Application Note

Amplification of Y-chromosomal short tandem repeats from skeletal material of the Napoleonic, Merovingian and Bronze Age time periods using the latest QIAGEN Investigator Argus Y-28 QS Kit

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Introduction

The analysis of DNA from human skeletal remains is a frequent and important challenge for a variety of different scientific fields. Forensic scientists, bioarcheologists as well as provenance researchers are faced with the task of identifying human individuals from their skeletal remains, be it to answer historical questions or to repatriate human remains to families following a crime, missing persons identification, war or other mass disasters. In some scenarios, the identification of human individuals based on autosomal short tandem repeats (STRs), also known as genetic fingerprinting, may not be sufficient. In these cases, the analysis of the sex chromosomes may yield further insight, as these allow for the analysis of familial relationships between individuals and may even indicate the biogeographic origin of skeletal remains. To address these questions, analysis methods that produce reliable results regardless of the sample material, the state of DNA preservation and the low amount of DNA are needed. This application note presents the results of the examination of Y-chromosomal STRs from different skeletal remains found in Germany from the bronze age to the Napoleonic time.

To obtain usable DNA profiles that could be compared to a database, the whole DNA analysis process needs to be optimized and all potential inhibitors removed or at least identified. This workflow has utilized QIAGEN® instrumentation and chemistry: MinElute® columns for purification, Investigator® Quantiplex® Pro RGQ for the quantification and quality assessment of the DNA extracts and finally amplification with the Investigator Argus Y-28 QS, the latest QIAGEN assay specifically targeting the Y-chromosome.

Materials and Methods

DNA samples

Samples from three different sets of skeletal remains with varying ages were analyzed and are presented in this study.

The first set consisted of the most recent samples from the early 19th century analyzed which were excavated in 2015 in Rödelheim, a district of Frankfurt am Main (Hesse, Germany). Historical and archaeological evidence suggests that the skeletons are the remains of Napoleonic soldiers, who most likely died due to a typhus epidemic between 1813 and 1815 (Hampel 2015).

The second set of from Boilstädt and were discovered during construction work near Gotha-Boilstädt (Thuringia, Germany). They were excavated between 2012 and 2013. The majority of the burials were dated to the early Middle Ages (1500 – 1300 YBP), the Merovingian era, based on archaeological findings (Tannhäuser 2023).

The third set, the oldest samples included in this study, are from the Lichtenstein cave near Osterode am Harz (Lower Saxony, Germany) and date back to the bronze age (3000 YBP) when the cave was used as a secondary burial site (Flindt & Hummel 2021). The skeletal remains were discovered in 1980 and excavated between 1993 and 2011 in several separate excavation campaigns.

Lysis and purification

All analysis steps followed established and published protocols (Mazanec 2022, Euskirchen et al. 2021, Flux et al. 2017, Hummel 2003), and the different steps are summarized in the following paragraphs.

Bone preparation

Tooth roots were separated from the tooth crowns and bone samples were sawn using a hand drill with a diamond saw blade. To remove possible surface contaminants, the tooth roots and bone fragments were placed in a 6% sodium hypochlorite solution for 10 to 15 minutes and then rinsed with bi-distilled water (Barta et al. 2013, Kemp and Smith 2005). Afterwards, all samples were mechanically pulverized in a bead mill at 24 Hz for approximately 10 to 20 seconds.

Decalcification and lysis

For the decalcification and lysis steps, 250 mg of tooth or bone powder was incubated under constant inversion in 3900 µL EDTA (0.5 M, pH 8.0, Invitrogen™) and 100 µL Proteinase K solution in Tris/HCl (pH 7.5, 0.01 mol/L, 600 mAnson-U/mL, Merck) for 18 hours at 56°C. After this incubation, 50 µL sodium dodecyl sulphate (10 mg/mL, Sigma-Aldrich®) was added to the suspension and incubated for 5 min at 65°C. The remaining solid substances were pelleted by centrifugation and the lysate was processed for DNA extraction and purification with MinElute spin columns (QIAGEN) and the QIAvac®-system (QIAGEN) following the manufacturer's protocol. For the sample series from Rödelheim and Boilstädt, the lysates underwent an organic DNA extraction using phenol and chloroform (Carl Roth GmbH + Co. KG) before the QIAvac-system (Flux et al. 2017).

For both protocols, approximately 3500 µL of lysate was mixed with five times the quantity of PB-Buffer (QIAGEN) and subsequently transferred to the MinElute tubes. Three washing steps using 700 µL PE buffer were performed, followed by three elution steps with 56°C ultrapure water (RNAse-Free Water, QIAGEN) and 5 min incubation each to a total elution volume of 60 µL. The DNA extracts were stored at –18°C.

DNA quantification

Samples were quantified with the Investigator Quantiplex Pro RGQ Kit, which provides quantification of human genomic DNA, male DNA and information about the DNA integrity of a sample using quantitative real-time PCR.

This assay consists of five specific targets to assess the quantity of human DNA (short DNA fragment), the quantity of male DNA (male-specific short DNA fragment), an internal control to detect inhibition and two longer DNA fragments (human DNA and male DNA) to give precise information about the degradation status.

The assay targets' fragment sizes are listed in Table 1.

Table 1.
Target size of the Investigator Quantiplex Pro RGQ Kit

Human target, small autosomal	91 bp
Human target, large autosomal	353 bp
Human male target, small Y-fragment	81 bp
Human male target, large Y-fragment	359 bp
Internal control (IC)	434 bp
Limit of detection	0.5* pg/µL – 200 ng/µL

*Stochastic effects might appear for the lower quantities.

It's important to note that the longer target fragments (autosomal and gonosomal) are utilizing the same region as their respective smaller fragments as this provides a fairer and more accurate assessment of DNA integrity.

The Investigator Quantiplex Pro RGQ Kit was prepared following handbook recommendations. An input volume of 2 µL of purified DNA was processed for each sample.

DNA quantification was carried out on a RotorGene® Q instrument and the results were analyzed using Q-Rex software.

All of the quantification results were exported from Q-Rex software and imported into the QIAGEN Quantification Assay Data Handling and STR Setup Tool. This tool is freely available on the QIAGEN website and can be found on the Investigator Quantiplex Kits page under the Additional Resources tab. The tool gives the user an overview of the data and presents a clear indication of potential mixture, degradation and/or inhibition.

Calculation of the mixture index, degradation index for male and human targets and inhibition index are performed by comparing the quantities of the different targets within each sample. Ratios are compared to the thresholds set up within the tool. For this study, the following thresholds were used:

- Mixture index (Human quantity/Male quantity): 2 (will flag when the human quantification result is two times or more than the male quantification result)
- Degradation index (Human quantity/Human degradation quantity): 10 (will flag when the small human target quantification is 10 times or more than the large target quantification)
- Degradation index for the male fragment (Male quantity/Male degradation quantity): 10 (will flag when the small male target quantification is 10 times or more than the large male target quantification)
- Inhibition Index (IC Shift): 1 (the average absolute value of the IC standard – IC sample)

STR analysis and CE control

Amplification was performed using the Investigator Argus Y-28 QS Kit. This kit contains 27 Y-chromosomal STR markers and an integrated Quality Sensor split across 6 dyes. It contains highly discriminating markers with 17 commonly used markers from AmpFLSTR™ Yfiler™ PCR Amplification Kit and 10 other commonly used Y-chromosomal markers, of which 6 are rapidly mutating.

In this study, the total PCR volume is 25 µL and consists of 7.5 µL Fast Reaction Mix 3.0, 2.5 µL Primer Mix and 7.5 µL of water. 7.5 µL of each DNA extract was added to the reaction for each sample. PCR cycling was performed according to the manufacturer's protocol (for the temperature) and by increasing the number of cycles from the recommended 31 to 35.

Table 2.
Cycling protocol for DNA samples

Temperature	Time	Number of cycles
96°C	12 minutes	–
96°C	10 seconds	35 cycles
61.5°C	1 minutes 25 seconds	
72°C	5 seconds	
68°C	5 minutes	–
60°C	5 minutes	–
10°C	∞	–

PCR products were stored at –18°C until further processing. Amplification success was checked via standard agarose gel electrophoresis on 2.5 % agarose gels (for protocol details, see Hummel 2003).

For capillary electrophoresis, the PCR product added varied between 0.1 and 2 µL (depending on the product intensity during agarose gel electrophoresis, Wittmeier and Hummel 2021). They were mixed with 12 µL Hi-Di formamide and 0.25 µL DNA Size Standard 24plex BTO from the Argus Y-28 QS Kit. For the denaturation, the reaction plates were incubated at 95°C for 3 min.

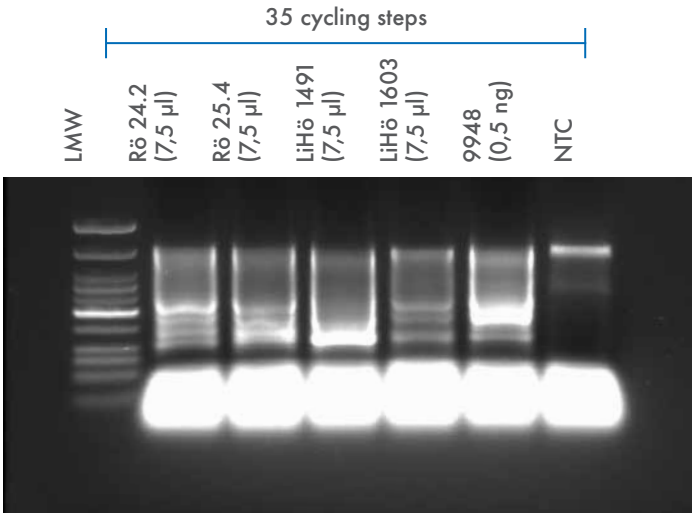

Figure 1.
Example of results with agarose gel electrophoresis.

The capillary electrophoresis was performed on a 3500 Series Genetic Analyzer (Applied Biosystems®) with a 15-second injection time at 1.2 kV, a 36 cm array and POP4™ Polymer (Applied Biosystems®) as the separation matrix. Data was analyzed using the GeneMapper 5 software (Applied Biosystems®) and an analytical threshold of 50 relative fluorescent units (rfu) was applied.

Results and discussion

Quantification and STR profile

Following the quantification, none of the samples were showing any signs of inhibition demonstrating a good extraction and efficient purification method. The results for the male fragment quantification and assessment are presented in Table 3.

Table 3.
Summary of the different individuals found in the Lichtenstein cave and the associated sample material

Sample / Individual	Skeletal set	Sample material	Male Short Fragment [ng/µl]	Male Long Fragment [ng/µl]	Male DI	Male Degradation Threshold	# of alleles recovered
916.08 PMb_M4	Lichtenstein cave	Tooth	1.75	0.02	109.63	Possible Degradation	27
3421_M3		Vertebra	1.08	0.21	5.20	Below Threshold	27
1491_M1		Vertebra	0.56	0.04	14.52	Possible Degradation	27
3609_M5		Sacrum	0.36	0.05	7.82	Below Threshold	27
1603_M9		Os coxae	0.09	0.00	23.49	Possible Degradation	27
58.02_M10		Femur	0.07	0.01	10.50	Possible Degradation	27
65.08_M11		Tibia	0.04	0.00	NA	Possible Degradation	21
322_M2		Tibia	0.03	0.00	NA	Possible Degradation	21
928.07_M14		Tooth	0.03	0.00	NA	Possible Degradation	13
56.01_M17		Femur	0.02	0.00	47.40	Possible Degradation	23
2402_M6		Os coxae	0.02	0.00	41.89	Possible Degradation	21

Table 3.
Summary of the different individuals found in the Lichtenstein cave and the associated sample material

Sample / Individual	Skeletal set	Sample material	Male Short Fragment [ng/µl]	Male Long Fragment [ng/µl]	Male DI	Male Degradation Threshold	# of alleles recovered
96	Boilstädt	Tooth	0.52	0.06	8.59	Below Threshold	27
90		Pars petrosum	0.21	0.03	7.64	Below Threshold	27
140		Tooth	0.16	0.00	58.04	Possible Degradation	26
2.1		Tooth	0.15	0.00	44.23	Possible Degradation	27
88		Tooth	0.10	0.00	36.22	Possible Degradation	26
118		Tooth	0.09	0.00	33.54	Possible Degradation	27
131		Tooth	0.07	0.00	53.13	Possible Degradation	27
152.2		Tooth	0.03	0.00	61.89	Possible Degradation	27
91.2		Tooth	0.02	0.00	30.13	Possible Degradation	22
89		Tooth	0.02	0.00	45.40	Possible Degradation	27
14.4	Rödelheim	Tooth	0.47	0.02	23.67	Possible Degradation	27
10.2		Tooth	0.44	0.02	22.04	Possible Degradation	27
18.1		Tooth	0.44	0.00	112.85	Possible Degradation	26
12.1		Tooth	0.43	0.03	12.72	Possible Degradation	27
6.4		Tooth	0.29	0.03	9.61	Below Threshold	27
24.5		Tooth	0.23	0.00	142.08	Possible Degradation	27
25.4		Tooth	0.14	0.00	30.82	Possible Degradation	27
18.3		Tooth	0.11	0.00	83.55	Possible Degradation	26
8.3		Tooth	0.04	0.00	NA	Possible Degradation	25
7.4		Tooth	0.03	0.00	9.99	Below Threshold	26
10.4		Tooth	0.03	0.00	33.39	Possible Degradation	26
7.1		Tooth	0.02	0.00	23.01	Possible Degradation	25
6.3		Tooth	0.02	0.00	41.71	Possible Degradation	24
20.2		Tooth	0.01	0.00	13.36	Possible Degradation	21
27.8		Tooth	0.01	0.00	NA	Possible Degradation	15
27.12		Tooth	0.01	0.00	NA	Possible Degradation	18
12.4		Tooth	0.01	0.00	NA	Possible Degradation	16
20.9		Tooth	0.01	0.00	73.12	Possible Degradation	17
13.1		Vertebra	0.01	0.00	NA	Possible Degradation	22
20.4		Tooth	0.01	0.00	NA	Possible Degradation	15
25.1		Tooth	0.01	0.00	NA	Possible Degradation	24
20.3		Tooth	0.01	0.00	NA	Possible Degradation	16
19.2		Tooth	0.01	0.00	18.56	Possible Degradation	23
6.1		Tooth	0.00	0.00	17.08	Possible Degradation	21
7.2		Tooth	0.00	0.00	14.14	Possible Degradation	23
9.3		Tooth	0.00	0.00	NA	Possible Degradation	15

For the bronze age samples from the Lichtenstein cave, 2 out of 11 show degradation "below threshold." The other samples show low degradation (with a ratio of 10.5) to high degradation (no long fragment quantified), which is defined in Table 3 as "NA=Not Applicable" in the QIAGEN data handling tool.

The same observation was made for the Boilstädt samples dating from the Middle Ages, 2 out of 10 show degradation "Below Threshold," and the other samples have a degradation ratio ranging from 30.13 to 61.89.

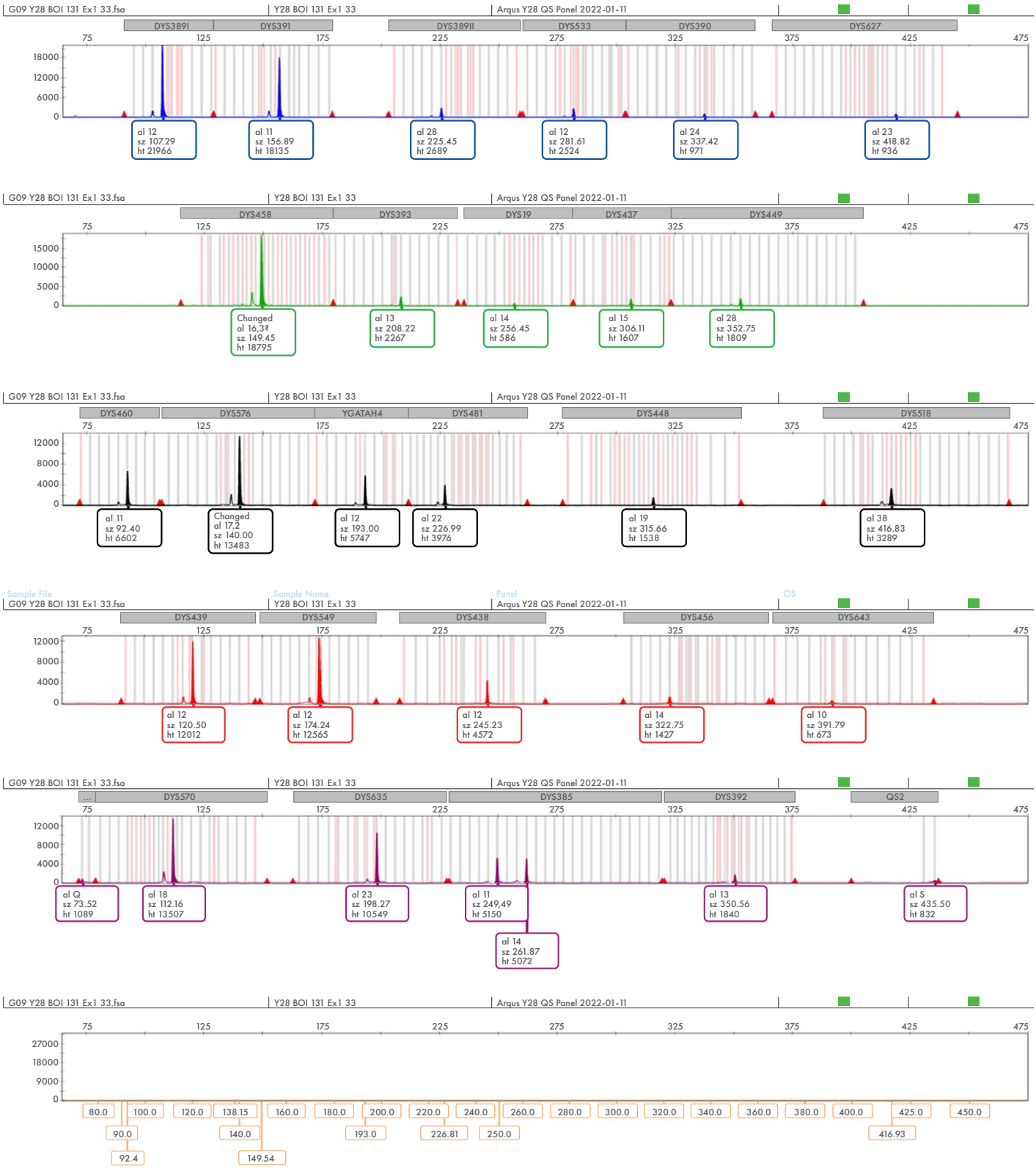

Figure 2.
Electropherogram from sample Boilstädt 131.

For the samples from the Rödelheim series, the most recent samples from the Napoleonic period, 2 out of 26 did not present any degradation of the Y-chromosomal DNA. However, this sample set appears to have higher DNA degradation than the other sample sets, as the amplification of the longer Y-fragment was successful for only one third (9 out of 26) of the samples. The smaller Y fragment was successfully amplified in 23 out of 26 samples.

Similar observations were also made for the quantification of the autosomal DNA fragments (data not shown).

The results of the quantification of human and male DNA were expected because ancient DNA was being analyzed. Ancient DNA is usually strongly degraded, which is reflected in the fragmentation of the DNA. Therefore, long DNA fragments are often not amplified. The degradation is not proportional to the age of the bones, but rather to the condition at which they were preserved. The oldest samples in the Lichtenstein cave, for example, were conserved at a low and stable temperature in a cave protected from sunlight and further weathering influences, which is ideal for DNA preservation.

The STR amplification of the Y-chromosomal DNA was successful for the ancient samples using an increased number of cycles, but some artifacts e.g. preferential amplification of shorter STRs and overamplified stutter bands were observed as well as drop-outs, particularly of higher molecular weight markers. Both Quality Sensors were amplified for all samples, confirming that no inhibitors were present in the reaction and that the DNA extraction method used was very efficient.

Conclusion

The Investigator Quantiplex Pro RGQ and Investigator Argus Y-28 QS are suitable for use with ancient DNA extracted from skeletal remains. By adding maximum volume of template DNA (15µL) and increasing the PCR cycles from the recommended 31 to 35, Investigator Argus Y-28 Kit generated DNA profiles even from the Bronze Age samples. The Investigator Quantiplex Pro RGQ results can also help further optimize this workflow by utilizing the latest features in our Data Handling Tool whereby PCR input amounts are adjusted based on the levels of inhibition and degradation.

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Ordering Information

Product	Contents	Cat. no.
Investigator Argus Y-28 QS (100) *Larger kit sizes are available. Please inquire.	For 100 reactions: Primer Mix, Fast Reaction Mix 3.0, Control DNA, Allelic Ladder, DNA Size Standard, Nuclease-Free Water	383625
Investigator Quantiplex Pro RGQ Kit (200)	For use on QIAGEN Rotor-Gene Q Real-Time Systems: Quantiplex Pro RGQ Reaction Mix, Quantiplex Pro RGQ Primer Mix, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387316
QIAprep 2.0 Spin Miniprep Columns (100)	100 spin columns	27115
RNAse-free Water	12 x 1.9 ml RNase-free water prepared without the use of diethylpyrocarbonate (DEPC)	129112
QIAvac Vacuum Systems	Vacuum manifold for processing 1–24 spin columns: includes QIAvac 24 Plus Vacuum Manifold, Luer Plugs, Quick Couplings	19413
Rotor-Gene Q	Real-time PCR cycler with 2 channels (green, yellow), laptop computer, software, accessories: includes Priority Package with software, installation, training, 3-year warranty on parts and labor, and 3 preventive maintenance visits	9001862

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