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TECHNICAL NOTE

A sensitive method to extract DNA from biological traces present on ammunition for the purpose of genetic profiling

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Abstract Exploring technological limits is a common practice in forensic DNA research. Reliable genetic profiling based on only a few cells isolated from trace material retrieved from a crime scene is nowadays more and more the rule rather than the exception. On many crime scenes, cartridges, bullets, and casings (jointly abbreviated as CBCs) are regularly found, and even after firing, these potentially carry trace amounts of biological material. Since 2003, the Forensic Laboratory for DNA Research is routinely involved in the forensic investigation of CBCs in the Netherlands. Reliable DNA profiles were frequently obtained from CBCs and used to match suspects, victims, or other crime scene-related DNA traces. In this paper, we describe the sensitive method developed by us to extract DNA from CBCs. Using PCR-based genotyping of autosomal short tandem repeats, we were able to obtain reliable and reproducible DNA profiles in 163 out of 616 criminal cases (26.5%) and in 283 out of 4,085 individual CBC items (6.9%) during the period January 2003–December 2009. We discuss practical aspects of the method and the sometimes unexpected effects of using cell lysis buffer on the subsequent investigation of striation patterns on CBCs.

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Abbreviations

STR	Short tandem repeat
PCR	Polymerase chain reaction
CBC	Cartridges bullets and casings
GSR	Gun shot residue

Introduction

Matching DNA profiles obtained from cartridges, bullets, and casings (CBCs) to a suspect—directly or as a hit in a suspect database—can be a very useful evidence because it can directly link the suspect to the crime involved. In most non-military situations, it is assumed that trace amounts of skin cells are transferred to cartridges while loading a magazine because this often requires substantial force. We specifically make this restriction to non-military situations because for military use, magazines are invariably loaded automatically and trace amounts of skin cells are not expected to be found. Especially the last items loaded into a magazine—thus the first to be fired—are considered as being most suitable for recovering biological material since more force has to be used for loading. Following shooting incidents, it is possible to retrieve a number of different categories of (parts of) ammunition at the crime scene: (1) the intact cartridge, often still in its magazine loaded into the firearm, (2) the empty casing thrown out of the firearm, and (3) the bullet (penetrating or non-penetrating). Apart from the penetrating bullets—which are likely to contain predominantly biological material from the victim—all parts of ammunition can hold biological material from the

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person who loaded the magazine. This person is often, but not always, also the primary suspect of the shooting incident.

For this reason, we developed a robust yet sensitive method to routinely isolate DNA from CBCs in 2003. In the forensic literature, we found a description of a method to type mitochondrial DNA [1] and two articles on short tandem repeat (STR) analysis on biological trace material from penetrating bullets [2, 3]. These methods were useful for linking these bullets to victims, but obviously, they cannot be used to link them to the suspect. We were unable to find literature describing a DNA extraction method specific for non-penetrating (parts of) ammunition.

There are several technical issues that need to be taken into account when analyzing CBCs for biological traces. First, the complete procedure should not interfere with other forensic analyses on the same ammunition. Casings always contain a small amount of gun shot residue (GSR) that should be secured prior to DNA analyses, but without contamination. Second, bullet and casing striations should be preserved for future analysis. Third, there are also some potential difficulties with the DNA quality. The amount of biological cell material on the ammunition is typically low, and because the intense heat during firing is detrimental to DNA, one often obtains degraded DNA fragments. Therefore, our aim was to develop an extraction procedure that yields as much DNA as possible while being non-interfering to subsequent analysis of CBCs. Initial results from a small pilot experiment suggested that reliable and reproducible DNA profiles could be obtained from CBCs. In this paper, we present the results of our final protocol for the DNA analyses on cartridges, bullets, and casings. We summarize our results obtained with this protocol from 616 criminal cases involving 4,085 individual CBC items collected between January 2003 and December 2009.

Materials and methods

DNA source material

In the Netherlands, from the moment investigators arrive at a crime scene up to DNA analyses in a forensic DNA laboratory, special precautions are routinely taken to prevent contamination with non-crime-related DNA material. Dutch crime scene investigators work according to the so-called forensic technical norms (FT norms) that describe all the necessarily procedures relating to handling forensic DNA trace material at a scene of crime. Examination gloves, face masks, and protective clothing should be worn when handling the CBCs by crime scene investigators. While processing CBCs, priority should be given to the DNA analysis over other analyses in order to minimize the risk of losing cells or contaminating cell material. Therefore, GSR from cartridge cases is secured at our DNA facility prior to the DNA analysis trajectory instead of at the physical chemistry department of the forensic laboratory routinely involved in investigating CBCs.

DNA extraction protocol

All DNA extraction procedures and PCR preparations are performed in a dedicated forensic pre-PCR facility. Negative controls are included at each step. The extraction is performed using the QIAamp® DNA Mini kit following the manufacturer's protocol (Qiagen) for bloodstains, but with some specific changes and additions, which we describe in more detail below. The protocol is flexible and leaves room for adjustments. In our hands, the time necessary to extract DNA from ten CBCs is approximately 4 h.

A single CBC is put into a sterile 10-ml round bottom tube (Greiner) with 400 μl of ATL buffer from the DNA Mini kit. By automatically rotating the tube under a suitable angle for 30 min at room temperature, we ensure that as much as possible, only the outer surface of the CBC comes in contact with the buffer. The optimal angle depends on the size of the CBC. This is most crucial for casings because they are open at one end; bullets and cartridges can be fully submerged. After 30 min, the lysis buffer is transferred to a 2-ml sterile screw cap tube. The CBC is taken out of the tube and the outer surface is swabbed with a dry sterile cotton swab (Copan Innovation) to secure remaining cell material and to minimize the oxidizing effect of the ATL buffer on the CBC. This swab is added to the 2-ml tube containing the lysis buffer. The screw cap tube containing buffer and cotton swab is incubated for 10 min at 85°C while shaking at 800 rpm. Next, 30 μl of proteinase K (20 mg/ml, Qiagen) is added, and the solution is incubated at 56°C while shaking at 800 rpm for 1 h. Then, 400 μl of AL buffer (Qiagen) is added and the solution vortexed for 10 s followed by a final incubation of 10 min at 70°C while shaking at 800 rpm. Four hundred microliters of absolute ethanol is added and the solution is vortexed briefly. In two rounds, the extraction solution is transferred to a QIAamp mini spin column and spun during 1 min at 6,000×*g* (8,000 rpm) in a micro-centrifuge in order to bind the DNA to the silica matrix inside the column. The DNA is then washed with 500 μl of wash buffer 1 (buffer AW1, Qiagen) and spun during 1 min at 6,000×*g*. Then, 500 μl of wash buffer 2 (buffer AW2, Qiagen) is added and the column is spun during 3 min at 20,000×*g* (13,000 rpm). The flow-through from the washing steps is discarded and the column placed into a clean collection tube (Qiagen). The DNA is eluted by loading 80 μl of sterile and pyrogene free water (Aqua Braun) on the column and centrifuging during 1 min at 6,000×*g*. The eluate is transferred back

onto the column and centrifugation is repeated for 1 min at 6,000×*g*. This final step is labor-intensive and can be omitted if the centrifuge has a so-called soft mode. By activating this "soft mode" function, start-up and braking times will be lengthened. This mode is developed to centrifuge sensitive samples. Switching to this mode and centrifuging 2 min at 6000×*g* is then the optional, and preferable, elution step.

PCR amplification of STRs

Prior to PCR amplification, we split the 80 μl of final DNA extract into two aliquots of 40 μl. One of these aliquots is stored for further possible contra-expertise DNA research, as is mandatory under Dutch Law. The remaining second aliquot is used for our routine protocol described here. Prior to 2008, no DNA quantification was performed. Since 2008, we use the Quantifiler Duo DNA quantification kit (Applied Biosystems) to infer DNA quality and quantity.

We routinely use the Powerplex® 16 system (Promega). This kit contains 15 autosomal STRs and facilitates a provisional gender typing by means of the Amelogenin locus. Our PCR setup uses a reaction volume of 12.5 μl, containing 1.25 μl 10× PCR buffer, 1.25 μl Primer mix, 0.40 μl Amplitaq Gold (Applied Biosystems), and 6.6 μl sterile and pyrogene-free water. We use 3.0 μl of DNA extract as PCR template. Decreasing the amount of water enables us to go up to 8.0 μl template DNA for low-concentrated DNA extracts. Negative controls and company-provided positive controls are included in the setup. Cycling conditions are as described in the forensic section of the Powerplex® 16 system manual, in some cases, when extended, the Powerplex® 16 system DNA profiles using the Identifiler ®or MiniFiler ™ (both Applied Biosystems) autosomal multiplex PCR kits.

Analysis of PCR products

STR variants are detected with an ABI3100 capillary gel electrophoresis system (Applied Biosystems). To do so, 1.0 μl of PCR product is transferred to an appropriate 96-well plate and mixed with 11.5 μl of sterile and pyrogene water and 0.5 μl of internal standard ILS600 (Promega). We used an 11-s and 3.0-kV injection. The raw data are analyzed with GenemapperID version 3.2 (Applied Biosystems) or GeneMarker 1.7 (Softgenetics).

Results and conclusion

Between January 2003 and December 2009, we processed 4,085 individual CBCs from 616 different crime cases by means of the above described protocol. Before further discussion, we first define how we interpret "reliable and reproducible DNA profile." For the purpose of this article, we use this term—abbreviated as DNA profile—for any number of individual DNA loci that could be genotyped reliably and reproducibly in a minimum of two, but often up to five, independent PCR reactions from a single sample source. In forensic case work on degraded DNA samples, it is often only possible to amplify the smaller loci because DNA fragments are typically in low quantity and/or highly degraded. It is our opinion that any number of reliably and reproducibly detected STR loci should be reported, although, of course, we are also aware of the limited value of a profile of a single or two STR loci. Especially for the purpose of this retrospective analysis, omitting such results is misleading. Hence, we did not restrict these success rate analyses to DNA profiles with a predefined minimum of STR loci. Full data are presented, allowing any reader to reanalyze our data using any predefined minimum loci cutoff value.

Since the success rates for cartridges and casings were rather similar, we combined their results (Tables 1 and 2 and Fig. 1). Over the entire period, the success rate per criminal case, defined as the number of criminal cases in which at least one DNA profile could be reported, varied between 16.5% (2006) and 52.6% (2003), with an average rate of obtaining a reproducible DNA profile in 26.5% of all criminal cases. Per CBC item, the rate of success varied between 3.8% (2008) and 13.9% (2003), with an average of 6.9%. Of the total number of 283 reproducible STR profiles, 45 (or 15.9%) were of mixed origin (contribution by a minimum of two persons). The remaining 238 (84.1%) where apparently from a single donor. In addition to these 283 profiles, we obtained eight profiles that could be attributed to one of the staff members handling CBC items in the laboratory.

The vast majority of the STR profiles (280, or 98.9%) contained reproducible STR genotypes of four or more STR loci (Fig. 1). A total of 51 profiles (18.0%) contained the full 15 STR locus Powerplex® 16 DNA profile. Of these, 44 were from a single donor and seven from a mixed DNA sample. In 33 (11.7%) samples, the 15 STR locus profile could be extended to 16 or 17 loci using either Identifiler® or MiniFiler™ kits.

It is difficult to put these numbers in a broader context simply because there are no other similar studies known to us. There is only one recently published study of Horsmann-Hall et al. [4] that provides some data in this respect. There are, however, many differences between that study and ours. Horsmann-Hall performed an exploratory study under strictly controlled conditions with known handlers of CBC items. Their preferred starting kit was MiniFiler™ kit, with an extension of a limited number of samples with the Powerplex® 16 system. Moreover, they

Table 1 Summary of the DNA profiling results of biological traces isolated from 4,085 CBCs in the period January 2003–December 2009

Year	*N* cases[a]	Succes rate per case[b] *N* (%)	*N* items[c]	Success rate per item[d] *N* (%)	*N* single contributor profiles[e] *N* (%)	*N* mixed profiles[f] *N* (%)	*N* profiles from staff members[g] *N*
2003	19	10 (52.6)	173	24 (13.9)	18 (75.0)	6 (25.0)	0
2004	50	14 (28.0)	337	38 (11.3)	25 (65.8)	13 (34.2)	0
2005	80	19 (23.8)	477	20 (4.2)	20 (100)	0 (0)	5
2006	85	14 (16.5)	419	19 (4.5)	17 (89.5)	2 (10.5)	0
2007	87	30 (34.5)	586	52 (8.9)	46 (88.5)	6 (11.5)	0
2008	131	24 (18.3)	811	31 (3.8)	26 (83.9)	5 (16.1)	1
2009	164	52 (31.7)	1,282	99 (7.7)	86 (86.9)	13 (13.1)	2
Combined	616	163 (26.5)	4,085	283 (6.9)	238 (84.1)	45 (15.9)	8

[a] *N* cases: annual total number of criminal cases in which at least one CBC was analyzed

[b] Success rate per case: the number of criminal cases in which at least one reliable and reproducible STR profile could be reported

[c] N items: annual total number of individual CBC items analyzed

[d] Success rate per item: the number of individual items from which a reliable and reproducible STR profile was reported

[e] *N* single contributor profiles: annual number of STR profiles of a single contributor and its percentage of the total annual number of individual items from which a reliable and reproducible STR profile could be obtained

[f] *N* mixed profiles: annual number of mixed STR profiles (of at least two contributors) and its percentage of the total annual number of individual items from which a reliable and reproducible STR profile could be obtained

[g] *N* profiles staff members: annual total number of profiles that could be retraced to a laboratory staff member. These totals are excluded from all summary statistics

studied a total number of 75 CBC items derived from a number of different experimental conditions. Finally, all of the 75 tested CBC items were known to be handled without gloves by a person. We analyze here, in retrospect, the results of a much larger number of CBC items (4,085) from 616 actual criminal cases under extremely variable and unknown conditions. Any direct comparison is misleading since, e.g., we simply do not know if each of the CBC items we tested had been handled without gloves by a person. In addition, our preferred starting kit was the Powerplex® 16 system, sometimes supplemented with MiniFiler™, or Identifiler ®. From Fig. 4a in [4], we

Table 2 Summary of number of STR loci per successfully obtained DNA profile isolated from 4,085 CBCs in the period January 2003–December 2009

No. of Loci	Year							Combined
	2003	2004	2005	2006	2007	2008	2009	
1	0	0	0	0	0	0	0	0
2	0	1	0	0	0	0	0	1
3	1	1	0	0	0	0	0	2
4	6	8	0	0	1	0	0	15
5	2	6	0	1	1	0	3	13
6	2	6	1	1	4	3	2	19
7	3	2	3	1	2	3	9	23
8	4	0	2	1	1	2	12	22
9	1	1	2	0	7	4	4	19
10	0	1	0	0	2	3	9	15
11	0	1	1	3	4	0	6	15
12	0	0	3	2	4	2	11	22
13	1	1	0	4	2	4	7	19
14	1	1	0	0	3	2	7	14
15	0	4	6	4	21	2	14	51
16	0	1	1	1	0	1	1	5
17	3	4	1	1	0	5	15	28

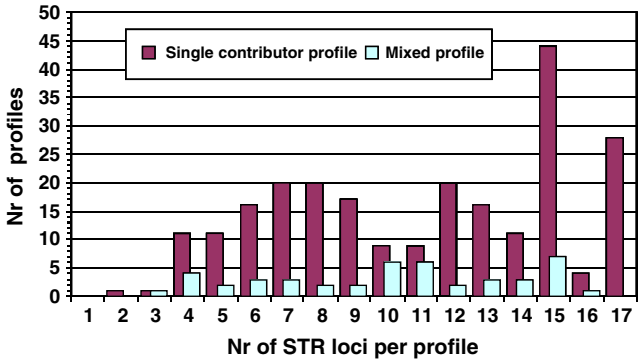

Fig. 1 Summary of the number of STR loci per successfully obtained DNA profile isolated from 4,085 cartridges, bullets, and casings (*CBC*) in the period January 2003–December 2009. Results are shown for single contributor profiles and mixed profiles separately

deduce that in 46% of the DNA extracts from 75 fired cartridge cases, an STR profile could be obtained under strictly controlled conditions using MiniFiler™. We obtained STR profiles in 283 (6.9%) of all 4,085 CBC items. Per profile, we were able to genotype an average number of 10.95 STR loci (not shown), ranging from 2 to 17 (Fig. 1).

In every criminal case, we tested all casings because it is impossible to infer which cartridge case was the result of the first shot (and thus most likely to contain sufficient biological material) or due to the last shot (potentially containing less biological material). Therefore, it is crucial to secure each item individually in order to prevent cross-contamination among different individual CBC items.

Protocol issues

Early on in our project, we noticed that oxidation had a negative effect on some of the CBCs after the lysis step (Fig. 2). This effect turned out to be due to the lysis buffer involved in the first isolation step. The CBC slowly oxidizes and the surface partially dissolves. This is clearly visible because the lysis solution colors blue due to the formation of copper ions. CBC items also color blue while lysing for the prolonged time. We thus decided to limit the first lysis step to 30 min. By subsequent swabbing of the CBCs after this lysis step followed by drying in air, we solved the oxidation problem. We also noticed that the severity of this effect strongly differs between different types of CBC batches. This is most likely due to different (largely unknown, at least to us) metal alloy compositions of these items. An alternative solution could simply be swabbing the outer surface of CBC items instead of our submerge approach. This was explored by us in the first phase of our project, and we found that it is consistently much less successful (results not shown), a result that was independently confirmed by a few cases in 2003 and 2004. In these cases, we received swabs that were taken by crime scene investigators instead of the original CBC items. We were unable to generate a DNA profile from any of these swabs.

Matching profiles

Since 2003, we were able to obtain DNA profiles in 163 out of 616 cases (Table 1). This is an overall success rate of ~26.5%. Although the success rate per item is much lower (283 out of 4,085 individual items, ~6.9%), it is still sufficiently high to render this method worthwhile since in many cases, multiple CBC items can be investigated. The DNA profile matches we obtained can be divided into four categories: (1) matches with a suspect, (2) matches with a DNA database trace profile, (3) a match with a victim, and (4) a match with people handling the trace material, e.g., lab technicians (these matches were not included in the summarizing statistics). Because of the almost complete lack of documentation of the relevance of the DNA results beyond our forensic laboratory, it was unfortunately impossible to systematically categorize all the 163 cases in which we successfully obtained and reported a reproducible DNA profile. We only have sporadic and unofficial information on the basis of which we conclude that matches of all four categories do occur. In the majority of criminal cases we handled, suspects were unknown prior to our DNA analyses (or reference samples of suspects and victims were not provided). Hence, all profiles could only be compared to the technicians performing DNA analysis.

Obviously, it is crucial to focus on these category (4) matches, i.e., profiles matching to laboratory staff members. For this purpose, DNA profile elimination databases have been built by the Netherlands Forensic Institute and by the

Fig. 2 The effect of lysis buffer on casings. *Left* a casing treated with lysis buffer. *Right* a casing that was untreated. Both casings came from the same batch

Forensic Laboratory for DNA Research (FLDO) for internal use only. Profiles of all relevant employees handling CBC items are entered into these databases in order to detect possible contamination before issuing the final report. We have observed eight category (4) matches over the entire period, both with technicians handling CBC items prior to DNA analysis while scanning for striations, as well as with technicians involved in photographing cartridges prior to DNA isolation, or actually working on the DNA analyses. These eight matches involved the following number of loci per profile: 6, 7, 8, 12 (twice), 13 (twice), and 14. As such, these category (4) matches are not relevant for the identification of possible suspects (presumably). However, these matches do indicate that the entire DNA profiling procedure is very sensitive and that despite all protective clothing regulations, it is still possible to contaminate items handled prior to DNA isolation under strictly controlled procedures. It also demonstrates the importance of using DNA profile elimination databases. Over the entire period 2003–2009, we have continually improved our internal laboratory procedures and adjusted our internal training protocols in order to eliminate these contaminations as much as possible. It is also important to note that for the purpose of this study, we use the Powerplex® 16 system (Promega) as the primary STR genotyping kit in order to obtain multiplex STR genotypes under standard conditions. In our hands, this kit is able to produce reproducible STR genotypes in as little as 40–60 pg of DNA without invoking any low copy number PCR methodology.

The results described here are the result of a retrospective analysis of 616 criminal cases involving CBC items that were routinely processed at the FLDO. It does not describe the results of an in-depth validation study aimed at finding the most optimal STR-PCR kit. This poses a number of restrictions on the possibility to use DNA extracts for research purposes. It is mandatory under Dutch Law to store 50% of the total DNA extract volume for contra-expertise purposes. Obviously, we are also aware that a number of manufacturers have recently released new short PCR amplicon multiplex PCR kits with an increased sensitivity. The use of these new kits will very likely increase the success rate of our method even further.

Conclusion

We describe a procedure for the extraction of DNA on cartridges, bullets, and casings. Subsequently, we present a retrospective analysis of results obtained by means of this method in 616 criminal cases involving 4,085 individual ammunition items. In our hands, this method was found to be reliable and sensitive, with a success rate (per case) of 26.5%. The success rate per item was 6.9%, and the average number of genotypes per profile was 10.9. If performed properly, the method has minimal influence on subsequent striation testing. We have routinely implemented this method for all our cases involving cartridges, bullets, and casings and are willing to share the finer details—if not already clear from this note—with those interested.

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