Application Note

Strategy for analysis of challenging bone samples using Investigator® Kits

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Short tandem repeat (STR) typing from skeletal remains is an extremely challenging task. Numerous abiotic factors (temperature and humidity at provenance and storage period) and biotic factors (e.g., microorganisms) can impair the analysis either by degradation or contamination of endogenous DNA or by inhibition of amplification. Therefore, sample selection is a critical step in the analysis of skeletal remains.

It is well established that dense and compact bones preserve DNA better than brittle and cancellous bones do (1). Generally, long bones and teeth are predominantly processed in forensic STR typing (2). In addition, petrous bone, one of the hardest bones in the human skeleton, shows high potential for STR typing in forensic (3, 4) and ancient DNA sciences (5). Because of the high prevalence of degradation and inhibition in bone samples and the impact these can have on STR typing, successful analysis requires surveillance of these events throughout the process. Here we present a strategy for analysis of challenging bone samples utilizing the Investigator Quantiplex® Pro Kit for quantification and the Investigator ESSplex SE QS Kit for highly sensitive amplification.

Methods

Sample preparation and DNA extraction

The sample set consisted of temporal bones from four skulls that were analyzed during missing-persons cases from 2011 to 2016 (Table 1, Figure 1). The petrous part of the temporal bones was isolated and decontaminated with commercial bleach (10%), water and ethanol (96%). Bones were ground in contamination-free disposable grinding chambers with a batch mill at 25,000 rounds per minute (rpm). 300 mg of ground bone powder was used for total demineralization with EDTA, proteinase K (PK), dithiothreitol (DTT) and Buffer ATL. After 20 h of incubation, the samples were concentrated on Microcon® DNA Fast Flow Centrifugal Filter Units. The lysate was collected and used for automated extraction.

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Table 1. Samples analyzed

Case	Skeletal element	Bone powder (mg)	Case year
1	Petrous bone	300	2011
2	Petrous bone	300	2011
3	Petrous bone	300	2012
4	Petrous bone	300	2016

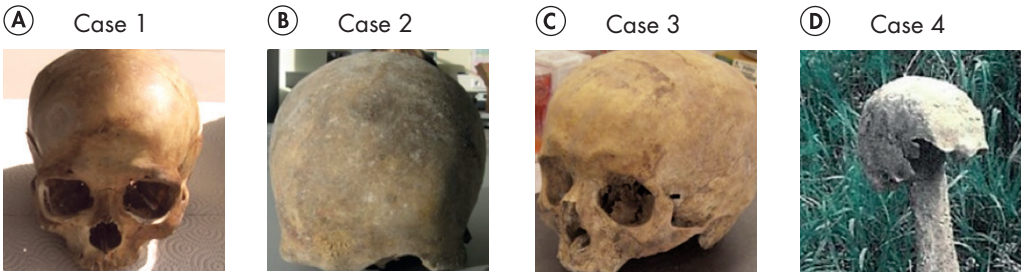

Figure 1. Overview of skull specimens.

DNA quantification with the Investigator Quantiplex Pro Kit

In all cases, DNA was quantified with the Investigator Quantiplex Pro Kit. Per reaction, 9 µl of Quantiplex Pro Reaction Mix was combined with 9 µl of Quantiplex Pro Primer Mix. 2 µl of DNA extract was added to a final volume of 20 µl per reaction. Control DNA was serially diluted, including 50 ng/µl, 1.8519 ng/µl, 0.0686 ng/µl and 0.0025 ng/µl dilutions. Amplification was carried out on the Applied Biosystems® 7500 Real-Time PCR System. Cycling started with an initial PCR activation step for 3 min at 95°C and was followed by 40 cycles of 5 s at 95°C for denaturation and 35 s at 60°C for combined annealing and extension. Data were collected during the second stage and were analyzed with the QIAGEN® Quantification Assay Data Handling and STR Setup Tool. The Investigator Quantiplex Pro Kit provides quantitative analysis of human and male DNA. The kit additionally determines the inhibition with an internal PCR control (IPC) and calculates a degradation index (DI) by computing the ratio of one long (353 bp) and one short amplicon (91 bp). Amplification targets are shown in Table 2.

Table 2. Overview of quantification targets

Target	Amplicon length (bp)	Dye channel	Ploidy	Copy number
Human target, small autosomal	91	FAM™	Diploid	Multi-copy
Male target	81	CY®5	Haploid	Multi-copy
Internal PCR control	434	JOE™	–	Synthetic fragment
Degradation target, large autosomal	353	TAMRA	Diploid	Multi-copy

Amplification using the Investigator ESSplex SE QS Kit

Amplification was accomplished using the Investigator ESSplex SE QS Kit in a reaction volume of 16 µl using 4.5 µl of Fast Reaction Mix 2.0 and 1.5 µl of Primer Mix. Up to 10 µl of DNA extract was added per reaction. Cycling started with 3 cycles of 30 s at 98°C, followed by 55 s at 64°C and 5 s at 72°C to activate hot-start DNA polymerase. This was followed by 27 cycles of 10 s at 96°C, 55 s at 91°C and 5 s at 72°C. Cycling finished with 2 min at 68°C. Interpretation of data is shown in Table 3.

Table 3. Interpretation of presence and absence of Investigator ESSplex SE QS Quality Sensor™ 1 (QS1) and 2 (QS2)

Allele peaks	QS1	QS2	Interpretation
Present	Present	Present	Successful profile
Absent	Present	Present	No DNA
Absent	Absent	Absent	Failed PCR
Ski-slope profile	Present	Dropdown	Inhibitors present
Ski-slope profile	Present	Present	Degraded DNA

Capillary electrophoresis

For capillary electrophoresis, 1 µl of amplified DNA product was combined with 12 µl Hi-Di formamide and 0.5 µl size standard BTO 550. Reaction plates were incubated for 5 min at 92°C for denaturation and analyzed on an ABI PRISM® 3130 Genetic Analyzer with an injection time of 16 s at 1.2 kV using a 36 cm long array with performance optimized polymer (POP) 7 as separation matrix. Data were analyzed with the GeneMapper® ID v3.2 software using a peak threshold of 50 relative fluorescent units (rfu).

Results

DNA quantification and degradation assessment

Quantification results are shown in Table 4.

Table 4. Quantification results of the Investigator Quantiplex Pro Kit

Case	Human DNA (ng/µl)	Male DNA (ng/µl)	Degradation DNA (ng/µl)	Degradation index
1	0.002	0.004	0.0004	5.0
2	0.003	0.005	0.0004	7.5
3	0.004	0.010	0.0009	4.4
4	0.019	0.025	0.0027	7.0

As expected, DNA amounts in bone samples were low. The degradation indices were elevated, indicating that DNA of the bone samples was degraded. Inhibition indices were not increased. Because DNA amounts were low, maximum input was used for subsequent STR amplification.

Amplification

Table 5. Amount of amplified alleles (maximum alleles = 34)

Case	Amplified alleles	Percentage of amplified alleles
1	28	83
2	31	91
3	34	100
4	32	94

The amount of amplified alleles (Table 5) correlated with the quantified DNA concentrations. The highest numbers of alleles were detected for cases 3 and 4, where DNA levels were comparably higher. STR profiling with the Investigator ESSplex SE QS Kit resulted in reportable profiles for all cases. QS results were concordant with observed IPC values and showed no significant interference of amplification by inhibition. Although QS were detectable, "ski-slope" profiles were observed for cases 1, 2 and 4 (Figure 2), showing that the DNA of the samples – as indicated by the increased DI – was degraded.

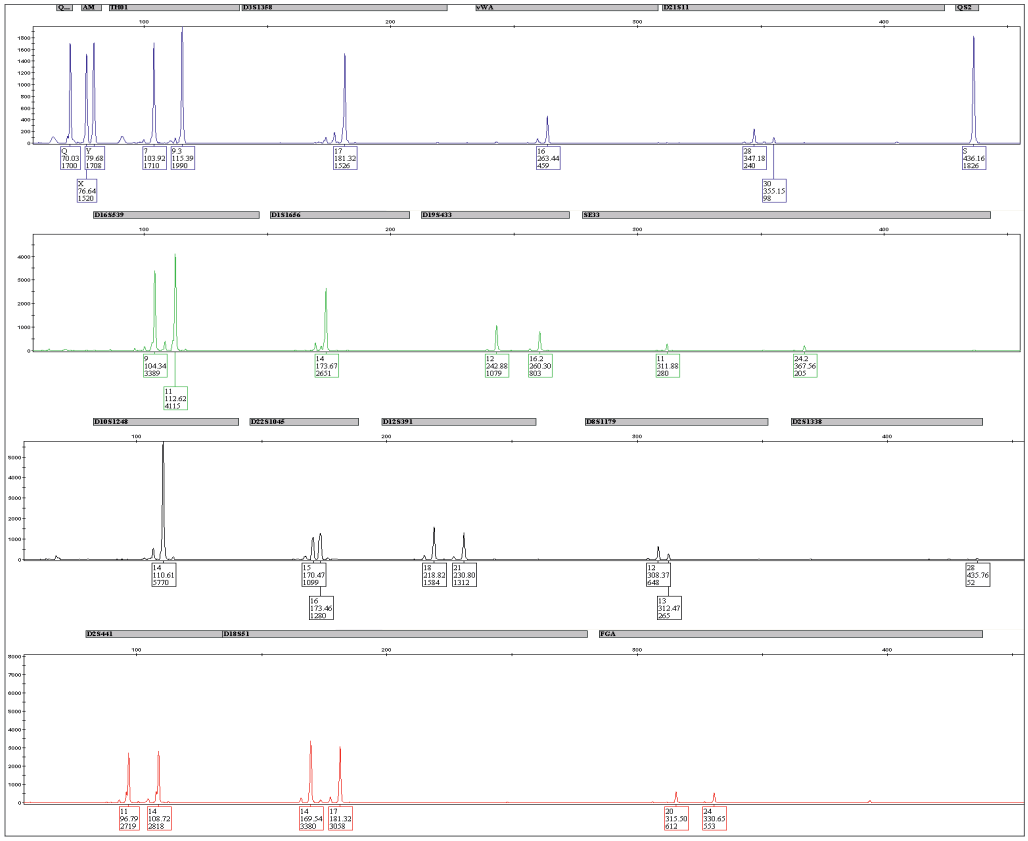

Figure 2. Electropherogram of case 4.

Conclusion

Use of the Investigator Quantiplex Pro Kit for challenging bone samples provides not only quantification of human and male DNA but also an assessment of the quality of the extracted DNA by amplification of two long targets, the 434 bp IPC and a 353 bp large autosomal human fragment. A DI can be calculated by dividing the quantity of the small autosomal target by the quantity of the large one. The DI reveals if the DNA in the sample is degraded or not while the IPC shows if an inhibition is present and whether the sample should be re-extracted.

Additionally, the Investigator ESSplex SE QS Kit provides a quality system with two Quality Sensors (QS1 and QS2) in the blue channel that can be used to monitor inhibition and degradation of the samples. Because Quality Sensors and the IPC use the same synthetic fragments, the possibility to get consistent results for inhibition monitoring during quantification and amplification is high. Furthermore, the Investigator ESSplex SE QS Kit performs a highly sensitive amplification resulting in reportable profiles in all analyzed bone cases. In summary, it can be concluded that the combination of the Investigator Quantiplex Pro Kit and the Investigator ESSplex SE QS Kit provides a powerful tool to monitor degradation and inhibition in challenging forensic samples, such as bones.

Summary

- The Investigator Quantiplex Pro Kit allows accurate quantification in low-template samples, such as bones
- The innovative degradation index shows if degradation occurred in samples
- The Investigator ESSplex SE QS Kit amplifies reproducible profiles with high sensitivity, especially for polymorphic SE33
- Quality Sensors QS1 and QS2 of the Investigator ESSplex SE QS Kit show if degradation or inhibition is present

References

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4. Kulstein, G., Hadrys, T., and Wiegand, P. (2017) As solid as a rock – comparison of CE- and MPS-based analyses of the petrosal bone as a source of DNA for forensic identification of challenging cranial bones. Int. J. Legal Med. doi:10.1007/s00414-017-1653-z.
5. Gamba, C. et al. (2014) Genome flux and stasis in a five millennium transect of European prehistory. Nature Communications **5**, doi: 10.1038/ncomms6257.

Ordering Information

Product	Contents	Cat. no.
Investigator Quantiplex Pro Kit (200)	For use on Applied Biosystems 7500 Real-Time Systems: Quantiplex Pro Reaction Mix, Quantiplex Pro Primer Mix, Quantiplex Pro Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387216
Investigator ESSplex SE QS Kit (100)	Primer Mix, Fast Reaction Mix including Taq DNA Polymerase, Control DNA, Allelic Ladder ESSplex SE QS, DNA size standard 550 (BTO) and Nuclease-Free Water	381575
Investigator ESSplex SE QS Kit (400)	Primer Mix, Fast Reaction Mix including Taq DNA Polymerase, Control DNA, Allelic Ladder ESSplex SE QS, DNA size standard 550 (BTO) and Nuclease-Free Water	381577

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