Improve interpretation results from bone samples

Reveal hidden traces of inhibitors using the GlobalFiler IQC PCR Amplification Kit

In this application note, we demonstrate:

- An efficient bone preparation method using bone chips as an alternative to bone powder
- Complementary methods for identifying inhibition in forensic samples with the Applied Biosystems™ Quantifiler™ Trio DNA Quantification Kit and GlobalFiler™ IQC PCR Amplification Kit
- Streamlined interpretation of results using a quantification control or short tandem repeat (STR) internal quality control (IQC)

IQC system in the amplification reaction: a useful tool to recognize inhibition in bone samples

A common workflow for bone processing incorporates an organic DNA extraction and purification method from approximately 1 g of bone powder followed by quantification using real-time PCR prior to genotyping with STR amplification chemistry. Inhibitors that have the potential to impact the quantification and STR amplification reactions can be present in the DNA extract even after purification. Due to the low volume of DNA sample (2 µL) per quantification reaction, there is a possibility that the concentration of inhibitor in this sample size may not affect amplification efficiency or be detected by the internal positive control (IPC) in the Quantifiler Trio kit at this step. Conversely, the STR amplification reaction may be partially or fully inhibited with a DNA sample size up to 15 µL per STR reaction, which results in 7.5 times the input of DNA and inhibitor. The IQC system in the GlobalFiler IQC kit can be used to confirm the presence of inhibitors in the DNA sample. Understanding the quality of a bone sample can promote workflow strategies to maximize allele recovery and assist with interpretation of challenging profiles.

Material and methods

A total of 41 bones (Table 1) from unique, unnamed skeletal remains of unknown age (post-mortem interval assumed to not exceed 100 years) obtained from a cemetery were analyzed for identification by the Nantes Atlantique Genetic Institute (IGNA).

Table 1. Bone types analyzed.

Femur	37
Ulna	2
Tibia	1
Humerus	1

Small chips were prepared from each bone using a cordless drill (Bosch™ Pro GSR 12V 15) with a metal drill bit (0.6 mm) as described in Figure 1. DNA was extracted and purified from each sample comprising 1 g of bone chips (about 4–6 drillings). The final volume of the DNA samples was between 50 µL and 70 µL.

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Bone specimen	
Partial bone scraping with a sterile scalpel	
Bleach decontamination (diluted to 2.5% v/v) of the surface area previously scraped, followed by a rinse with sterile water and drying with a sterile compress	
Slow drilling to produce bone chips	
4–6 drillings per thick bone, such as femur, generated ~1 g of bone chips	
Bone chips 3–4 mm long were collected on sterile paper placed under the bone	

Figure 1. Bone chip preparation workflow.

DNA samples were quantified with the Quantifiler Trio DNA Quantification Kit on an Applied Biosystems™ 7500 Real-Time PCR System for Human Identification (HID) and analyzed with Applied Biosystems™ HID Real-Time PCR Analysis Software v1.2, according to the manufacturer's recommendations. This kit contains an IPC to detect the potential presence of inhibitors in each sample. STR typing was performed with the GlobalFiler IQC PCR Amplification Kit on the Applied Biosystems™ HID Veriti™ 96-Well Thermal Cycler for 29 PCR cycles, according to the manufacturer's recommendations.

The GlobalFiler IQC kit contains two IQC markers to confirm successful amplification and electrophoresis. The IQC markers can also help distinguish between inhibited and degraded samples. PCR products were separated on an Applied Biosystems™ 3500xL Genetic Analyzer for HID with Applied Biosystems™ 3500xL Data Collection Software 3.1. Samples were injected at 3 kV for 24 sec. The profiles were analyzed using Applied Biosystems™ GeneMapper™ *ID-X* Software version 1.4. Figure 2 provides an overview of the complete workflow—from sample preparation to DNA analysis.

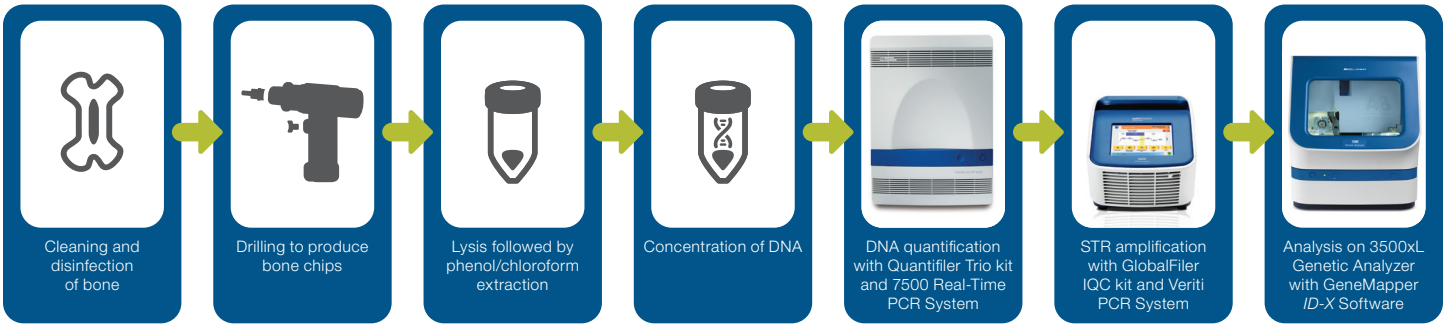

Figure 2. Bone sample workflow using traditional capillary electrophoresis.

Quantifiler Trio and IPC results and interpretation

DNA concentrations ranged from 0 to 369 pg/µL, and degradation to some extent was indicated by degradation indexes (DI) >1 across most samples with interpretable values. Results for each sample are listed in Table 2. DNA quantity per amplification reaction was 1 ng, when possible. For the majority of samples, due to the low quantity of DNA, the maximum volume of sample (15 µL) was added to the amplification reaction.

Table 2. GlobalFiler IQC results with associated Quantifiler Trio results from 41 bone samples.

Sample	DNA concentration (pg/µL)	C_t of IPC	Degradation index	IQCS	IQCL	Number of valid loci out of 24 (♂) or 22 (♀)	
						Initial data analysis	Post review by DNA expert
Sample 15	369	27.70	2	+	+	18	21
Sample 21	308	27.67	11	+	+	12	14
Sample 18	293	28.26	1	+	+	22 (♀)	22 (♀)
Sample 19	244	27.96	5	+	+	15	19
Sample 05	155	27.77	16	+	+	9	11
Sample 13	140	28.02	1	+	+	21 (♀)	22 (♀)
Sample 40	110	27.69	3	+	+	20	23
Sample 37	93	27.77	1	+	+	24	24
Sample 27	67	27.73	2	+	+	22	24
Sample 16	45	27.54	2	+	+	21 (♀)	21 (♀)
Sample 09	165	27.80	2	+	±	16	19
Sample 23	100	27.80	10	+	±	12	14
Sample 22	72	27.84	2	+	±	18	20
Sample 25	67	27.67	2	+	±	16	20
Sample 14	41	27.50	4	+	±	15	15
Sample 35	35	27.93	1	+	±	20	23
Sample 11	27	27.81	1	+	±	17	20
Sample 24	62	27.99	3	+	–	1	4
Sample 10	57	27.86	2	+	–	12	14
Sample 31	48	27.65	2	+	–	14	16
Sample 02	44	27.68	2	+	–	9	12
Sample 36	44	27.65	2	+	–	14	14
Sample 32	43	27.53	1	+	–	15	15
Sample 28	39	27.79	1	+	–	11	12
Sample 38	36	27.72	4	+	–	8	11
Sample 20	20	27.91	9	+	–	8	8
Sample 17	11	27.93	3	+	–	2	3
Sample 01	8	27.50	2	+	–	8	10
Sample 06	2	27.84	3	+	–	1	1
Sample 03	0	27.89	NI	+	–	0	0
Sample 12	0	28.52	NI	+	–	0	0
Sample 26	0	28.23	NI	+	–	0	0
Sample 29	0	27.92	NI	+	–	0	0
Sample 04	27	27.61	9	±	–	0	0
Sample 07*	3 (1/5)	28.21 (1/5)	NI	±	–	0	0
Sample 34*	3 (1/10)	27.89 (1/10)	NI	±	–	1	1
Sample 08	0	27.86	NI	±	–	0	0
Sample 41	0	27.98	NI	±	–	0	0
Sample 33*	3 (1/10)	27.81 (1/10)	NI	–	–	0	0
Sample 30	0	28.09	NI	–	–	0	0
Sample 39	0	27.84	NI	–	–	0	0

* Inhibited sample in Quantifiler Trio data using IPC.

NI : Not interpretable

+ : No inhibition of the PCR IPC

± : Partial inhibition of the PCR IPC

– : Full inhibition of the PCR IPC

The IPC in the Quantifiler Trio kit is used to detect the potential presence of inhibitors in each sample. During IGNA's laboratory validation, a set of criteria was established for IPC interpretation guidelines. Using a range of DNA inputs from whole blood samples, an IPC C_t of 27.52 ± 2 was determined to be the expected IPC C_t result of a typical reaction without impact from inhibitors. Three samples resulted in undetermined IPC results, indicating inhibitor presence in 7% of the samples. IPC results did not indicate inhibition in 93% of the samples. The 3 samples in which the IPC did not amplify (sample 07, sample 33, and sample 34) were diluted and re-quantified. A 5- or 10-fold dilution resulted in IPC amplification without indication of inhibition. Therefore, the minimal dilution for each sample without evidence of inhibition was carried into the STR reaction for a total DNA input of 45 pg for each of these samples.

Threshold determination for the GlobalFiler IQC markers

The GlobalFiler IQC kit contains two IQC markers to confirm successful amplification and electrophoresis. The IQC markers can also help distinguish between inhibited and degraded samples. To evaluate amplification performance using the IQC markers, thresholds for both the small IQC (IQCS) and the large IQC (IQCL) targets were established. Eleven negative controls and 11 positive controls were amplified under the same conditions as the bone DNA samples. The fluorescence (in relative fluorescence units, RFU) of each IQC peak was analyzed. Results are shown in Table 3, with the final thresholds being set at 4,000 RFU for the IQCS and 6,000 RFU for the IQCL.

IQC system results

Inhibition was detected in 4 times more samples at the IQCL marker than at the IQCS marker. Figure 3 details the comparative results. Likewise, the larger autosomal markers were more affected by inhibition and showed lower peak heights or more allelic dropout than the smaller markers.

Overall, inhibition based on the IQC results was detected in 76% (N = 31) of the bone samples tested, compared to 7% (N = 3) when the IPC was used for the detection. When the IQCS indicated inhibition of the PCR reaction (N = 8), the resulting profile was negative or resulted in a single valid locus. Of the 8 samples showing some level of inhibition at the IQCS marker, inhibition of 3 samples was also detected at the quantification stage. Despite amplifying dilutions of these 3 samples, the IQC system was robust in reacting to inhibitors present in the PCR reaction at both the IQCS and IQCL markers.

Of the 41 samples, 4 samples generated valid results at all loci and neither IQC marker indicated the presence of inhibitors. When inhibition was indicated, the number of valid loci was reduced, which could eventually reduce the success rate for bone identification. Table 2 details quantification, degradation, inhibition, and amplification results for all 41 samples. Figure 4 depicts electropherograms from 3 samples.

Table 3. Intensity of the IQCS and IQCL peaks from positive and negative template controls.

Replicate	Positive template control (0.75 ng of DNA/PCR)		Negative template control	
	IQCS (RFU)	IQCL (RFU)	IQCS (RFU)	IQCL (RFU)
Average	**6,025**	**10,162**	**5,580**	**10,306**
Minimum	4,666	7,126	4,666	6,942
Maximum	7,281	12,952	8,904	17,004
Standard deviation	809	1,720	1,213	2,510

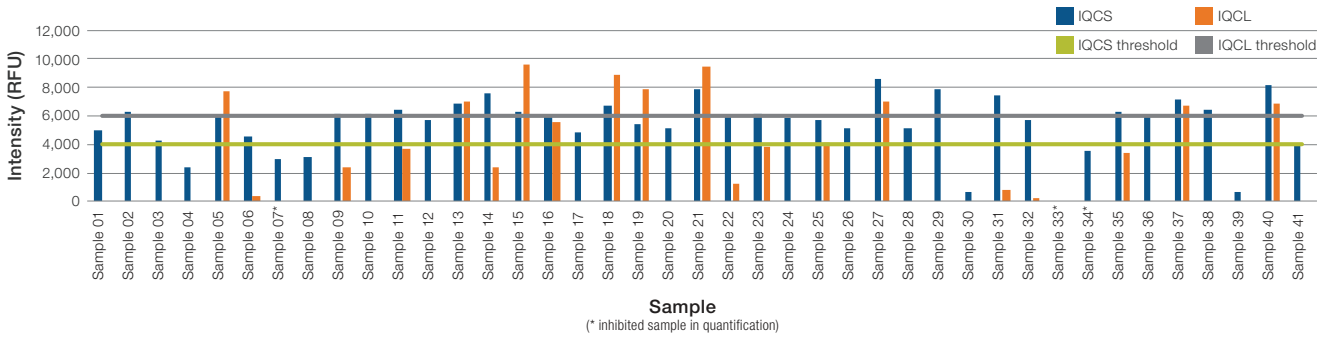

Figure 3. Intensity of the IQCS and IQCL peaks by bone sample.

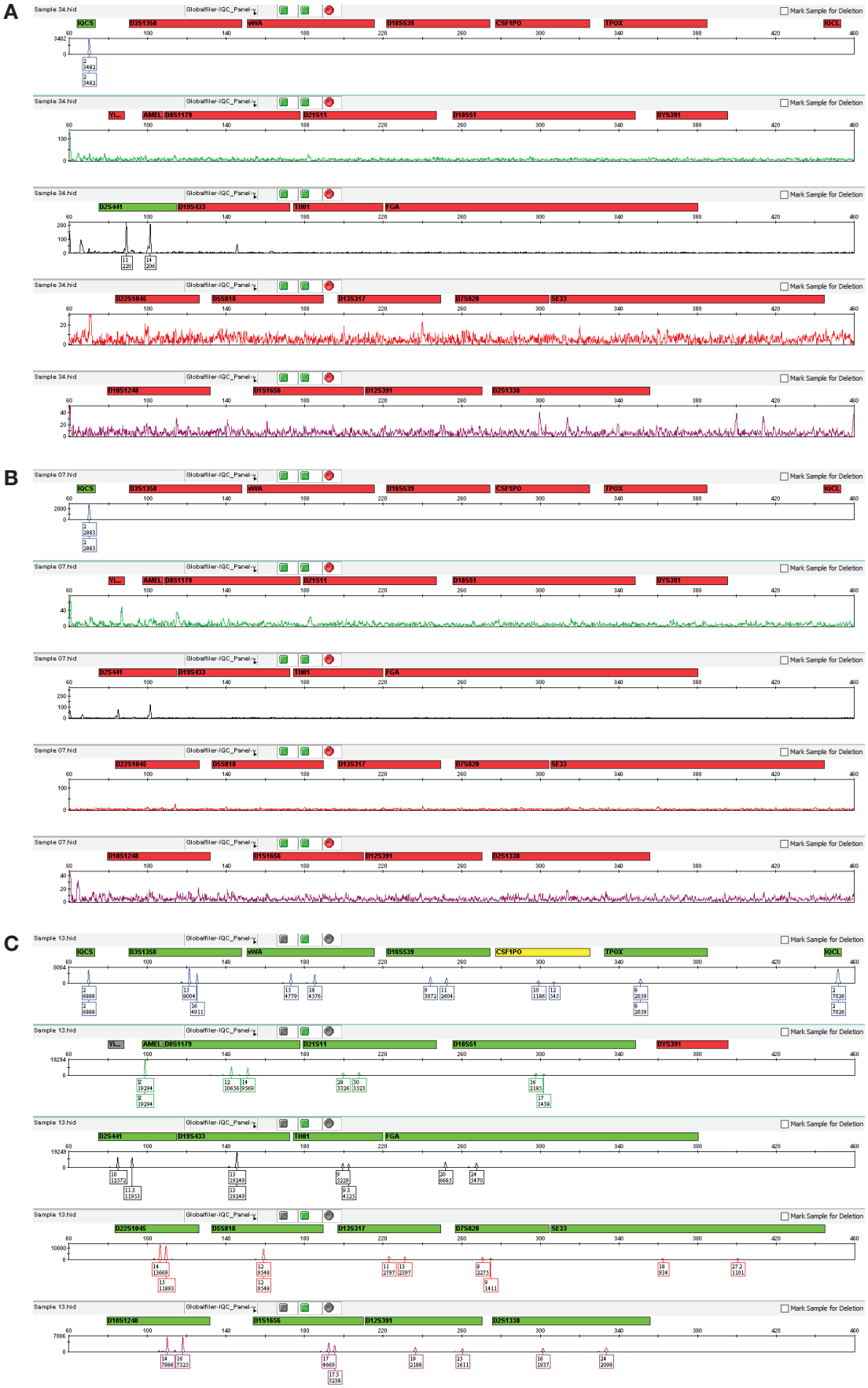

Figure 4. GeneMapper *ID-X* Software v1.4 electropherograms for three samples. Sample 34 **(A)** shows partial inhibition at the IQCS marker, full inhibition at the IQCL marker, and autosomal STR results at D2S441. Sample 7 **(B)** shows partial inhibition at the IQCS marker, full inhibition at the IQCL marker, and no autosomal STR results. Sample 13 **(C)** shows a full profile suitable for identification, with neither quality control marker indicating inhibition.

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Conclusion

Preparation of bone samples using an efficient process of drilling bone chips rather than turning bone into a fine powder can generate DNA profiles of sufficient quality for human identification.

DNA analysis from bone samples, regardless of the preparation method, can be challenging due to the presence of inhibitors co-extracted with DNA, and the additive impact of sample degradation. The internal positive control (IPC) used in the Quantifiler Trio kit may not be sensitive enough to detect low inhibitor concentrations in the quantification reaction volume; therefore, inhibitory effects on the STR PCR reaction are still possible, although not indicated at the quantification stage.

The internal quality control small and large (IQCS and IQCL) markers used in the GlobalFiler IQC kit offer an additional, reliable approach to detecting inhibition in the STR PCR reaction. When inhibition is indicated by the IQC system, strategies that can improve STR allele recovery by overcoming inhibition, such as reamplification with a diluted sample, or additional purification, can be considered.

The interpretation of results to maximize allele recovery is made more decisive with information about the samples being tested. Both the Quantifiler Trio and GlobalFiler IQC kits provide details about the quality of the sample that can promote allele recovery.

The information, data, and opinions in this application note are provided by Franck Jaffrédo, forensic scientist from IGNA. The parameters, procedures, and limitations regarding the internal validation of quantitation and amplification chemistries along with genetic analyzers are unique to each testing laboratory.

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