*J Forensic Sci,* July 2009, Vol. 54, No. 4
doi: 10.1111/j.1556-4029.2009.01066.x
Available online at: www.blackwell-synergy.com

*Kathrin Müller,*[1] *Drs.; Günter Braunschweiger,*[2] *Ph.D.; Rachel Klein,*[1] *Ph.D.; Erich Miltner,*[1] *M.D.; and Peter Wiegand,*[1] *Ph.D.*

# Validation of the Short Amplicon Multiplex Q8 Including the German DNA Database Systems*

**ABSTRACT:** We have developed a concept to enable the analyzing of degraded stains with limited DNA template quantity. Therefore we have constructed a short tandem repeat (STR) multiplex including the German DNA database systems (Q8). The amplicon lengths are smaller than 280 bp. For the validation of Q8 over 50 degraded samples were investigated. Amplifications were performed with "low copy number" PCR, the number of PCR cycles was increased to 33 and the reaction volume was decreased to 12.5 μL. Compared with the MPX2 and Nonaplex kit, the average success rate was increased using the Q8 kit by approximately 20% and 30%, respectively. The efficiency of a sensitive STR multiplex with reduced amplicon lengths was confirmed in comparing the success rates of Q8 for typing degraded samples and samples with limited amount of DNA template while partial profiles were observed with the majority of the samples using commercially available kits.

**KEYWORDS:** forensic science, DNA typing, short amplicon multiplex, DNA degradation, low copy number, German DNA database systems, D3S1358, FGA, D8S1179, D18S51, D21S11, TH01, VWA, SE33, amelogenin

Nowadays, more and more difficult biological stains are taken up at crime scenes, i.e., stains polluted with environmental contaminants or stains with little and highly degraded DNA. These stains may be hard to analyze with commercially available kits (1–7). Inhibitors which are present in many stains can disrupt the PCR reaction unless they cannot be separated from the DNA during the extraction. Weak stains with highly degraded DNA are less successful to analyze due to the long amplicon lengths produced by the commercial short tandem repeat (STR) multiplex kits. To increase the success rate especially in typing such problematic stains, a sensitive STR multiplex with shortened amplicon lengths is useful. As there are several thousand copies of mtDNA in a single cell, mtDNA typing of these "poor" stains would increase the number of identifiable stains dramatically compared with the currently used nuDNA typing. However, there are two drawbacks: first, mtDNA markers are considerably less polymorphic than STRs. Second, the results cannot be compared with the national German DNA database STR systems (SE33, D21S11, von Willebrand factor A [VWA], TH01, human alpha fibrinogen [FGA], D3S1358, D8S1179, and D18S51) and amelogenin, which allows to search for a suspect without the need of gaining DNA samples for comparison. So there is great interest in new STR concepts, sensitive for typing weak stains with highly degraded DNA and stains polluted with environmental contaminants.

Sensitive STR kits with shortened amplicon lengths increase the success rates of polluted stains and stains with little and degraded DNA while avoiding the problems of decreased polymorphy and the lack of an existing database. We have developed such a STR concept including the eight German DNA database systems and amelogenin (8).

In the first step we tested and optimized the STR multiplex Q8 on different cell lines and on stains with sufficient DNA like blood, saliva, and sperm. In the second step we improved the concept for using it with bones, hairs, and skin cell mixtures. We have analyzed stains from crime scenes with the Q8 concept which gave no or incomplete profiles with commercial PCR kits.

For the validation of the Q8 concept over 50 biological stains with little and highly degraded DNA were examined using low copy number (LCN) PCR (9–16). The sensitivity of LCN PCR is usually increased by raising the number of cycles or by use of nested primer PCR. Because of the low amounts of DNA and the increased number of cycles, allele dropout as well as sporadic contamination and false alleles caused by slippage artefacts during the first PCR cycles may be observed with LCN PCR. To minimize PCR errors the number of cycles has to be optimally adjusted to the limited amount of DNA template. Gill (9) showed that the optimum for less than 100 pg DNA was 34 cycles. More cycles result in increased artefact production but not in increased PCR sensitivity. Besides increasing the number of cycles, other approaches can raise the sensitivity of LCN PCR such as reduction of PCR volume, rise of injection time (13), and optimization of the PCR reagents. As the first PCR cycles are decisive for the PCR result, the initial limited DNA template quantity in LCN PCR can lead to different alleles. Therefore the same sample has to be analyzed more than once to interpret the LCN DNA profiles. An allele cannot be scored unless it is present at least twice in replicate samples. The interpretation of LCN DNA profiles was previously performed and described in the greatest detail possible (9,11,15). Kloostermann and Kersbergen (16) suggested amplifying the DNA using the standard 28 cycles for the SGM Plus kit. If a low level result is obtained, then more Taq polymerase is added to the remaining PCR and six more cycles of PCR are performed to carry out LCN typing. The normal sensitivity using the standard protocol of 28

[1]Institute of Legal Medicine, University Hospital Ulm, Ulm, Germany.
[2]genFOR GmbH, Altenahrer Strasse 9, 53501 Grafschaft-Gelsdorf, Germany.

*Study promoted by BMBF funding program BioChance Plus (D.2503).

Received 16 April 2008; and in revised form 8 Sept. 2008; accepted 12 Sept. 2008.

cycles could be increased after LCN methods were used. LCN DNA is mainly suitable in skin cells (14), bones (9,15), telogen hairs, or hair shafts (17). DNA degradation accompanied by the natural development of that tissue or caused by environmental influences makes the STR typing more difficult using commercially available multiplex STR kits due to the wide size range of PCR products generated. Short amplicon multiplex kits produce a higher success rate with degraded DNA samples. Our goal was not only to create a kit for typing stains with little amount of DNA but also for typing degraded samples by selecting primers as close as possible to the STR repeat region. For comparison purposes, PCR was additionally carried out with the commercially available, well-established kits, MPX2 and Nonaplex.

## Materials and Methods

### *DNA Sample*

DNA from weak and highly degraded stains, such as hairs, bones, and skin cell mixtures were extracted using the GEN-IAL protocol (GEN-IAL GmbH, Troisdorf, Germany) according to Junge et al. (18). These extracts from about 50 samples were used to validate the Q8 (Table 1). Hair and bone extracts were additionally purified with silica as described (17).

### *PCR Amplification*

PCRs were performed using the STR multiplex according to Wiegand et al. (2) plus the loci SE33, D8S1179, D18S51, and D21S11.

TABLE 1—*The number and origin of the individual examined stains.*

| Skin | Bone | Hairs | Vomit | Feces | Blood | Saliva |
|---|---|---|---|---|---|---|
| 33 | 2 | 3 | 1 | 1 | 2 | 12 |

TABLE 2—*STR primer sequences used for the Q8.*

| Locus | Modified STR Primer Sequence (5′ to 3′) |
|---|---|
| Amel | |
| F | FAM-CCTGGGCTCTGTAAAGAATAGTG |
| R | AGCTTAAACTGGGAAGCTGGTGG |
| D3S1358 | |
| F | ACTGCAGTCCAATCTGGGT |
| R | FAM-GAAATCAACAGAGGCTTGCA |
| FGA | |
| F | GAACTCACAGATTAAACTGTAAC |
| R | FAM-TGATTTGTCTGTAATTGCCAGC |
| THO1 | |
| F | JOE-GTCACAGGGAACACAGACTC |
| R | ATTCCCATTGGCCTGTTCCT |
| VWA | |
| F | AGAATAATCAGTATGTGACTTGGATTG |
| R | JOE-CAGATGATAAATACATAGGATGGATG |
| SE33 | |
| F | JOE-GAAAGAGACAAAGAGAGTTAG |
| R | ACATCTCCCCTACCGCTATAG |
| D8S1179 | |
| F | TET-TTTTTGTATTTCATGTGTACATTCGT |
| R | GATTATTTTCACTGTGGGGAATAGA |
| D18S51 | |
| F | TET-GCACTTCACTCTGAGTGAC |
| R | GGAGATGTCTTACAATAACAG |
| D21S11 | |
| F | TET-AAATATGTGAGTCAATTCCCCAAG |
| R | GTTGTATTAGTCAATGTTCTCCAG |

STR, short tandem repeat.

Primer sequences and the labeling dyes are listed in Table 2. All primers were diluted to a concentration of 100 pmol/μL. We used the following concentrations for each primer pair for the multiplex: amelogenin, 4.1 μM; TH01, 5.5 μM; D3S1358, 4.6 μM; VWA, 15.1 μM; FGA, 11.5 μM; SE33, 11.5 μM; D8S1179, 7.4 μM; D18S51, 5.5 μM; D21S11, 6.8 μM. Thermal cycling conditions were 11 min at 95°C, followed by 33 cycles of 93°C for 1 min, 59°C for 1 min, and 72°C for 30 sec. After the thermal cycling, a final extension step of 72°C for 40 min and a final cooling down to 15°C was carried out. The samples were amplified in a 12.5 μL reaction volume consisting of 1x PCR Buffer II (Applied Biosystems, Darmstadt, Germany), 1.6 mM $MgCl_2$, 2 U AmpliTaqGold polymerase (Applied Biosystems), 3 μg bovine serum albumin (Sigma, Munich, Germany), 0.1 mM of each nucleotide, and 0.9 μL of the primer mix. The $MgCl_2$ and the polymerase concentration was increased at the amplification of hair extracts because of the inhibition effect of melanin as described (17).

Additionally, customary PCR kits with different amplicon lengths were used (MPX2, Serac, Bad Homburg, Germany; Mentype® Nonaplex$^{QS}$, Biotype, Dresden, Germany) (Table 3) according to the manufacturer instructions but the number of PCR cycles was also changed to 33 for comparison purposes. For analysis and data interpretation the samples were amplified twice.

### *Analysis on ABI 310 Genetic Analyzer*

Electrophoresis was carried out on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) with the polymer POP4 using the Genescan software. Samples were prepared with 10 μL Hi-Di formamide (Applied Biosystems), 0.5 μL GeneScan™ 500 ROX™ Size Standard (Applied Biosystems), and with 1 μL of PCR product. The STR samples were run using the module C which performs an electrokinetic injection onto the single capillary for 5 sec at 3000 V. The STR alleles were then separated at 15000 V for *c.* 20 min with a run temperature of 60°C.

## Results and Discussion

A mini-STR concept improves the investigation of weak and highly degraded stains (1–3). However, even more factors play an important role in successful typing apart from the amplicon length. The labeling fluorescent dyes, the primer binding sites, the PCR components, the number of PCR cycles and finally the PCR program influences the quality of the STR concept as well. We have tested all variations of PCR conditions and components to find the best conditions for a successful typing of stains polluted with environmental contaminants and stains with little and degraded DNA.

We tested a series of primers with different sequences that are not only located near the repeat region but also promote the amplification of the locus. Several primers were tested for every locus with the aim

TABLE 3—*Comparison between the amplicon lengths of Q8 and the commercial kits MPX2 and Nonaplex.*

| PCR Kits/Systems | Q8 (bp) | MPX2 (bp) | Nonaplex (bp) |
|---|---|---|---|
| Amel | 96, 102 (FAM) | 102, 108 (FAM) | 83, 86 (FAM) |
| D3S1358 | 106–143 (FAM) | 106–143 (NED) | 152–190 (HEX) |
| FGA | 153–204 (FAM) | 153–204 (NED) | 190–243 (NED) |
| TH01 | 76–100 (JOE) | 120–144 (JOE) | 92–116 (HEX) |
| VWA | 104–149 (JOE) | 124–168 (FAM) | 108–153 (NED) |
| SE33 | 164–282 (JOE) | 206–323 (FAM) | 206–323 (HEX) |
| D8S1179 | 74–116 (TET) | 256–296 (JOE) | 105–146 (FAM) |
| D18S51 | 124–191 (TET) | 242–310 (NED) | 282–351 (FAM) |
| D21S11 | 213–254 (TET) | 204–244 (JOE) | 187–227 (FAM) |

TABLE 4—*Comparison between the success rates of the individual systems of the Q8 and the (well established) commercial kits MPX2 and Nonaplex.*

| System/Kit | Amel (%) | D3S1358 (%) | FGA (%) | D8S1179 (%) | D18S51 (%) | D21S11 (%) | TH01 (%) | VWA (%) | SE33 (%) | Ø (%) | Ratio (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Q8 | 85 | 79 | 76 | 86 | 76 | 62 | 85 | 77 | 74 | 78 | 100 |
| MPX2 | 92 | 65 | 70 | 61 | 43 | 61 | 82 | 69 | 35 | 64 | 82 |
| Nonaplex | 61 | 63 | 41 | 54 | 30 | 43 | 74 | 60 | 49 | 53 | 68 |

Peaks were valued if they had reached at least the 50 rfu (relative fluorescence units) threshold.

of reducing the amplicon length. The most sensitive primers were combined to the Q8 multiplex. The amplicon lengths were reduced compared with commercial kits up to a maximum amplicon length of 180 bp (Table 3). After the Q8 multiplex was developed and first tested on cell lines, telogene hair and hair shafts from known persons were analyzed. The LCN typing results from the hair extracts were compared with buccal swabs from the hair donors as reference samples. The investigation of stains with degraded DNA showed that the success rate of the individual systems increased with the shortness of the amplicon length (Table 4 and Fig. 1). An allele was scored when it was present twice in replicate samples. Only if this criterion was met, the amplification was regarded as successful. Before LCN DNA typing the degraded samples were amplified using the Q8 multiplex with 30 and 33 cycles. If a true increase in sensitivity was observed using 33 cycles, LCN typing was carried out simultaneously with the Q8, MPX2, and Nonaplex kit for comparison purposes. Only few samples did not demonstrate an increased sensitivity with 33 versus 30 cycles, presumably because there was no DNA in the sample or the DNA was highly degraded. About 50 samples were amplified using all three kits.

Furthermore, weak stains can be analyzed quite well with Q8 due to the sensitive primer binding sites we chose. To improve the sensitivity of PCR typing, the Q8 primers were labeled with the sensitive fluorescent dyes 6-carboxyfluorescein (6-FAM), 6-carboxy-2′,7′-dimethoxy-4′,5′-dichlorofluorescein (JOE), and 4,7,2′,7′-tetrachloro-6-carboxyfluorescein (TET).

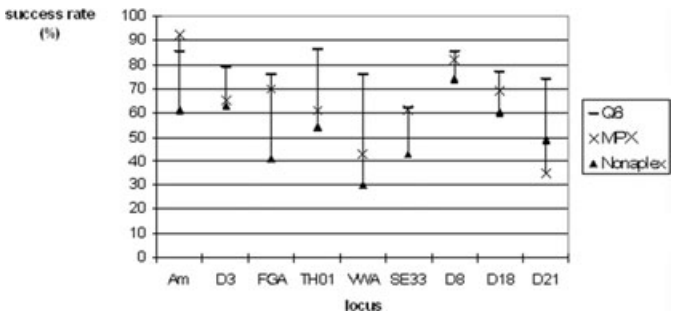



FIG. 1—*Success rate of the individual loci from 54 samples, see Table 1 (Am: amelogenin; D3: D3S1358; D8: D8S1179; D18: D18S51; D21: D21S11). FGA, human alpha fibrinogen; VWA, von Willebrand factor A.*

Finally, the Q8 concept was further optimized by adding the PCR components to the PCR reaction in different quantities to find out the best composition and concentrations as listed in Materials and Methods. Taq polymerase and $MgCl_2$ concentration had to be increased with amplification of hair samples (17). An increased concentration of Taq polymerase raised furthermore the typing success of degraded blood samples. Because there are all sorts of inhibitors present in the different samples taken up at crime scenes, it is difficult to find a PCR additive that would neutralize the inhibition effect of the many different contaminants and would thereby increase the typing success significantly. PCR volume reaction studies have proven that a reduced volume PCR increases the success for typing degraded stains with limited DNA template quantity. Stochastic effects seem to be reduced as the initial DNA was less diluted and the PCR components were concentrated. The PCR volume was halved to 12.5 μL. Additionally, the PCR program was changed until it was optimally adjusted to the Q8 primers. Because there were many substances having an inhibitory effect against the Taq polymerase, there was the idea for testing the Time Release PCR method, allowing the enzyme to activate slowly during cycling. The pre-PCR activation step could be omitted, and a minimum of 10 additional cycles could be added. There was no increase in the success rate using the Time Release PCR method. The Taq polymerase, mainly in the inactivated condition, seems not to be more insensitive against inhibitory substances, the typing success of samples with inhibitory substances was similiar using the normal PCR method as described in Materials and Methods.

Finally, different numbers of cycles were tested, and an amplification of 33 cycles has proven to be the best approach for weak stains with degraded DNA. Due to the increased number of PCR cycles, some stains from crime scenes could be analyzed. By the use of LCN PCR with 33 cycles, a profile, competent for the registration form, could be produced and an offender could be convicted on account of a hit in the database (Fig. 2). The electropherograms from the commercial kits using standard amplification conditions and then additional cycles for the same DNA template showed also an increased sensitivity using LCN typing (data not shown).

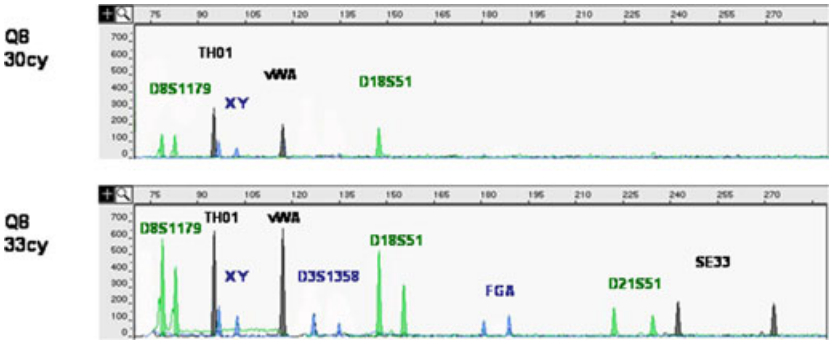
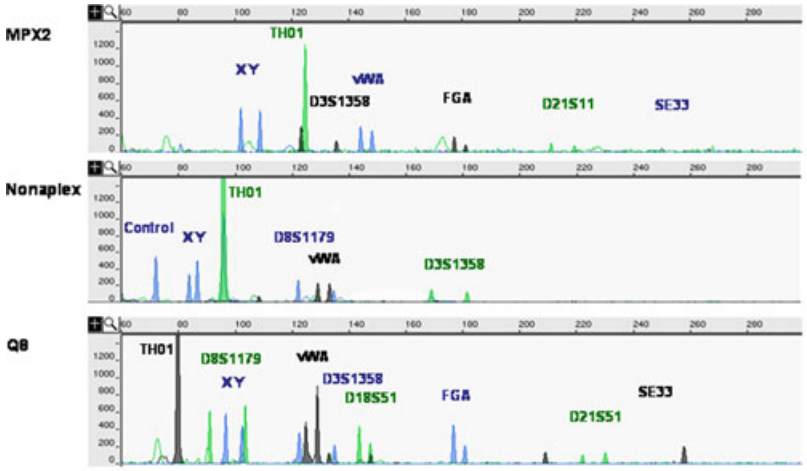



FIG. 2—*Short tandem repeat profile from a degraded blood stain using the Q8 concept with 30 cycles and with 33 cycles.*




| kitlocus | Amel | D3S1358 | FGA | D8S1179 | D18S51 | D21S51 | TH01 | vWA | SE33 |
|---|---|---|---|---|---|---|---|---|---|
| MPX2 | X,Y | 15,18 | 22,23 | | | 28,30 | 6 | 16,17 | 18 |
| Nonaplex | X,Y | 15,18 | | 12,15 | | | 6 | (11),16,17 | |
| Q8 | X,Y | 15,18 | 22,23 | 12,15 | 12,13 | 28,30 | 6 | 16,17 | 18,30.2 |

FIG. 3—*Short tandem repeat profiles from feces. PCR was carried out with the MPX2, Nonaplex, and Q8 kit.*

Compared with the success of the short amplicon multiplex Q8 for typing degraded samples, partial profiles were observed with the majority of the samples using the commercially available kits, MPX2 and Nonaplex. One exemplary result of our concept is shown in Fig. 3. The feces extract was simultaneously amplified with the MPX2, Nonaplex, and Q8 kit. There was concordance between the commercial kits and the Q8 multiplex. More loci could be analyzed with the use of the Q8 compared with the two commercial kits, MPX2 and Nonaplex. The data interpretation of LCN DNA typing is more complicated because of stochastic effects like allele drop-out, allele drop-in, increased stutter, and heterozygote imbalance. The stochastic effects increase with the amplicon length. Therefore the replicate analysis produced a discordant result in the longer systems more frequent than in the shorter ones.

In summary, the STR multiplex Q8 generated better typing results than the commercial kits. Q8 is a very sensitive multiplex, so weak stains can be analyzed quite well in combination with LCN PCR. Furthermore, the typing of highly degraded DNA samples such as bones and telogene hairs is enabled with this concept. As the Q8 contains the eight DNA database systems, a comparison with the DNA database is possible.

## References


1. Wiegand P, Kleiber M. Less is more—length reduction of STR amplicons using redesigned primers. Int J Legal Med 2001;114:285–7.
2. Wiegand P, Klein R, Braunschweiger G, Hohoff C, Brinkmann B. Short amplicon STR multiplex for stain typing. Int J Legal Med 2006;120:160–4.
3. Butler JM, Shen Y, McCord BR. The development of reduced size STR amplicons as tools for analysis of degraded DNA. J Forensic Sci 2003;48:1054–64.
4. Grubwieser P, Mühlmann R, Berger B, Niederstätter H, Pavlic M, Parson W. A new ''miniSTR-multiplex'' displaying reduced amplicon lengths for the analysis of degraded DNA. Int J Legal Med 2006;120:115–20.
5. Chung DT, Drábek J, Opel KL, Butler JM, McCord BR. A study on the effects of degradation and template concentration on the amplification efficiency of the STR miniplex primer sets. J Forensic Sci 2004; 49(4):733–40.
6. Dizdaroglu M. Oxidative damage to DNA in mammalian chromatin. Mutat Res 1992;275:331–42.
7. Lindahl T. Instability and decay of the primary structure of DNA. Nature 1993;362:709–15.
8. Müller K, Klein R, Miltner E, Wiegand P. Q8—a short amplicon multiplex including the German DNA database systems. Forensic Sci Int: Genetics 2007;1(2):205–7.
9. Gill P. Application of low copy number DNA profiling. Croat Med J 2001;42:229–32.
10. Meyerhans A, Vartanian JP, Wain-Hobson S. Strand specific PCR amplification of low copy number DNA. Nucleic Acids Res 1992; 20(3):521–3.
11. Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, et al. Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Res 1996;24(16):3189–94.
12. Akbari M, Hansen MD, Halgunset J, Skorpen F, Krokan HE. Low copy number DNA template can render polymerase chain reaction error prone in a sequence-dependent manner. J Mol Diagn 2005;7(1):36–9.
13. Budowle B, Hobson DL, Smerick JB, Smith JAL. Low copy number—consideration and caution. Proceedings of the Twelfth International Symposium on human identification, Oct 9–12. Biloxi, MS: Promega, 2001. Available at: http://www.promega.com/geneticidproc/ussymp12proc/contents/budowle.pdf (accessed on February 17, 2008).
14. Murray C, Lowe A, Richardson P, Wivell R, Gill P, Tully G, et al. Use of low copy number (LCN) DNA in forensic inference. Proceedings of the Twelfth International Symposium on human identification, Oct 9–12. Biloxi, MS: Promega, 2001. Available at: http://www.promega.com/geneticidproc/ussymp12proc/contents/murray.pdf (accessed on February 17, 2008).
15. Butler JM. Introduction to low copy number (LCN) DNA testing issues. Part of Forensic DNA Workshop at the MAAFS meeting, May 3–5. Richmond, VA: Mid-Atlantic Association of Forensic Scientists, 2006. Available at: http://www.cstl.nist.gov/biotech/strbase/pub_pres/LCNintro_MAAFSworkshop_May2006.pdf (accessed on February 17, 2008).
16. Kloostermann AD, Kersbergen P. Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci. J Soc Biol 2003;197(4):351–9.
17. Müller K, Klein R, Miltner E, Wiegand P. Improved STR typing of telogen hair root and hair shaft DNA. Electrophoresis 2007;28:2835–42.
18. Junge A, Lederer T, Braunschweiger G, Madea B. Validation of the multiplex kit genRESMPX-2 for forensic casework analysis. Int J Legal Med 2003;117:317–25.



Additional information and reprint requests:
Peter Wiegand, Ph.D.
Institut für Rechtsmedizin
Universitätsklinikum Ulm
Albert-Einstein-Allee 11
89081 Ulm
Germany
E-mail: peter.wiegand@uni-ulm.de