodiversity
56	Network (GGBN; <u>http://www.ggbn.org</u> ). GGBN is a network of institutions dedicated to
57	preserving genetic resources, but also to advancing the data model for tissues, DNAs,
58	RNAs, and similar resources and their standardizations (Droege et al., in press).
59	We present a simple, cost-effective agarose gel electrophoresis method for
60	qualitatively analyzing genomic DNA (gDNA) extractions (for genomic analyses) that

61	can provide a quantifiable value of gDNA. Our method requires only basic molecular
62	laboratory equipment (standard TBE gel rigs, UV imaging station, etc.); thus it does not
63	rely on any expensive reagents or more expensive analytical quantification equipment
64	(e.g. Spectrophotometers, Automated Electrophoresis Systems, Tape Stations, etc.).
65	Studies that explore the impact of tissue preservation on DNA quality often
66	measure DNA concentration or test whether particular loci will PCR-amplify and
67	sequence using traditional Sanger methods (e.g. Dawson et al., 1998; Vink et al., 2005;
68	Yodder et al. 2006; Erkens et al., 2008; Frampton et al., 2008; Gaither et al., 2011;
69	Moreau et al., 2013). However, these approaches are limited, as even fragmented DNA
70	may amplify and produce high quality Sanger sequencing products, particularly when
71	these products are in the size-range for Sanger methods [e.g. ~500–1000 base-pairs (bp)].
72	DNA concentration can be increased by adding more material (i.e. tissue) or combining
73	multiple extractions from the same material, and does not provide any information about
74	size. For genomic quality DNA, it is preferred to be mostly intact (whole chromosomes
75	and organelle genomes), particularly if the intention is to sequence entire genomes, as the
76	assembly of degraded gDNA (non-randomly) sheared gDNA prior to library preparation
77	can be problematic for most NextGen platforms (Chen et al., 2015). Therefore, in order
78	to assess the quality of their DNA, many researchers use agarose gels with high
79	molecular-weight DNA ladders to visualize size and quality (Williams, 2007; Gaither et
80	al., 2011), in addition to more sophisticated methods such as spectrophotometry,
81	flourometry, or automated electrophoresis methods.
82	Here, we propose a standardized, simple method for electrophoresing genomic
83	DNA on agarose gels with the standard $\lambda$ phage <i>Hin</i> dIII ladder. The size of the gDNA

84	can be assessed by comparison to any of six bands in the <i>Hin</i> dIII ladder (2,027 bp, 2,322
85	bp, 4,361 bp, 6,557 bp, 9,416 bp, and 23,130 bp), and from this comparison, the percent
86	of gDNA greater than a given band size can be calculated from a regular gel image. We
87	recommend this method to standardize quality assessment of tissues collected and
88	reporting by biorepositories and data aggregators such as GGBN. We suggest use of the
89	~"9 kb" (= 9,416 bp) size marker as a working standard, because it is substantially longer
90	than standard NextGen reads (e.g., Illumina, etc.), and given current technologies, would
91	be an appropriate minimum for long read sequencing. However, any size marker can be
92	used to quantify gDNA, hence the method is referred to as "greater than $X$ kb."
93	We present our method with a case study exploring preservative methods within
94	field collection workflows that yield DNA of sufficient quality and quantity suitable for
95	genomic sequencing. Within this case study, fresh, field collected tissue of a fish
96	(Morone americanus) and a crab (Callinectes sapidus) were used to test if DNA quality is
97	dependent on treatment and time until preservation. Alternative preservative solutions,
98	temperature, and time were used as variables. Immediate cryopreservation was used as a
99	benchmark for comparison as most researchers to date believe that freezing tissue at
100	ultra-cold temperatures, such as -80°C or -190°C (liquid nitrogen), is the best
101	preservative method for yielding genomic quality DNA.
102	We address four questions in this study: 1. Can DNA quality (in terms of
103	fragment length) be measured quickly, consistently, and economically? 2. How does
104	preservation method (buffers vs. temperature) affect DNA quality? 3. How does time
105	since death affect DNA quality? 4. How does storage temperature (in various buffers)
106	affect DNA quality?

107	
108	Materials & Methods
109	
110	Evaluating Preservation Methods 1.0
111	
112	Tissue Collection 1.1
113	Muscle tissue was collected from two species: Morone americanus (white perch,
114	hereafter "fish") and Callinectes sapidus (blue crab, hereafter "crab") at the Smithsonian
115	Environmental Research Center (SERC) in Edgewater, MD. The crabs were euthanized
116	using liquid nitrogen asphyxiation (held above liquid nitrogen, which depletes oxygen),
117	and the fish were euthanized with MS222, following our ACUC protocols. Individual
118	fish were filleted immediately after death. Fillets were cut into small strips (~0.5 cm x 5
119	cm) and immediately submerged in liquid nitrogen (LN $_2$ ). Individual crabs were
120	dismembered upon death and claws were immediately submerged in $LN_2$ . Two
121	experiments were conducted ("Time" and "Temperature"; see below), and because this
122	involved processing nearly 500 samples, all samples were submerged into $LN_2$ within five
123	minutes after death to reduce postmortem tissue degradation. Each experiment only used
124	the tissue collected from a single individual. For both experiments, each treatment
125	combination consisted of ten replicates.
126	
127	Time Experiment 1.2
128	Fillet strips and claws were thawed and sub-sampled; samples were weighed to
129	the nearest mg before being subjected to one of seven preservation treatments: 1 ml 95%

130	EtOH, 1 ml salt-saturated DMSO-EDTA buffer (modified from Seutin et al., 1991), 1 ml
131	DNAzol (Molecular Research Center), 1 ml of RNA <i>later</i> (Ambion), 300 $\mu$ l M2 tissue
132	digestion buffer (Autogen, Inc.), frozen at -20° C, frozen in $LN_2$ ( $\approx$ -190°C). The
133	DMSO-EDTA buffer we use is a slight modification of the tissue buffer used by Seutin et
134	al. (1991); we use 25% of DMSO, instead of 20% (with 25% of 0.5M EDTA, 50% sterile
135	$H_20$ , saturated with sodium chloride). Before being subjected to the preservation
136	treatment, all samples were allowed to sit at room temperature for one of three time
137	periods: preserved immediately after thawing (<10 minutes total thaw time), 3 hours after
138	thawing, and 24 hours after thawing. After 14 (crab) or 20 (fish) days, all sample tubes
139	were moved into $LN_2$ and stored until DNA was extracted.
140	
141	Temperature Experiment 1.3
142	Tissue samples were placed into one of five preservatives: 1 ml 95% EtOH, 1 ml
143	DMSO-EDTA, 1 ml DNAzol, 1 ml RNA <i>later</i> , 300 $\mu$ l M2 lysis buffer and then stored for
144	15 (crab) to 21 (fish) days at one of five temperatures: room temperature (RT), 4°C, -20°
145	C, -80° C, and ~-190° C (LN <sub>2</sub> ). Subsequently, all sample tubes were stored in liquid
146	nitrogen until DNA was extracted.
147	
148	DNA Extraction 1.4
149	All samples were digested overnight in 300 $\mu$ l AutoGen M2 and 300 $\mu$ l M1 buffer
150	(including Proteinase K). DNA was extracted from 300 $\mu$ l (½ of the digested amount) of
151	each digested sample by an AutoGen Prep 965 automated DNA extractor (AutoGen Inc.)

using the manufacturers standard animal tissue (phenol-chloroform) extraction method,

and then dried. Samples were eluted in either  $100 \ \mu l$  (fish) or  $50 \ \mu l$  (crab) R9 DNA resuspension solution (AutoGen Inc.).

155

156 DNA Quantification 1.5

157	All sample extractions were quantified through fluorescence, using a BioTek
158	Synergy HT Multi-Mode Microplate Reader and Quant-iT dsDNA Assay Kit, broad
159	range (Invitrogen, Cat# Q33130). Eight $\mu$ l of eluted DNA was added to 200 $\mu$ l of buffer
160	and 1 $\mu$ l of reagent in an opaque black 96-well microplate (Corning, Cat# 3915) and
161	mixed thoroughly. Ten $\mu$ l of seven solutions with known dsDNA concentrations (0, 10,
162	20, 40, 60, 80, and 100 ng/ $\mu$ l) were each added to two wells on every plate to calculate
163	standard concentration curves. After sitting at room temperature for three minutes,
164	samples were excited at 485 nm and ensuing fluorescence was read at 528 nm. Each
165	sample was read twice, with five minutes between reads, and reads were averaged.
166	Duplicate fluorescence values from concentration standards were averaged, and a general
167	linear model of these fluorescence values versus total known DNA amounts was
168	calculated using the program R, with the intercept constrained to run through the origin.
169	This model was then used to calculate the total ng of DNA in each sample. The DNA
170	concentration of the extraction was calculated by dividing total DNA by eight (the
171	volume of sample used). We also calculated the total DNA extracted by multiplying
172	fluorescent sample total DNA by 12.5 (for fish, because it was eluted in 100 $\mu$ l) or 6.25
173	(for crab, because it was eluted in 50 $\mu$ l). Finally, we calculated a DNA extraction yield
174	(ng DNA/mg Tissue) by dividing the total DNA extracted by the weight of each tissue

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175 samp	le, and multip	lying by two	(because	only half	of each diges	st was used in the
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- 176 extraction).
- 177

#### 178 <u>Statistical Analyses 1.6</u>

- 179 Treatment differences were evaluated separately for each species (fish, crab) and
- 180 experiment (Time, Temperature). The program R was used to run two-factor Analyses of
- 181 Covariance (ANCOVA) on the quality and quantity datasets separately. Analyses
- 182 included a time or temperature by preservative interaction term and used tissue weight as
- 183 a covariate. If weight was non-significant, it was removed and the same ANCOVA, but
- 184 without the covariate, was run. In total, 14 analyses were conducted for each experiment.
- 185 Therefore, a Bonferroni correction of  $\alpha = 0.004$  was used as a measure of significance.
- 186

187 Gel Quantification: Greater than X kb 2.0

188

189 <u>Gel electrophoresis protocol 2.1</u>

190 Extracted gDNA for all samples was visualized on a 1% agarose Tris-Borate-

191 EDTA (TBE) gel. Five  $\mu$ l of each gDNA extract was loaded into the gel, and

192 electrophoresed at 45 volts for 2.5 hours in 1X TBE buffer. To estimate gDNA fragment

193 length, 0.5–1 ug of *Hin*dIII ladder was loaded into wells on each side of the DNA-loaded

194 wells. After electrophoresis, gels were stained for 30 minutes in a solution of Ethidium

195 Bromide (EtBr; at a final concentration of  $0.5 \mu g/ml$ ) and 1X TBE buffer. Gels were

subsequently de-stained (to reduce background staining) in H<sub>2</sub>O for 15 minutes. Finally,

197 gDNA was visualized, photographed and images were stored as TIFF files using a

198 Syngene Gene Gene Gene Gene Bio Imaging System.

199 Additionally, because EtBr is carcinogenic, and many labs are moving away from 200 its use and replacing it with safer methods, we also optimized the gel electrophoresis 201 protocol with GelRed<sup>™</sup> (BioTium). For this method, we recommend a 0.7% agarose 202 Tris-Borate-EDTA (TBE) gel, run for  $\sim 2.5$  hours at 45 volts in 1X TBE buffer. When using GelRed<sup>TM</sup>, we recommend loading a few wells of diluted *Hin*dIII ladder in various 203 204 amounts (e.g. 1:24, 1:49, and 1:99) because the GelRed<sup>™</sup> can cause wide smearing in the 205 HindIII ladder bands (Fig. 1). For either staining method, it is important to have HindIII 206 ladder on either ends of the gDNA, such that it "brackets" the gDNA samples on either 207 end for post-scoring of the gel images (Fig. 1). 208 209 Scoring of the Gels 2.2 210 Specific, step-by-step instructions can be found on the online Supplementary Information 211 Appendix I.

212

213 To visualize and score the gel images, we used the program ImageJ v1.48 (W. 214 Rasband, NIH). ImageJ is an open-source, Java based program in the public domain, 215 available at http://rsb.info.nih.gov/ij. Gel image files are opened in ImageJ, with the 216 wells of the gel at the top. The gel image is color inverted to enhance visualization of the 217 bands and make density curves positive, rather than negative (see below). The image is 218 made level with respect to the top band in each ladder on either side of the gDNA 219 samples, the background is subtracted to remove smooth continuous backgrounds from 220 the gel images. Next, a vertical box is drawn encompassing the entire length of the lane

221 containing *Hind*III ladder, starting below the well, above the  $\sim 23$  kb band, and to the 222 extent of the gDNA (Fig. 1A). Vertically, the box should encompass the entire length of 223 the smallest fragments of the gDNA lane on the gel with the greatest range. 224 The *Hin*dIII ladder box on the left is selected as the first lane. If using multiple 225 dilutions of *Hind*III ladder, select the dilution that has the clearest bands on both sides of 226 your gDNA samples. The box over the *Hin*dIII ladder is then moved to the first gDNA 227 lane, and this lane is selected as the next to be analyzed (note: when moved, the original 228 box stays in place and a new one is placed over the next lane). The horizontal location of 229 this box must be carefully selected, so that the bands (or smears) of gDNA encompass the 230 entire width of the box. The program automatically adjusts the vertical placement to be 231 level with the first box. Then, the box from the first gDNA lane is moved to the next 232 gDNA lane to be scored (typically, the next one to the right), again carefully selecting the 233 location of the new lane horizontally, centering the box on the gDNA. Additional gDNA 234 lanes are selected from left to right, by dragging the previous box from the left to the 235 right, and selecting "Next Lane," until all desired lanes are included. The last lane 236 selected must be the HindIII ladder to the right of gDNA lanes (Fig. 1A). An "Intensity 237 Plot" is then created of the selected lanes, including the *Hin*dIII ladder lanes. 238 The Intensity Plot opens in a new window and is rotated 90° clockwise from the 239 gel orientation (i.e. the leftmost lane becomes the top intensity plot). A straight line is 240 drawn for the DNA size threshold (Fig. 1B) from the apex of the ladder threshold peak 241 (e.g. "9 kb") on the first ladder (top of the Intensity Plot) to the apex of the ladder 242 threshold peak on the second ladder (bottom of the Intensity Plot). This separates the

243 intensity curves of each lane into a region greater than the threshold peak (Fig. 1C) and a

244 region less than the size of the threshold peak (Fig. 1D). All regions must be closed to be 245 measurable; if the right side of the intensity curve does not meet the vertical line at the 246 right side of the plot (leaving this region open), the Straight tool is used to draw a vertical 247 line connecting the right part of the curve to the border of the plot. 248 The Wand tool is then used to select and measure the area of a region under the 249 Intensity Plot, on the left side of the vertical line (>9 kb) and to the right of the vertical 250 line (<9 kb). Once selected, the area is automatically calculated and presented in a 251 Results table (Fig. 1E). If a dark imperfection appears in the gel that is clearly not part of 252 the gDNA (Fig. 1A, gDNA II), a peak is recorded in the Intensity Plot. Similar to closing 253 areas to measure (described above), one can eliminate the erroneous peak by using the 254 Straight tool (Fig. 1F), and recalculate the area (Fig. 1G). The numbers in the Results 255 window are the areas of the curve greater and less than the size of the threshold peak, 256 respectively (Fig. 1E). These data are then copied and pasted into a data processing file 257 and the percentage of the area greater than the chosen peak (e.g.  $\sim 9$  kb) is easily 258 calculated by dividing the area to the left (> 9 kb), by the total area. 259

260 <u>Scoring Tests 2.3</u>

We ran two analyses to test the repeatability (the variation obtained when one person measures samples repeatedly using the same methods) and reproducibility (the variation obtained when multiple people measure samples repeatedly using the same methods) of our gel scoring method. In the first analysis, two co-authors (CM and KSM) each independently scored the same gel image (FishTime <10 min) consisting of 40 lanes of gDNA, 10 times. For each scoring, the entire process was repeated, starting with

267	opening the raw image in ImageJ. Additionally, each scoring process was timed to give
268	an estimate of method efficiency. The results of the 20 scored gel images (consisting of
269	800 scored gDNA lanes) were analyzed using a Gage repeatability and reproducibility
270	ANOVA (Gage R&R) using the spreadsheet devised by J. Muelaner
271	( <u>www.muelaner.com/quality-assurance/gage-r-and-r-excel/</u> ). In order to test the
272	consistency of dDNA in the gels, for the second analysis we ran seven gDNA samples,
273	four crab (from Time <10 min) and three fish (from Temp = $4^{\circ}$ C), each multiple times
274	on two different gels. Each crab sample was run three times on each gel, while each fish
275	sample was run four times on each gel. The gel images were also independently scored
276	by two co-authors (DM and KSM), and the results of this test were also analyzed using a
277	Gage R&R.
278	
279	Results
279 280	Results
279 280 281	<b>Results</b> Time Experiment
279 280 281 282	<b>Results</b> <i>Time Experiment</i>
279 280 281 282 283	Results <i>Time Experiment</i> Figure 2A shows the fish gDNA extractions run out on a gel with the <i>Hin</i> dIII ladder from
279 280 281 282 283 283	Results         Time Experiment         Figure 2A shows the fish gDNA extractions run out on a gel with the HindIII ladder from the seven preservation methods at time <10 mins after death. The DMSO-EDTA and
279 280 281 282 283 283 284 285	Results         Time Experiment         Figure 2A shows the fish gDNA extractions run out on a gel with the HindIII ladder from the seven preservation methods at time <10 mins after death. The DMSO-EDTA and DNAzol buffers have the greatest percent of gDNA > 9 kb (72% and 87%, respectively),
279 280 281 282 283 284 285 286	Results <i>Time Experiment</i> Figure 2A shows the fish gDNA extractions run out on a gel with the <i>Hin</i> dIII ladder from the seven preservation methods at time <10 mins after death. The DMSO-EDTA and DNAzol buffers have the greatest percent of gDNA > 9 kb (72% and 87%, respectively), consistent with the gel patterns showing the largest bands of gDNA, with little streaking
279 280 281 282 283 284 285 286 287	Results <i>Time Experiment</i> Figure 2A shows the fish gDNA extractions run out on a gel with the <i>Hin</i> dIII ladder from the seven preservation methods at time <10 mins after death. The DMSO-EDTA and DNAzol buffers have the greatest percent of gDNA > 9 kb (72% and 87%, respectively), consistent with the gel patterns showing the largest bands of gDNA, with little streaking or smearing in the lanes, indicating very little fragmented DNA. Figure 2B shows the
279 280 281 282 283 284 285 286 287 288	Results <i>Time Experiment</i> Figure 2A shows the fish gDNA extractions run out on a gel with the <i>Hin</i> dIII ladder from the seven preservation methods at time <10 mins after death. The DMSO-EDTA and DNAzol buffers have the greatest percent of gDNA > 9 kb (72% and 87%, respectively), consistent with the gel patterns showing the largest bands of gDNA, with little streaking or smearing in the lanes, indicating very little fragmented DNA. Figure 2B shows the results of the fish gDNA extractions from samples preserved at room temperature in 95%

290	The gDNA degrades through time resulting in little to no high molecular weight DNA
291	after 24 hours (Fig. 2B).

292	Figure 3 shows the gel image of the crab gDNA for time <10 mins., for seven
293	different preservation methods. The EtOH, DMSO, and DNAzol gDNAs have the
294	greatest percentage of gDNA > 9kb ( $93\%$ , $93\%$ , $100\%$ , respectively), and show less
295	smearing and larger fragment size than RNAlater, -20°C, -190°C, and M2.
296	Average measures of quantities, concentrations, and quality (as measured by $\%$ of
297	DNA > 9,416 bp) are shown for each trial for the time experiment for the fish and crab
298	gDNA extractions in Table 1. Figures 4 and 5 show the quality of fish and crab gDNA
299	(% > 9  kb), respectively, for seven different preservation methods over three time
300	periods. DNA quality varies greatly in both taxa at time <10 mins., but all methods show
301	degradation in quality of gDNA over time for both fish and crab tissues.
302	
303	
304	Temperature Experiment
305	
306	Figure 6A shows the fish gDNA extractions electrophoresed on a gel with the
307	HindIII ladder from five different preservation methods at room temperature. Note the
308	DMSO-EDTA, DNAzol, and RNAlater buffers have the greatest percentage of gDNA >
309	9kb (89%, 98%, 100%, respectively), and show the largest bands of gDNA with little
310	streaking or smearing in the lanes, indicating very little fragmented gDNA. Figure 6B
311	shows the results of the fish gDNA extractions electrophoresed on a gel (with the HindIII

312 ladder) from the five different temperatures the tissue were stored at while in the DMSO-

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313	EDTA buffer. Figure 7 shows the gel image of the crab gDNA for the different
314	preservation methods stored at room temperature. Average measures of quantities,
315	concentrations, and quality (as measured by $\%$ of DNA > 9,416 bp) are shown for each
316	trial for the temperature experiment for the fish and crab gDNA extractions in Table 2.
317	Figures 8 and 9 show the quality of fish and crab gDNA ( $\% > 9$ kb), respectively, for the
318	different preservation methods over the five temperatures at which tissues were stored.
319	
320	
321	ANCOVA Statistics
322	
323	Table 3 shows the results of the analysis of covariance statistics for the fish and
324	crab, Time and Temperature experiments, for both quality (% of $gDNA > 9 kb$ ) and
325	quantity (ng gDNA/mg tissue) of gDNA. In the fish Time Experiment, time,
326	preservation method, and the interaction variable all significantly affected DNA quality,
327	while only time had a significant affect on quantity. For both quality and quantity, the
328	covariate weight was marginally insignificant. In the crab Time Experiment, all factors
329	(time, preservative, time x preservative, weight) significantly affected gDNA quality. The
330	interactive term was significant only for gDNA quantity, although preservation method
331	was just marginally insignificant. For the fish Temperature Experiment, only
332	preservation method significantly affected gDNA quality, while temp, preservation
333	method and weight all affected quantity. Finally, in the crab Temperature Experiment,
334	only preservation method significantly affected either quality or quantity, although
335	weight was marginally insignificant for both (Table 3).

336	
337	Repeatability and Reproducibility
338	
339	The first (single gel) test resulted in the repeatability variation (the % of total variation
340	that is attributable to the scorer, i.e. the variability among the 10 scores a single co-author
341	gave the same sample) of 3.99% and reproducibility variation (the % of total variation
342	attributable to differences in the way the co-authors scored the same sample) of 5.71%.
343	The total Gage Repeatability and Reproducibility variation was 6.97%. The second
344	(multiple gel) test had a repeatability variation of 22%, a reproducibility variation of
345	18.1%, and a total Gage R&R variation of 28.5%.
346	
347	Discussion
348	
348 349	Greater than X kb
348 349 350	Greater than X kb
348 349 350 351	<i>Greater than X kb</i> Here we demonstrate a simple, consistent, and efficient method for determining the size
348 349 350 351 352	<i>Greater than X kb</i> Here we demonstrate a simple, consistent, and efficient method for determining the size and quality of genomic DNA that does not require expensive equipment or reagents.
348 349 350 351 352 353	Greater than X kb Here we demonstrate a simple, consistent, and efficient method for determining the size and quality of genomic DNA that does not require expensive equipment or reagents. Previous studies have presented the effects of different preservation conditions on DNA
348 349 350 351 352 353 354	Greater than X kb Here we demonstrate a simple, consistent, and efficient method for determining the size and quality of genomic DNA that does not require expensive equipment or reagents. Previous studies have presented the effects of different preservation conditions on DNA without providing an objective metric for genomic quality as we have done (e.g., Gaither
<ul> <li>348</li> <li>349</li> <li>350</li> <li>351</li> <li>352</li> <li>353</li> <li>354</li> <li>355</li> </ul>	Greater than X kb Here we demonstrate a simple, consistent, and efficient method for determining the size and quality of genomic DNA that does not require expensive equipment or reagents. Previous studies have presented the effects of different preservation conditions on DNA without providing an objective metric for genomic quality as we have done (e.g., Gaither et al., 2011; Camacho-Sanchez et al., 2013). We propose this method as a heuristic
<ul> <li>348</li> <li>349</li> <li>350</li> <li>351</li> <li>352</li> <li>353</li> <li>354</li> <li>355</li> <li>356</li> </ul>	Greater than X kb Here we demonstrate a simple, consistent, and efficient method for determining the size and quality of genomic DNA that does not require expensive equipment or reagents. Previous studies have presented the effects of different preservation conditions on DNA without providing an objective metric for genomic quality as we have done (e.g., Gaither et al., 2011; Camacho-Sanchez et al., 2013). We propose this method as a heuristic standard for biodiversity biobanking facilities and the genomic community, which may
<ul> <li>348</li> <li>349</li> <li>350</li> <li>351</li> <li>352</li> <li>353</li> <li>354</li> <li>355</li> <li>356</li> <li>357</li> </ul>	Greater than X kb Here we demonstrate a simple, consistent, and efficient method for determining the size and quality of genomic DNA that does not require expensive equipment or reagents. Previous studies have presented the effects of different preservation conditions on DNA without providing an objective metric for genomic quality as we have done (e.g., Gaither et al., 2011; Camacho-Sanchez et al., 2013). We propose this method as a heuristic standard for biodiversity biobanking facilities and the genomic community, which may desire an initial assessment of DNA quality before requesting tissue samples. Using this

359	or any other large-sized DNA marker, and quantified using simple plots in the free
360	software ImageJ (W. Rasband, NIH: http://rsb.info.nih.gov/ij.).
361	We tested whether a single, or multiple researchers could reliably score a gel
362	image similarly multiple times using a using a Gage Repeatability and Reproducibility
363	ANOVA. The Gage R&R evaluates repeatability by the amount of variation attributable
364	to a single measurer through multiple measures of the same sample, and evaluates
365	reproducibility by the amount of variation attributable to differences between measurers.
366	The analysis also calculates an overall measure of repeatability and reproducibility (the
367	total Gage R&R), where most guidelines consider any total Gage R&R values under 10%
368	to be acceptable, and any total values under 30% to be acceptable under certain
369	conditions (Pan, 2006). For this test, the repeatability variation was 3.99%, the
370	reproducibility variation was 5.7%, and the Total Gage R&R was 6.97%. Both
371	researchers gave the same sample image similar quality scores (though not surprisingly,
372	quality scores were slightly more different between researchers than were scores by the
373	same researcher) to an extent acceptable to most quality control applications.
374	We conducted a second test to evaluate quality scores from samples run multiple
375	times on a gel, and on separate gels. Our Gage R&R variability was much higher for this
376	experiment, with a repeatability variation of 22%, a reproducibility of 18%, and a Total
377	Gage R&R variation of 28.5%. There are many factors that can affect these scores in
378	addition to measurer variability, such as variation in image quality (camera exposure,
379	focus, dynamic range), variation in gel staining (length of time, mixing of stain, type of
380	stain), and pipetting variation. Minor nuances between gel runs can result in slight

discrepancies of quality scores, but in our case most values of the same sample were
within ~5% of individual scores.

383 For the purposes of demonstrating the method, we chose the 9.416 bp ( $\sim$ "9 kb") 384 size marker from *Hin*dIII as the standard, and reported the percentage of genomic DNA 385 greater than 9 kb, with the recognition of 50% or more of the gDNA being greater than 9 386 kb as a candidate indicator of "genomic quality." Although other fragment sizes could be 387 chosen, in our experience of legacy biorepository samples, many gDNA extractions will 388 fail to meet a higher standard. NextGen sequencing techniques are capable of much 389 longer reads than 9 kb (Loman and Quinlan, 2014); therefore, threshold measures of 390 genomic quality will be useful to the field of biodiversity genomics. 391 Moreover, this method does not depend on the choice of fragment size as a 392 threshold for "genomic quality." One could as well pick the HindIII 564, 2,027 or other 393 fragment sizes as a standard. From the point of view of a biodiversity tissue and DNA 394 repository, whose samples may be been collected years ago and under difficult field 395 conditions, or whose future samples may require difficult field conditions, we propose 396 that 9 kb is, given current technology, a pragmatic value. Whatever the standard chosen, 397 the threshold percentage of gDNA also implies that the extraction will contain fragments 398 much larger than the actual threshold value. Importantly, the "DNA Threshold" and the 399 "Percent above Threshold" standards (http://terms.tdwg.org/wiki/GGBN\_Data\_Standard -400 GGBN\_Gel\_Image\_Vocabulary) provide a computable number for comparative values. 401 These values coupled with the gel images allow the researcher to reach their own 402 conclusions on the quality of gDNA for their specific needs.

18

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403	Of course, genomic DNA of many small organisms, such as certain arthropods,
404	nematodes, meiofauna, and other microscopic organisms is generally difficult to visualize
405	on agarose gels, yet suitable amounts of genomic sequence data can be successfully
406	amplified from such organisms (e.g. Blaimer et al., 2015). We also realize that
407	"degraded" gDNA, < 9 kb for example, can still be used for myriad analyses (e.g.
408	sequence capture, ultra-conserved elements, etc.), including complete genome
409	sequencing, such as the Neanderthal genome (Prüfer et al., 2014). Indeed, ancient DNA
410	rarely exceeds 100 bp. However, as biodiversity scientists seek to preserve samples from
411	all major clades of the tree of life, from all biomes, practical and economical field
412	techniques must be developed, and in turn, the effectiveness of such techniques should be
413	quantitative.
414	Whole genome sequencing will advance technically to use very long fragment
415	sizes, as longer reads provide higher quality assemblies (Schatz et al., 2010). Therefore,
416	for plants and animals that can easily be visualized on an agarose gel, we recommend the
417	"greater than X kb" method as a standard for biodiversity biobanking laboratories to
418	report the quality of gDNA extracts.
419	Typically, most library preparation methods to date begin with shearing gDNA to
420	sizes compatible with the maximum size range of most NextGen sequencing platforms

421 (e.g. 300–500 bp). Therefore, one might question why we should be concerned with

422 large pieces of intact gDNA prior to library preparation. Mechanical shearing, or

423 sonication, shear gDNA randomly across the genome, whereas degradation can cause

424 shearing in non-random places, and in the same places repeatedly, possibly leading to

425 biased NextGen results (Zackin and Ge 2010; Choi et al., 2002). Furthermore, the use of

426	large insert mate-	pair libraries	up to 25 kb	can increase	the efficiency	of genomic
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427 structure analyses (van Heesch et al., 2013).

428

429 Preservation Methods

430

431	In our tests, salt-saturated DMSO/EDTA buffer and DNAzol are better at
432	preserving high-quality (> 9 kb) gDNA than other methods such as direct storage in
433	liquid nitrogen (-190° C) or -20° C storage (Fig. 2A and Fig. 3; Table 3). Saturating
434	tissues with storage buffer immediately is also important, as significant DNA degradation
435	can occur, even within three hours time after death (Fig. 2B and Figs. 4–5; Table 3).
436	Temperature appears to have less of an effect on tissue preservation for overall size-
437	quality of gDNA (Figs. 8–9; Table 3). Therefore, time before preservation and
438	preservation method (buffer vs. frozen), and interactions between these factors, have the
439	biggest influence on gDNA quality when measured as size, for both the fish and crab
440	tissue samples (Table 3).
441	Currently, many genetic researchers working on non-model organisms are under
442	the impression that directly freezing fresh tissue is the best way to preserve gDNA, and
443	the faster and colder the method of preservation, the better (e.g. Wong et al., 2012).
444	Liquid nitrogen can be expensive, and both liquid nitrogen and dry ice can be difficult to
445	obtain and transport in certain countries and under remote field conditions. Our results
446	show that putting tissue directly into buffers, such as the salt-saturated DMSO/EDTA or
447	DNAzol is actually better than directly into liquid nitrogen or -20° C storage, without any
448	buffer for fish (Figs. 4; Table 1), and putting tissues directly into buffers or liquid

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449	nitrogen alone is far better than -20° C storage for the crab (Fig. 5); albeit all of our
450	samples were flash-frozen first in liquid nitrogen without any buffers prior to treatment.
451	This is good news for molecular biologists collecting field samples of genomic
452	material. Salt-saturated DMSO/EDTA is easy and inexpensive to make in the lab and is
453	more easily transported than reagents such as 95% EtOH. We suspect, based on our
454	observations, that the best method of preservation is to allow fresh tissue material to soak
455	in the salt-saturated DMSO/EDTA buffer for approximately 1 hr. (depending on amount
456	of tissue) and then preserving it in liquid nitrogen, or -20° C for transportation and/or
457	long term storage.
458	Interestingly, 95% EtOH appears to be just as good as DMSO for preserving
459	DNA quality in crab tissue (Fig. 5), but not so for fish (Fig. 4). Ethanol can cause
460	extensive, crude dehydration of animal tissues, which may cause fragmentation of gDNA
461	(Gaither et al., 2011). Some research supports that ethanol performs better in invertebrate
462	tissues (Williams, 2007), perhaps in insects because it can more easily penetrate the
463	cuticle and exoskeletons. Our crab tissue was removed from the shell prior to
464	preservation, which may have improved the performance of EtOH in our study.
465	Regardless, the DMSO/EDTA buffer and 95% EtOH each performed better than direct
466	cryopreservation for the fish and crab tissues, respectively.
467	Challenges of sufficient amounts of total DNA extracted and concentration (ng
468	DNA/mg tissue) can be overcome by increasing the amount of starting material, and/or
469	combining extractions from several separate extractions of the same starting material
470	source. Here, we have shown that time since death is the biggest factor in gDNA
471	concentration for the fish, but this does not seem to be a factor for the crab tissue (Table

472 3). Temperature is important for the preservation of fish tissue in terms of quantity

- 473 (Table 3).
- 474 Our study was limited to a small number of organisms, one vertebrate, the white
- 475 perch (Morone americana) and one marine invertebrate, the blue crab (Callinectes
- 476 *sapidus*). Ideally, we would like to see our methods tested for a variety of organisms,
- 477 including vascular plants, algae, and terrestrial invertebrates. Ultimately, we would like
- 478 to see tests of methods of genomic DNA preservation for all groups of life.

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488	
489	Supplemental Information
490	Supplemental information for this article can be found online at (to be included upon
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#### Tables

Table 1. Time Experiment. Average quantities of Extracted DNA (ng), DNA extraction yield (ng DNA/mg tissue digested; see text for method of calculation) and Quality (% DNA > 9,416 bp) of genomic DNA extracted from tissues of two species of fish (*Morone americana*) and crab (*Callinectes sapidus*) held in one of 21 treatments: three times prior to preservation (<10 min, 3 hrs, 24 hrs), then stored in seven preservative methods (95% EtOH, DMSO-EDTA, DNAzol, RNAlater, M2, -20°C, and -190°C) for a minimum of 14 days. Mean and standard deviation (SD) values are shown.

			< 10 mi	in			3 hrs							24 hrs						
	Extracted DNA (ng)		Extraction (% Yield (ng (% DNA/mg Tissue)		Quality (% > 9416 bp)		Extracted DNA (ng)		Extraction Yield (ng DNA/mg Tissue)		Quality (% >9416 bp)		Extracted DNA (ng)		Extraction Yield (ng DNA/mg Tissue)		Qualit (% > 9 bp)	ty 416		
				S		S				S				a D		S		S		
	Mean	SD	Mean	D	Mean	D	Mean	SD	Mean	D	Mean	SD	Mean	SD	Mean	D	Mean	D		
	Fish: <i>N</i>	1orone a	mericana																	
EtOH	186	28	78	10	6	3	209	49	83	18	4	4	98	14	40	7	1	2		
DMSO	183	57	81	20	72	10	171	94	71	40	25	8	13	5	6	2	0	0		
DNAzol RNAlate	244	170	108	71	87	5	157	25	63	12	34	8	14	4	6	2	0	0		
r	182	17	81	8	7	6	180	36	72	20	0	0	86	37	38	18	0	0		
M2	214	44	101	21	35	20	174	31	79	13	4	4	84	18	40	8	3	5		
-20°C	236	37	102	15	7	5	166	29	69	11	1	2	110	45	49	19	2	5		
-190°C	191	44	84	15	3	2	165	24	70	10	0	2	137	43	59	18	0	0		
Crab: Callinectes sapidus																				
EtOH	97	48	22	10	93	5	369	312	79	64	46	36	303	94	61	17	1	1		
DMSO	38	11	8	3	93	13	136	85	30	15	48	22	28	15	5	2	0	0		
DNAzol RNAlate	34	28	8	7	100	0	130	108	28	23	20	10	14	4	3	1	0	0		
r	140	74	28	14	81	17	231	126	52	30	53	37	184	33	35	7	0	0		
M2	119	88	34	26	75	18	250	114	64	29	56	32	31	40	8	10	0	0		
-20°C	275	56	58	12	17	16	278	143	70	43	26	37	196	66	43	7	0	0		
-190°C	209	112	44	23	72	16	381	214	90	47	49	36	102	141	19	26	0	0		

Table 2. Temperature Experiment. Average quantities of Extracted DNA (ng), DNA extraction yield (ng DNA/mg tissue digested, see text for method of calculation) and Quality (% DNA > 9416 bp) of genomic DNA extracted from tissues of two species fish (*Morone americana*) and crab (*Callinectes sapidus*) held in one of 25 treatments: five storage temperatures (Room Temperature = RT, 4°C, -20°C, -80°C, and -190°C) x five preservative methods (95% EtOH, DMSO-EDTA, DNAzol, RNAlater, M2) for a minimum of 14 days. Mean and standard deviation (SD) values are shown.

			RT			4°C						-20°C						
	Extracted		Extrac	tion	Quali	ty	Extracted		Extraction		Quality		Extracted		Extraction		Quality	
	DNA (	(ng)	Yield	(ng	(% >9	416	DNA (ng)		Yield (ng $(\% > 9416)$		DNA	(ng)	Yield (ng		(% > 9416			
			DNA/	/mg	bp)				DNA/	mg	bp)				DNA/	'ng	bp)	
			Tissu	ıe)					Tissu	le)					Tissu	ıe)		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fish	: Morone	america	ina															
EtOH	233	129	150	69	46	18	229	87	157	54	23	12	188	38	120	23	41	11
DMSO	375	170	248	96	89	10	295	116	196	66	89	8	309	155	208	108	94	6
DNAzol	298	177	194	109	98	4	297	114	192	74	83	15	294	143	202	90	96	6
RNAlater	173	127	125	102	100	0	169	44	109	23	59	14	160	28	111	13	81	6
M2	161	73	110	52	82	4	180	33	134	18	30	17	188	87	142	64	80	10
Crab	: Callinect	tes sapic	lus															
EtOH	1238	354	430	112	52	35	889	190	329	81	62	24	804	432	291	159	39	14
DMSO	888	367	320	155	79	6	388	162	141	54	37	20	727	345	257	98	45	27
DNAzol	643	252	242	100	90	10	705	436	257	177	77	27	511	371	171	110	NA	NA
RNAlater	641	175	229	56	62	23	704	242	271	108	55	11	399	131	142	50	54	7
M2	582	166	222	67	84	15	606	282	227	105	73	21	750	194	268	81	59	19

#### Table 2 (continued).

	-80°C						-190°C						
	Extracted DNA (ng)		Extraction Yield (ng DNA/mg Tissue)		Quality (% >9416 bp)		Extracted DNA (ng)		Extraction Yield (ng DNA/mg Tissue)		Quality (% > 9416 bp)		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Fish: Morone americana													
EtOH	159	31	105	17	49	24	306	105	209	71	32	17	
DMSO	372	192	263	132	95	13	488	244	326	189	82	20	
DNAzol	447	184	307	127	100	0	609	238	403	157	40	42	
RNAlater	184	78	125	57	77	10	174	61	117	35	71	23	
M2	164	45	116	31	88	6	269	66	172	33	63	13	
Curt	<i>C</i> -11:		1										
Crab: Callinectes sapidus							0.42	222	220	07	~~	24	
EtOH	1264	580	415	173	24	4	942	222	320	87	11	24	
DMSO	864	472	308	186	24	3	715	284	241	101	49	28	
DNAzol	920	270	332	110	28	3	432	213	151	72	91	12	
RNAlater	772	667	289	271	36	16	532	320	178	90	65	24	
M2	848	474	309	171	78	23	1024	273	336	93	60	28	



Table 3. Summary of ANCOVA results for Time and Temperature experiments. Covariance was tested for between Time, Preservation method (Pres.), and Weight (Wt.) for the Time experiments and Temperature (Temp.), Preservation method (Pres.), and Weight (Wt.) for the Temperature experiments. Interactions (Int.) were tested for between Time and Temp. and Pres., if weight was not significant it was removed. A Bonferroni correction of  $\alpha = 0.004$  is used. Significant results are shown in boldface font.

	Time Experiment					Temperature Experiment					
Fish:		Time	Pres.	Wt.	Int.	Т	emp.	Pres.	Wt.	Int.	
Morone americana	Quality	<0.001	<0.001	0.0074	<0.001	(	0.0408	<0.001	0.2079	0.8923	
	-	<0.001	<0.001		<0.001	(	0.0339	<0.001		0.9260	
	Quantity	<0.001	0.0452	0.0743	0.0220	<	<0.001	0.0026	<0.001	0.7179	
		<0.001	0.0505		0.043						
Crab:											
Callinectes sapidus	Quality	<0.001	0.0022	0.0010	0.0014	(	0.0197	<0.001	0.0607	0.5840	
						(	0.0081	<0.001		0.5550	
	Quantity	0.6902	0.0099	0.5916	<0.00191	(	0.7545	<0.001	0.0129	0.0568	
		0.7591	0.0135		0.0015	(	0.4273	<0.001		0.0481	



Figures

Figure 1. Scoring a gDNA gel in ImageJ. A) An inverted gDNA gel image with the *Hin*dIII ladder in wells 1–2 (wells labeled on bottom) and 7, and gDNA samples (I–IV) in wells 3–6. The bands in the *Hin*dIII ladder are labeled in well 1; note the ~2 kb bands are visible in this lane, but not in the more diluted lanes (2 and 7; see text). B) The Plot results of the six yellow boxes scored in A), with the Straight tool line spanning the ~9 kb peaks of the two *Hin*dIII ladders (wells 2 and 7, top and bottom, respectively), with the four gDNA samples in the middle. C) The area calculated for > 9 kb. D) The area < 9kb. E) The Results box, with values 1 and 2 for the areas > 9 kb and < 9kb for gDNA sample I, respectively. F) The Straight tool line used to eliminate peak cause by flaw in gel for gDNA sample II. G) The calculated area < 9kb with the erroneous peak removed. The percent of gDNA > 9kb is calculated by dividing the area > 9kb (C) by the total area below the curve (C + D).



Figure 2. Gel image of extracted gDNA from the white perch, *Morone americana* for the Time Experiment. The ladder in the left- and right-most lanes is the *Hin*dIII with bands at 564, 2027, 2322, 4361, 6557, 9416, and 23130 bp. A) Samples in all tissue storage treatments for Time-since-death <10 minutes: 1) in EtOH; 2) in salt saturated DMSO/EDTA preservation buffer ("DMSO"); 3) submerged in DNAzol Reagent (Invitrogen); 4) submerged in RNAlater (Ambion); 5) submerged in M2 tissue digestion solution (Autogen); 6) held at -20°C with no preservation solution; 7) submerged in liquid nitrogen ( $\approx$ -190°C) with no preservation solution. B) Samples in Time-since-death treatments for EtOH tissue storage treatment are shown for the three different time periods (< 10 mins., 3 hrs., 24 hrs.).



Figure 3. Gel image of extracted genomic DNA from the blue crab, *Callinectes sapidus*, for the Time Experiment, showing all tissue storage treatments for Time-since-death <10 minutes. The *Hin*dIII ladder is shown in the left- and right-most lanes of the gel.



Figure 4. Graph of Fish Time Experiment. Quality of gDNA extracted from the white perch, *Morone americana*. Quality is shown as the mean % of gDNA > 9,416 bp on the y-axis. Preservation treatments are differentiated by symbols (see lgend). Time-since-death treatments are shown on the x-axis for the three time periods tissue samples sat before preservation. See Table 1 for exact values of each method.



Figure 5. Graph of Crab Time Experiment. Quality of gDNA extracted from the blue crab, *Callinectes sapidus*. Quality of extracted gDNA is shown as the mean % of DNA > 9,416 bp on the y-axis. Preservation treatments are differentiated by symbols. Time-since-death differentiated are shown on the x-axis for the three time periods tissue samples sat before preservation. See Table 1 for exact values of each method.



Figure 6. Gel image of extracted gDNA for the white perch, *Morone americana*, for the Temperature Experiment. Prior to DNA extraction, tissue was stored for 14–20 days in one of five solutions (EtOH, DMSO, DNAzol, RNAlater, M2) and kept at one of five temperatures: Room Temperature, -20°C, -80°C, -190°C. A). Showing all tissue storage buffer treatments for tissue storage at Room Temperature. B). Showing all tissue storage temperature treatments for DMSO-EDTA salt buffer.



Figure 7. Gel image of extracted genomic DNA for the blue crab, *Callinectes sapidus* for preservation Temperature Experiment, showing all tissue storage buffer treatments for tissue storage at room temperature.



Figure 8. Graph of Fish Temperature Experiment. Quality of gDNA extracted from white perch, *Morone americana*. Quality of extracted gDNA is shown as the mean % of gDNA > 9,416 bp on y-axis. Preservation solutions are differentiated by symbols (see legend), with the different preservation temperatures on the x-axis. See Table 2 for exact values of each temperature.



Figure 9. Graph of Crab Temperature Experiment. Quality of gDNA extracted from the blue crab, *Callinectes sapidus*. Quality of extracted gDNA is shown as the mean % of gDNA > 9,416 bp on the y-axis. Preservation treatments are differentiated by symbols. Temperatures are shown on the x-axis for the five temperatures tissue samples were held after preservation. See Table 2 for exact values of each temperature.

#### Supplementary Information: Mulcahy et al., PeerJ

Protocol for using ImageJ to determine relative band size of DNA from gel images

- 1. **Download Program**: Download Java program ImageJ from http://rsb.info.nih.gov/ij
- 2. **Open Image**: Open raw image file in ImageJ with  $\Re$  or using the menu (File>Open).
- 3. **Invert Image**: Invert the image with shift+i. This is not necessary, but I find that it is easier to see the limits of bands with an inverted image, and it makes the density curves that we will create positive rather than negative. It also makes Step 4 (image leveling) significantly easier.
- 4. Level Image: Make sure lanes in your image are horizontally level using the menu (Image>Transform>Rotate). This brings up a dialog that allows you to rotate the image by degrees (positive numbers for clockwise rotation, negative numbers for counterclockwise rotation). To determine if rotation is necessary, select the Preview box, set the Angle to 0, and increase the Grid Lines value. This allows you to compare your lanes to the horizontal and vertical lines of the grid. Change the Angle value until the lanes in your image are horizontally level (i.e. you want the top bands in both ladders to be level).
- 5. **Subtract Background**: Subtract background from image using the menu (Process>Subtract Background). A pop-up window will open. Make sure that the box for Light Background is checked, as well as the box for Preview. Choose the Rolling ball radius that gives the whitest background without degrading DNA signal. Usually the default (50.0 pixels) works fine, but you may want to play with this. If you use too low of a value, you start to lose dark pixels at the edges of the DNA bands, but you want the lowest value obtainable without losing dark pixels.
- 6. Select Rectangle Tool: Select the Rectangle tool from the toolbar
- 7. **Draw Box Encompassing Ladder**: Draw a rectangle on the lane of the first ladder. The bands of the ladder should cross the entire width of the drawn box, so make the box as narrow as possible. Vertically, the box should encompass the entire DNA band of the lane on your gel with the greatest vertical range (but do not include the well in the box). In other words, there should be no white space on the left or right sides between the DNA band and the sides of the drawn box, but there should be white space between the longest band and the top of the box, and white space between the shortest band and the bottom of the box. If you make a mistake in drawing boxes or selecting lanes and need to start over, this can be done via the menu (Analyze>Gels>Reset)
- Select Box as First Lane: Select this lane as the first lane to be analyzed using #1. This can also be accomplished using the menu (Analyze>Gels>Select First Lane)
- 9. Select Next Lane: Drag the box to the next lane to be measured, and select this lane as the next to be analyzed using ₩2. This can also be accomplished using the menu (Analyze>Gels>Select Second Lane). When you drag the box to the

new lane, carefully select the location of the new lane horizontally, attempting to center the box on the bands (as in Step 8). You do not have to be as careful with vertical placement of the box: ImageJ will automatically align new boxes vertically as you select them.

- 10. **Select all Lanes:** Repeat Step 9 for all lanes that you wish to analyze. Make sure that the last lane selected is the last ladder. It doesn't matter which ladder is selected first, and which is selected last, as long as they are the first and last selected.
- 11. **Create Intensity Plot**: Create intensity plot of the selected lanes using ₩3. This can also be accomplished using the menu (Analyze>Gels>Plot). If you need to remake the intensity plot, close the current plot, make the gel image the active window, and use the menu (Analyze>Gels>Re-Plot Lanes). This will give you a new, clean intensity plot.
- 12. **Draw Line for DNA Size Cutoff:** From the toolbar, choose the Straight tool. Draw vertical line on the intensity plot from the apex of the ladder peak of choice on the first ladder (top of page) to the apex of the ladder peak of choice on the second ladder (bottom of page). This separates the intensity curves of each lane into a region greater than the size of the peak chosen and a region less than the size of the peak chosen.
- 13. Close All Regions to Measure: All regions under the intensity curves must be closed to measure area, but you will notice that the right side of the intensity curve does not meet the vertical line at the right side of the plot, leaving this region open. To fix this, with the Vertical tool still selected, draw a vertical line from the furthest right part of the curve on the first ladder to the furthest right part of the curve on the last ladder. This may have to be repeated on the left side of the curve if it doesn't meet the left side of the plot. You will know if this is necessary if, during Step 14, the Wand tool highlights more than just the area under the curve.
- 14. Select Region Above Cut-off for First Measurement: Choose the Wand tool from the toolbar. Select the region under the intensity curve on the left side of the vertical line from Step 12 for your first sample.
- 15. Measure First Region: Some users may find that an area measurement is automatically taken when the region is selected using the Wand tool. If a new box (labeled "Results") appears when you select a region, then measurements are automatic. If no "Results" box appears, you need to tell ImageJ to measure each region with ૠm, or using the menu (Analyze>Measure). The number in the results window is the area of the curve greater than the size of the peak chosen. If you need to measure multiple areas (i.e. the curve reaches the lower limit of the graph, essentially splitting up the region), select each while holding shift, then measure after all regions have been selected. If measurements are automatic, you cannot select multiple regions before measuring. Instead, measure each region separately and add them together to get the area of the entire curve
- 16. **Select and Measure Region Below Cut-Off**: Repeat Steps 14 and 15 with the region under the intensity curve on the right side of the vertical line. This is the area of the curve less than the size of the peak chosen, and should appear in the "Results" window, below the first value. Note that ImageJ numbers the

measurements sequentially as they are made, so keep track of which value belongs to each curve area measurement.

- 17. Measure All Regions: Repeat Step 14 through Step 16 for each sample.
- 18. **Save Results**: Save the Results table with ℜs or using the menu (File>Save As). By default, ImageJ saves the table as a tab-delimited file with an excel (.xls) extension.
- 19. **Calculate Proportion:** For each sample, add the two portions (left and right) to get the total area under the curve. Divide the first area value (area to the left of the peak) by the total area to obtain the % of area under the curve greater than the size chosen.

Notes:

This protocol is for usage on a Mac, usage on a PC is similar (replace  $\mathbb{H}$  with Ctrl for keyboard shortcuts).

The ImageJ users guide can be viewed at <u>http://rsb.info.nih.gov/ij/docs/guide/index.html</u> or downloaded using <u>http://rsb.info.nih.gov/ij/docs/guide/user-guide.pdf</u>. This protocol was based on the video tutorial found at

http://imagejdocu.tudor.lu/doku.php?id=video:analysis:gel\_quantification\_analysis All video tutorials can be accessed at <u>http://imagejdocu.tudor.lu/doku.php?id=video:start</u>