

INTRODUCTION

Genetic-based bovine parentage identification is seeing rapid uptake, driven by increasingly affordable and comprehensive approaches. To increase adoption, the processes of tagging, collection, transport, storage and genotyping of bovine samples must be integrated, more robust, and more cost-effective. Prionics has devised a simple method of tagging and sample collection (TypiFix™ Sample Collector). Biomatrix has developed a novel approach to long-term, ambient temperature sample storage (DNAgard®). Illumina now offers a comprehensive suite of bovine genotyping arrays (GoldenGate Bovine Net Merit Panel, Infinium Bovine SNP50 DNA Analysis BeadChip and Infinium Bovine HD BeadChips). When combined, these three elements converge to produce a desirable system from sample collection to genetic information.

The current study evaluates elements of the desired system. Specifically, the study measures the effect of shipping and storage temperature, duration of storage and DNA extraction method on the ability to generate high quality genotypes. Genotyping results are compared among the various conditions using Illumina's GoldenGate Bovine Net Merit Panel, Infinium BovineHD BeadChip, as well as Infinium Bovine SNP50 DNA Analysis BeadChip.

METHODS

Sample Collection and Storage:

Bovine ear punches were collected from 42 cows by a large ear notcher, subsequently cut with a 6mm biopsy punch 3 times, each 6mm punch was cut in half with a scalpel and then stored in DNAgard (350 µl) or in the absence of protectant ("unprotected"). Additionally, two 3mm samples of each animal were collected using the TypiFix™ Sample Collector system for immediate storage in Prionics' protectant ("TypiFix™").

Ear Punch	Storage Format	Storage Temperature
A	Unprotected	Ambient Temperature
B	TypiFix™	Ambient Temperature
C	DNAgard	Ambient Temperature
D	Unprotected	-80 C
E	Unprotected	Shipping Simulation*
F	TypiFix™	Shipping Simulation*
G	DNAgard	Shipping Simulation*
H	Unprotected	-80 C

*Shipping simulation cycle

Temperature	Time
Ambient Temperature	3 days
45 C	2 days
Ambient Temperature	2 days
-20 C	3 days
Ambient Temperature	2 days
45 C	2 days
Ambient Temperature	Hold

Samples were then stored at ambient temperature or subjected to a two week temperature cycle (simulated shipping, followed by storage at ambient temperature). Positive control samples were stored frozen at -80 C.

DNA isolation:

After 2 weeks (14 days) and 2 months (60 days) of storage, DNA was extracted from the ear punch samples by one of two methods: 1) QIAGEN's QIAamp DNA Mini kit referred to as "Column" or 2) Tissue lysis followed by phenol:chloroform:isoamyl alcohol extraction referred to as "Phenol" (Current Protocols in Molecular Biology (1998) 2.2.1-2.2.3). For DNAgard-stored ear punches: tissue and formulation were processed in QIAamp isolations (Appendix A, page 18 DNAgard Handbook); ear punches were removed from the formulation prior to processing by method "2" (page 7, DNAgard Handbook). DNA was eluted/ resuspended in 100 µl final volume.

DNA analysis:

The DNA recovery from each sample was quantified using the Quant-iT PicoGreen kit (Invitrogen). The integrity of 200 ng of isolated DNA was analyzed via gel electrophoresis (0.8% agarose, 1xTAE) against a 1 kb DNA ladder (New England Biolabs).

Genotyping:

A subset of bovine individuals were randomly selected for genotyping. These samples were normalized to be as close to 50ng/ul as possible. DNA from TypiFix Sample Collection System required additional steps, e.g. concentrating samples, to achieve as best as possible the concentration and quantity necessary to successfully genotype. The DNA isolated from ear punches stored under the above conditions was analyzed on Illumina's Infinium (Bovine SNP50 array and Bovine HD array) and Golden Gate Platforms (Bovine 3K array).

RESULTS

Table 3. DNA Yield After Storage (µg)

Storage Time	2 Weeks		2 Months		
	Phenol	Column	Phenol	Column	
Stored Ambient	Unprotected	A 13.8 ± 11.9	2.1 ± 1.9	2.0 ± 1.2	1.3 ± 0.6
	TypiFix	B 8.6 ± 3.6	9.0 ± 1.4	13.5 ± 7.5	8.1 ± 4.3
	DNAgard	C 8.9 ± 6.7 ¹	15.8 ± 4.5	31.8 ± 3.4 ²	8.8 ± 3.0
-80 C control	D 29.9 ± 6.4	8.3 ± 3.5	30.6 ± 4.1	11.3 ± 4.6	
Simulated Ship Then Stored Ambient	Unprotected	E 1.4 ± 1.3	1.4 ± 0.8	1.8 ± 2.1	2.1 ± 2.2
	TypiFix	F 4.8 ± 6.0	5.1 ± 4.5	10.8 ± 9.9	5.4 ± 4.1
	DNAgard	G 29.7 ± 4.2 ²	18.7 ± 4.9	33.3 ± 7.8 ²	24.5 ± 3.3
	-80 C control	H 34.5 ± 10.7	11.3 ± 2.4	36.8 ± 1.8	11.5 ± 3.1

1 = Ear punch and DNAgard formulation processed for DNA isolation
2 = Ear punch, only, processed for DNA isolation

Table 3. DNA yield after 2 weeks and 2 months of storage. Ear punches from the same bovine were stored in four conditions (unprotected; desiccated in TypiFix Sample Collector; DNAgard solution; frozen at -80 C) and stored at ambient temperature or -80 C (rows A to D, and H) or subjected to a 14 day shipping cycle, followed by 46 day storage at ambient temperature (rows E to G). Genomic DNA was isolated using the QIAamp DNA Mini kit or by a standard procedure involving phenol-chloroform extraction. DNA yield is presented as the average of six replicates (separate bovine individuals) standard deviation.

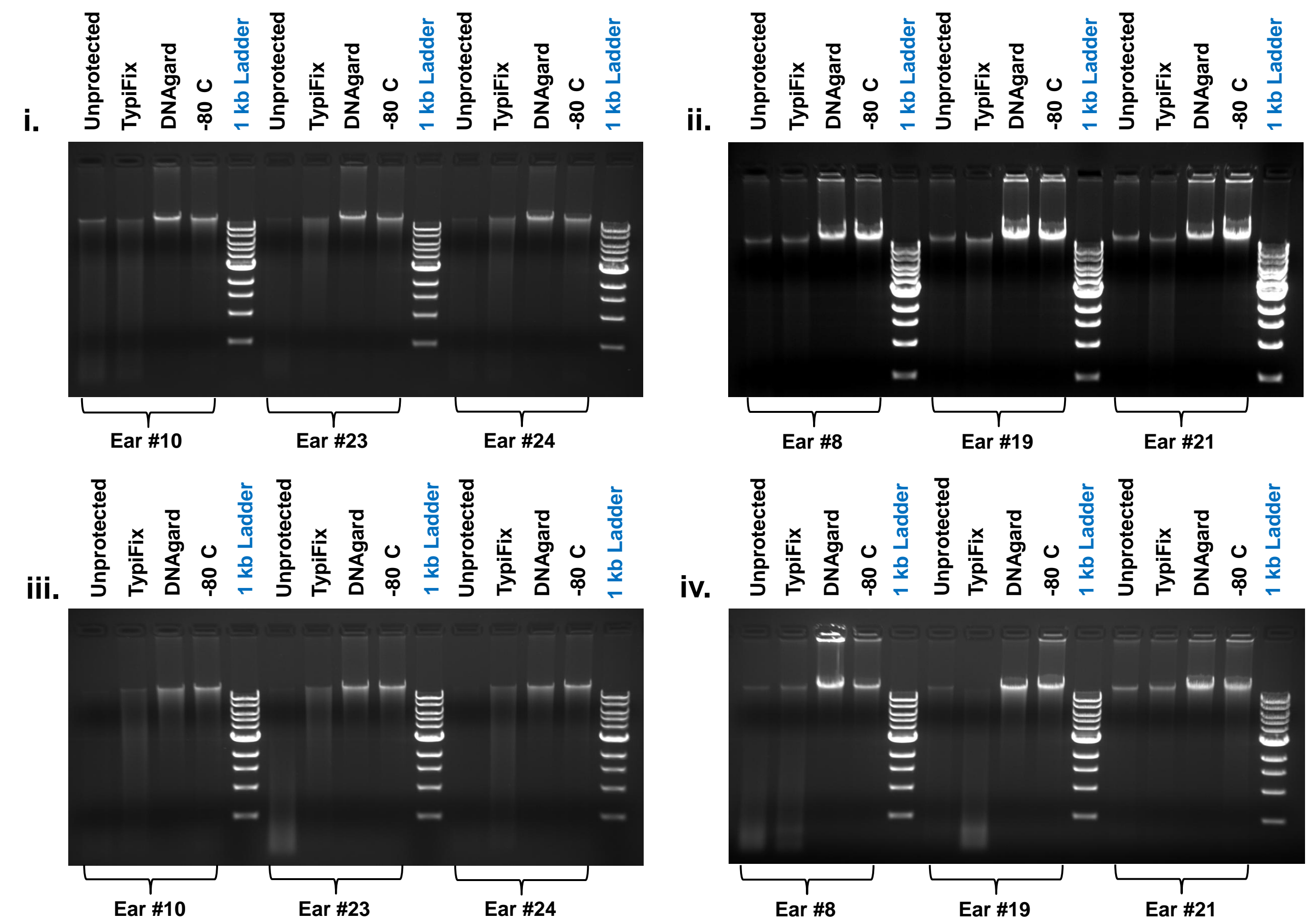


Figure 1. DNA integrity after 2 months of storage. Ear punches from the same bovine were stored in four conditions (unprotected; TypiFix Sample Collector with protectant; DNAgard solution; frozen at -80 C) and stored for 2 months at room temperature (i and ii) or subjected to a 14 day shipping cycle, followed by storage for 46 days at room temperature (iii and iv). Genomic DNA was isolated using the QIAamp DNA Mini kit (i and iii) or by a standard procedure involving phenol-chloroform extraction (ii and iv). Gel analysis of 200 ng of each sample reveals significantly higher amounts of intact, high molecular weight genomic DNA in frozen controls and DNAgard-stored samples relative to unprotected and TypiFix-stored samples.

Table 4. Shipping Simulation Experiment Average Call Rates (%)

Storage Time	DNA Isolation Method	2 Weeks		2 Months		
		Phenol	Column	Phenol	Column	
Unprotected	E	Bovine 3K	66.29 ± 7.44	37.44 ± 20.51	44.40 ± 20.00	56.41 ± 38.99
		Bovine SNP50	95.65 ± 1.63	60.85 ± 6.27	69.47 ± 24.00	69.74 ± 25.87
		Bovine HD	96.43 ± 2.27	68.64 ± 8.03	70.83 ± 21.44	75.37 ± 19.82
TypiFix	F	Bovine 3K	79.37 ± 16.50	98.39 ± 1.36	99.19 ± 0.05	99.25 ± 0.05
		Bovine SNP50	93.21 ± 10.39	99.62 ± 0.02	99.72 ± 0.03	99.73 ± 0.04
		Bovine HD	92.82 ± 10.96	99.67 ± 0.07	99.71 ± 0.01	99.68 ± 0.04
DNAgard	G	Bovine 3K	99.17 ± 0.18	99.18 ± 0.02	99.06 ± 0.14	99.18 ± 0.10
		Bovine SNP50	99.75 ± 0.04	99.53 ± 0.18	99.72 ± 0.03	99.75 ± 0.02
		Bovine HD	99.72 ± 0.02	99.61 ± 0.20	99.73 ± 0.02	99.73 ± 0.01
-80°C control	H	Bovine 3K	99.25 ± 0.02	99.24 ± 0.03	98.25 ± 1.54	99.23 ± 0.05
		Bovine SNP50	99.74 ± 0.02	99.37 ± 0.40	97.49 ± 3.80	99.75 ± 0.01
		Bovine HD	99.72 ± 0.01	99.26 ± 0.71	97.33 ± 4.15	99.73 ± 0.01

Table 5. Average Concordance (%)

Illumina Array	Bovine 3K	Bovine SNP50	Bovine HD
Unprotected	89.93	92.01	93.15
TypiFix	99.19	99.43	99.44
DNAgard	99.94	99.99	99.99
-80°C control	99.94	100	100

Table 4 & 5. SNP genotyping results for samples subjected to simulated shipping cycle. DNA recovered from ear punches stored for 2 weeks (14 days) or 2 months (60 days) under four conditions (unprotected; TypiFix Sample Collector with protectant; DNAgard solution; frozen at -80 C) were analyzed with normalized DNA sample input to achieve successful genotyping on all three genotyping arrays (Illumina). Shown are the average call rates standard deviation of DNA samples from three separate ears (Table 4) and the average concordance values (Table 5) of all 24 samples for each storage type (both ambient temperature storage and shipping experiments) when paired with -80 C positive controls. For comparison, concordance was also determined for duplicate -80 C controls from each ear (samples "D" and "H") and the average presented.

Storage time	DNA Isolation method	2 Weeks						2 Months						
		Phenol			Column			Phenol			Column			
Store Ambient	Unprotected	A	+++	---	+++	---	---	---	---	---	---	---	---	---
	TypiFix	B	+++	++	+++	---	+++	---	+++	+++	+++	+/ -	+++	+++
	DNAgard	C	+++	+++	++	++	+++	+++	+++	+++	+++	---	+/ -	+++
	-80 C	D	+++	+++	+++	---	+++	++	+++	+++	+++	+++	+/ -	+++
Simulated Ship then Ambient	Unprotected	E	---	---	---	---	---	---	---	---	---	---	---	---
	TypiFix	F	---	++	---	++	+++	+++	+++	+++	+++	+++	+++	+++
	DNAgard	G	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	++
	-80 C	H	+++	+++	+++	---	+++	+++	+++	+++	+++	---	+++	++

Table 6. Combined genotyping results. Normalized DNA from ear punch sample was scored based upon its performance in the three array types. Scores are based upon quality assessments using the following metrics: call rates, GenCall quality scores (please refer to Illumina Infinium Genotyping Data Analysis technote). In green, the samples that passed (+++ for the 3 arrays, ++ for 2 arrays) and in red the samples who failed (--- for the 3 arrays, -- for 2 arrays). In grey, the undetermined results (1 clear pass and 1 clear failure).

SUMMARY / CONCLUSION

Summary

Recovery of high yields of intact genomic DNA from bovine ear punch samples is of increasing importance as sample genotyping and sequencing become standard practice in the cattle industry. Our data demonstrates the importance of selecting the appropriate storage method to achieve DNA protection and yield required for downstream applications, especially in cases where both genotyping and next generation sequencing (which can require up to 30 µg DNA) are to be performed. Our data indicates that the proper DNA isolation method for ear punch samples is also critical in recovering sufficient genomic DNA for genotyping analysis. DNA is degraded rapidly (within 14 days) in unprotected ear punch samples. Our results show that high quality DNA can be recovered from samples stored frozen or stored in DNAgard at ambient temperature and that significantly higher yields can be achieved when using a phenol extraction-based method compared with a common column-based method. Genotyping results on Illumina's three array types indicate the importance of preserving genomic DNA in ear punch samples during transport and storage: unprotected ear punch samples almost always resulted in failed genotyping. Passed samples out of 24 total samples based on performance scores for each storage method were as follows: 2 for unprotected, 16 for TypiFix, 19 for DNAgard, 18 for frozen. Samples stored in DNAgard resulted in the highest pass rates across all genotyping arrays.

Next steps

- Genotyping analysis of ear punch samples after 6 months and 1 year of storage.
- Potentially next generation sequencing of samples with sufficient yields at various time points.

DNAgard

- generally high DNA yield (similar to recovery from frozen controls; Table 3)
- recovered DNA is intact (Figure 1)
- 19 of 24 samples (79.1%) resulted in clear passing metrics for all three arrays (whereas 18 of 24 frozen control samples (75.0%) resulted in clear passing metrics for all three arrays). Samples 10 and 23 appeared to have global failure and/or undetermined results including the -80 C control.

TypiFix

- Although generally lower DNA yield (relative to frozen controls; Table 3) and partially degraded (Figure 1), genotyping was generally successful across all three array types when DNA sample input is normalized.
- 16 of 24 samples (66.6%) resulted in clear passing metrics for all three arrays.
- 2 failed samples (row F-Ear13, row F-Ear15) correlates with low DNA concentration (< 50 ng/µl) as well as degradation (Figure 1)

Unprotected

- low DNA yield (Table 3) and degraded (Figure 1) with the exception of row A-Ear13 and row A-Ear15
- 22 of 24 samples resulted in failing metrics in at least two of the arrays.