*Quarterly Reviews of Biophysics*

**www.cambridge.org/qrb**

# How reliable is PCR? A mini review

Helen Konrad[1] and Andreas Beyer[2]

[1]Institut für Rechtsmedizin, Universitätsklinikum Essen, Essen, Germany and [2]Fachbereich 8, Westfälische Hochschule Gelsenkirchen, Bocholt, Recklinghausen, Germany

**Review**

**Cite this article:** Konrad H and Beyer A (2025). How reliable is PCR? A mini review. *Quarterly Reviews of Biophysics*, **58**, e15, 1–15
https://doi.org/10.1017/S0033583525100024

Received: 25 March 2025
Revised: 02 May 2025
Accepted: 06 May 2025

**Keywords:**
DNA analysis; PCR; pseudoscientific critique; reliability of PCR; thermostability of DNA

**Corresponding author:**
Andreas Beyer;
Email: andreas.beyer@w-hs.de

**Abstract**

The question of whether PCR is reliable sounds strange at first. However, looking at the scientific literature from the 1950s and 60s, one will find many publications on the physicochemistry of DNA that have been forgotten meanwhile. Quite a few of these studies have shown that DNA is thermolabile, which consequently raises the question of whether this thermolability is relevant in the context of PCR, namely in the denaturation phase. However, it can be shown that this is not the case: losses due to thermal hydrolysis are irrelevant for the performance of contemporary PCR protocols and their specificity as well as for the significance of their results. There is now a huge amount of scientifically verified and published data on technical and molecular aspects of PCR, a small selection of which we quote here. In addition, we present some primary data that also clearly demonstrate the reliability of PCR.

## Table of contents

**Introduction–the early days** **1**
**Nature tells a different story: hyperthermophilic prokaryotes** **2**
**Consideration of some contemporary PCR methods** **2**
RT-PCR (reverse transcription PCR) and SMART-libraries 2
qPCR: measuring the efficiency parameter 2
Sanger cycle sequencing 3
Next generation sequencing (NGS) with bridge amplification PCR 3
Assessing error sources in PCR 5
Forensic DNA analysis: STR-PCR 5

**Denaturation–renaturation and $C_0t$ analysis** **8**
**A simple experiment** **8**
**Loop-mediated isothermal amplification (LAMP): a modern alternative to PCR** **9**
**Coming back to the paper by Serpieri and Franchi** **9**
**Conclusion: PCR is reliable** **9**
**Materials and methods** **10**
Forensic DNA typing 10
PCR 10
qPCR 11

## Introduction–the early days

Recently, two authors (Serpieri and Franchi, 2024) analyzed the scientific literature on the physicochemistry of DNA between 1947 and 1961 as well as some publications on PCR/DNA denaturation until the 1980s. Some of the studies cited contradict each other, but a number of scientific publications show the fragility of DNA at higher temperatures. There are indeed a couple of results indicating heat-induced (hydrolytic) DNA fragmentation:

- Viscosity drop after heating indicates DNA fragmentation (e.g., Goldstein and Stern, 1950).
- Heating alters gDNA so that the ethanol precipitation produces a flocculent rather than fibrous precipitate, indicating a change from its pre-heating behaviour. (Goldstein and Stern, 1950).
- Heating leads to thixotropy in DNA solutions, while untreated DNA does not show this phenomenon, which indicates irreversible (physico-)chemical changes (Zamenhof and Chargaff, 1950).
- Heated DNA loses its ability to transform bacteria, as shown for the uptake of antibiotic resistance genes by *Haemophilus influenzae* (Zamenhof *et al.*, 1953), indicating loss of its integrity.

© The Author(s), 2025. Published by Cambridge University Press.

- Serpieri and Franchi report further arguments for heat-induced depolymerisation, e.g. observed changes in UV absorption and changes in DNA dye staining behaviour (for refs. see Serpieri and Franchi, 2024).
- For more aspects (and refs.) see Serpieri and Franchi (2024).

Overall, there is indeed some experimental data in favour of DNA degradation by heat-induced depolymerisation. Some of the effects have been reported to occur already just above 80 °C. Since denaturation in PCR takes place at approx. 95 °C, it is reasonable to ask whether the DNA is fragmented during this step. Surprisingly, there seems to be little systematic research on this topic; one example is Gustafson *et al.* (1993), who also report DNA damage at high temperatures. We therefore want to take a closer look at this question.

## Nature tells a different story: hyperthermophilic prokaryotes

The assumption that DNA is already significantly damaged at over 80 °C is not compatible with what nature tells us. There are hyperthermophilic prokaryotes that not only withstand temperatures above 100 °C but even grow under such conditions: *Pyrolobus fumarii* grows up to 113 °C (Blöchl *et al.*, 1997). *Geogemma barossii* ('Strain 121') grows at 121 °C and tolerates 130 °C (Kashefi *et al.*, 2003). *Methanopyrus kandleri* can survive and reproduce at 122 °C (Takai *et al.*, 2008). The latter two species can be grown in a running laboratory autoclave without any problems.

Of course, the conditions in a living cell are quite different from those for "naked" DNA in solution: DNA integrity is continuously monitored and maintained by enzymes: cells take various measures to protect their DNA (Daniel and Cowan, 2000; Grosjean and Oshima, 2007), amongst others specific chaperones (e.g. Kampmann and Stock, 2004) and adapted DNA repair systems, which surprisingly do not appear to be exceptional (Marshall and Santangelo, 2020). Nevertheless, even in the presence of these repair and maintenance systems, it seems very unlikely that the molecular biology of a cell should be able to constantly counteract the complete destruction of its genome more than 40 °C above the critical temperature.

## Consideration of some contemporary PCR methods

A large number of PCR variants now exist (e.g. Coen *et al.*, 2003: Unit 15 in Ausubel et al.). It is not necessary to cover them all: in the following paragraphs, we will focus on some of these that are particularly significant in terms of DNA stability.

### *RT-PCR (reverse transcription PCR) and SMART-libraries*

RT-PCR is the means of amplifying RNA targets. Since virtually all DNA-dependent DNA polymerases are unable to accept an RNA template, RNA must be converted to cDNA by reverse transcriptase before it can be used as a template for PCR. A very intelligent combination of reverse transcription and PCR is the method for generating SMART cDNA libraries (Zhu *et al.*, 2001). This technique has been widely used to create cDNA libraries as part of genome sequencing projects in order to map exons (and thus genes) to genomic data (e.g. Wiemann *et al.*, 2001). Since TaqPol has no proof-reading activity (Tindall and Kunkel, 1988; Cline et al., 1996), an increased error rate (causing point mutations) of about $10^{-4}$ is to be expected in SMART libraries. (The value actually determined varies: it depends on assay methodology, reaction conditions, and template sequences, as outlined by Potapov and Ong 2017.) These errors are automatically detected during the mapping of the cDNA sequences to the genomic data. If further errors and artefacts were to occur during PCR, as claimed by Serpieri and Franchi (2024), they would easily have been detected during these data processing steps. Zhu *et al.* (2001) systematically compared the quality of a SMART library and a conventionally generated cDNA library (with and without a PCR step, respectively). They found that the SMART library was superior in every respect. The German cDNA consortium (Wiemann *et al.*, 2003, Wellenreuther *et al.*, 2004, Bechtel *et al.*, 2007) has cloned, sequenced, and mapped over 2,200 different ORFs from human cDNA. Their quality was verified and the proportion of artefacts was low – despite the complex and tricky SMART technology. Baross *et al.* (2004) have developed and tested a method for the targeted isolation of cDNA clones. After cDNA synthesis, the target ORFs were amplified by PCR with gene-specific primer pairs flanking the ORFs. The clones were then sequenced to investigate reverse transcription and amplification artefacts. In this way, the authors were able to determine the probability of obtaining error-free clones as a function of their length. High-quality cDNA libraries have been established for plant transcriptomes, too (e.g. Cartolano *et al.*, 2016). Meanwhile, the SMART method has been tuned in a way that the recovery and analysis of RNA from a single cell is possible (e.g. Hagemann-Jensen *et al.*, 2022). In all such cases, temperature-induced artefacts as described by Serpieri and Franchi (2024) have not been found.

### *qPCR: measuring the efficiency parameter*

In the 1990s, a PCR variant was developed for the quantification of target DNA or (m)RNA molecules: the qPCR (Higuchi *et al.*, 1993, Heid *et al.*, 1996, Kubista *et al.*, 2006, Dorak, 2006, VanGuilder *et al.*, 2008). This method allows the quantification of the initial template by quantitatively detecting the amplicons in each individual cycle – that is: in real time. Meanwhile, quite a few variants exist (for a short overview see Dymond, 2013) utilizing specific (e.g. TaqMan) or non-specific (e.g. SYBR green) detection methods. It is possible to precisely determine the efficiency parameter for qPCR (e.g. Svec *et al.*, 2015, Tellinghuisen, 2021). This parameter provides information about the efficiency of the amplification reaction, that is how close the PCR process comes to an exact duplication of amplicons per cycle. The mathematical formulation of the efficiency parameter $E$ is:

$$m_{(n)} = m_0 \bullet (1 + E)^n$$

$m$: mass of amplicons; $m_0$: starting material (with respect to the target amplicon);
$\underline{m}_{(n)}$: mass of amplicons after $n$ cycles; $n$: number of cycles;
$(1 + E)$: base of the exponential function; $E$: efficiency parameter.

In the case of $E = 1$, the PCR performance would be perfect because each cycle (before the process approaches the plateau) exactly doubles the number of amplicons. The crucial point: if DNA damage were to occur, as claimed by Serpieri and Franchi (2024), then acceptable $E$ values would not be achievable (also see below: next section). qPCR has been thoroughly tested and the pitfalls – which of course exist as with any method – are well known (Bustin and Nolan, 2004), but DNA fragmentation is not one of these. For quantitative PCR methods, a detailed guideline catalogue

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

has been created ("MIQE qPCR & dPCR": Bustin *et al.*, 2009), in which all challenges, pitfalls, and solutions are dealt with.

In forensics (see below), qPCR is routinely used to quantify template DNA. Systematic studies have been carried out on various kits (e.g. Poetsch *et al.*, 2016 – see below: Forensic DNA analysis: STR-PCR) So, qPCR has become a very powerful and reliable tool (Valasek and Repa, 2005).

### Measuring the *E* parameter of a qPCR system in a practical course

qPCR is meanwhile a very well-established method that can be performed by students. The Molecular Biology degree programme at the Westphalian University of Applied Sciences offers a qPCR practical course in which students are tasked with preparing and carrying out a qPCR to detect GMO gDNA. They are then asked to manually calculate the efficiency of the qPCR system (see Materials and methods section). Figure 1 shows the regression line.

Such a value for *E*, close to 1.0, cannot be achieved if the DNA is significantly damaged during the denaturation phase. Note that the entire amplicon must remain intact, as not only the primer binding sites but also the binding site of the TaqMan probe must be present and intact.

### qPCR in virus detection: the case of SARS-CoV2

Early in the COVID-19 pandemic, qPCR detection systems for SARS-CoV2 were developed. Quite a few studies assessed the fidelity, sensitivity, and specificity of these qPCR systems (Chan *et al.*, 2020; Nagura-Ikeda *et al.*, 2020; Winichakoon *et al.*, 2020; Yang *et al.*, 2020; Lawrence *et al.*, 2022). The values determined were between *satisfactory* and *very good.* The main factor influencing the fidelity was not the PCR system itself, but the sample source and quality. Moreover, the sensitivity depends in particular on the expression rate of the transcripts targeted by the qPCR system (Chan *et al.*, 2020). Quite a few studies have compared different qPCR systems (e.g. Freire-Paspuel *et al.*, 2021a, 2021b; Freire-Paspuel *et al.*, 2022; Salamanca-Neita *et al.*, 2022): their performance was *good* to *excellent.* A reliable diagnostic system cannot function if the DNA is thermolabile to any significant degree.

### *Sanger cycle sequencing*

In 1977, Sanger *et al.* developed the enzymatic chain termination method of DNA sequencing. About a decade later, the enzymatically produced fragments were labeled with dyes rather than with radionuclides, allowing the process to be automated (Chait *et al.*, 1988). Around the same time, the cycle sequencing method was invented, which is a "one-primer PCR," so to speak (Murray, 1989, Blakesley, 1993). Templates that can be used are plasmid or phage clones as well as PCR products that – after removal of the PCR primers – can be sequenced directly (Coen *et al.*, 2003: Unit 15.2 in Ausubel et al.; Mardis and McCombie, 2017). Cycle sequencing is usually carried out with 20–30 cycles. It only leads to linear amplification, as only a single primer is used. Artefacts such as those claimed by Serpieri and Franchi (2024) can therefore not be compensated for by exponential amplification. Provided that the template DNA is clean, the primers have no secondary binding sites, and the cycle conditions (especially the annealing temperature) are appropriate, cycle sequencing typically provides clear signals without background. Figure 2 shows an arbitrarily selected example (human VPS4A: Beyer *et al.*, 2003). This is not compatible with the assumption of major DNA damage due to fragmentation during the denaturation steps.

Some publications deal with the reasons for poor Sanger sequencing electropherograms (e.g. Al-Shuhaib and Hashim, 2023). There are also troubleshooting guides by sequencing core facilities such as those at the Center for Computational and Integrative Biology of Massachusetts General Hospital in Harvard (https://dnacore.mgh.harvard.edu/new-cgi-bin/site/pages/sequencing_pages/seq_troubleshooting.jsp). In none of those sources, heat-induced hydrolysis of the DNA during PCR is cited as one of the reasons for poor results in Sanger Sequencing and noisy peaks in the electropherogram.

### *Next generation sequencing (NGS) with bridge amplification PCR*

MPS (massively parallel sequencing) approaches were developed in the 1990s and have been commercially available since around 2005 (Margulies *et al.*, 2005; see also: Voelkerding *et al.*, 2009). These

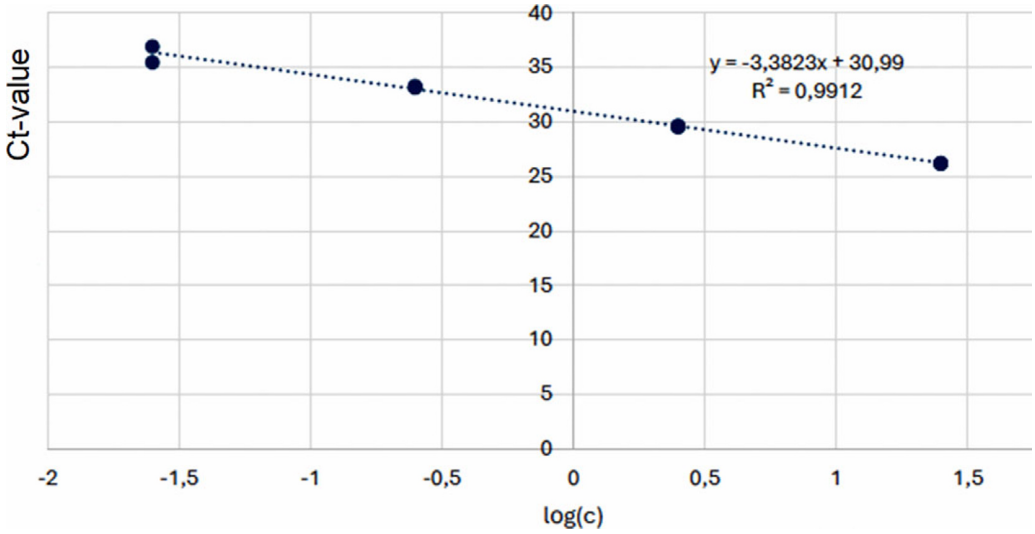



**Figure 1.** Regression straight line from a TaqMan qPCR and the associated *E* parameter (duplicate determination). Determined E parameter = 0,975.

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

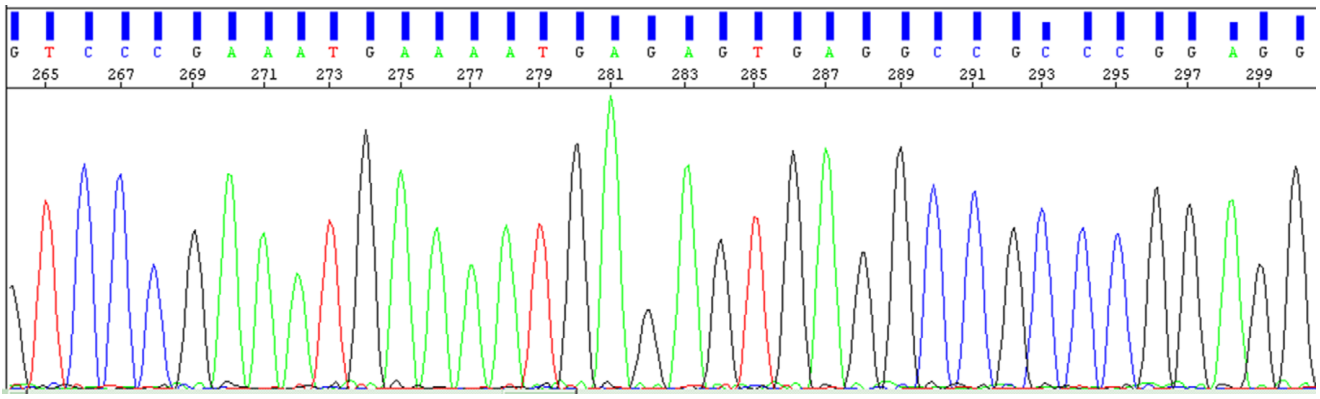



**Figure 2.** Image section of an electropherogram from a Sanger cycle sequencing reaction (from Beyer *et al.*, 2003). The electropherogram shows clear, clean signals with extremely low background. There are no signs of DNA fragmentation or other damage caused by the repeated denaturation steps.

technologies usually involve a PCR-based amplification step that starts from single (!) molecules (Anderson and Schrijver, 2010). Two examples that will be briefly explained here are *Bridge Amplification/Polony PCR* and *Emulsion/Droplet PCR.*

First step in the development of both methods was the "DNA colony generation principle," invented and developed in 1996 by Pascal Mayer and Laurent FarinelliIn (Glaxo-Welcome's Geneva Biomedical Research Institute, GBRI: patents WO 9844151 and WO 9844152). For both methods, the prepared DNA – usually gDNA – is fragmented down to a defined size and subsequently adapters are ligated to the (blunt) ends of these fragments. These adapters carry target (binding) sequences for PCR and sequencing primers. (The adapters are more complex than described here: they also carry index sequences that allow parallel sequencing of different samples in a single run. This is omitted here for simplicity.) The fragments are then amplified by PCR. The main difference from a standard PCR is the fact that the PCR primers are immobilized, that is covalently attached to a surface. Therefore, the ends of the immobilized fragments have to find the immobilized primers by diffusion (and not vice versa, as in a standard PCR process). In this way, clusters of – in each case identical – PCR-generated fragments are formed. These clusters of PCR-generated DNA colonies are called *polonies.* (An early paper on the principle in the context of genome sequencing is Mitra *et al.*, 2003.)

- Bridge amplification/polony PCR – the Solexa/Illumina technology
  In this case, the primers are attached to the surface of a so-called *flow cell.* The DNA fragments (fitted with adapters) are applied to the flow cell in a defined concentration so that polonies are formed in a defined density. After the PCR process, these polonies sit on the surface of the flow cell like little forest islands and here, the sequencing reaction takes place. (Figure 3).
- Emulsion/droplet PCR: the 454 technology
  Here, the primers are attached to the surface of beads. These beads are mixed with the DNA fragments (with adapters ligated to both ends), TaqPol, reagents, and buffer. This solution is mixed with oil and vortexed thoroughly so that an emulsion is formed. Most micelles include a single (or no) bead and most beads have bound a single (or no) DNA fragment. The PCR process is performed in this emulsion so that the bridge amplification takes place on the surface of the beads. Finally, most of the beads have polonies, each of which emerged from an individual DNA fragment. After the PCR, the beads are applied to PicoTiter™ plate where the sequencing reaction is performed.

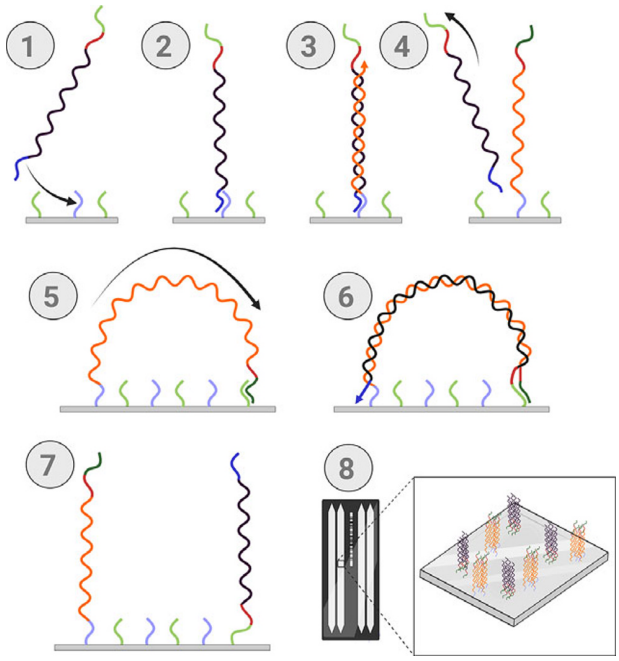



**Figure 3.** Simplified sketch of the bridge amplification principle. Steps 5-7 are repeated in the process of colony-PCR. 8 shows the result: polonies on the surface of the flow cell. Note that each polony/cluster emerges from a single DNA molecule after some rounds of amplification and thus also some rounds of denaturation.

With such PCR-based technologies the 1,000 genomes project (https://www.internationalgenome.org/) has sequenced over 2,000 human genomes. They were able to identify over 88 million sequence variants in our genome (Sudmant *et al.*, 2015; 1,000 Genomes Project Consortium, 2015). The project was continued: until 2022 over 110 Million single nucleotide variants and over 14 Million InDels have been identified and verified (Byrska-Bishop *et al.*, 2022).

The quality of the obtained data has been assessed thoroughly (Belsare *et al.*, 2019). Different classes of error were examined, and of course, it turned out that the data was not error-free. One of the error classes concerns incorrect SNPs caused by errors produced by polymerase, sequencing, or data processing. If, however, DNA indeed displayed "scarce resilience and significant loss of structural integrity when DNA is heated at temperatures above 90 °C, even for 1 minute," as claimed by Serpieri and Franchi (2024), this would have had extreme impact on the error rate, which was never observed. Within the human genome project, the overall base

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

accuracy was high, with an average of one base error per 651,000 base pairs, equating to an error rate of about 0.00015% mainly due to errors such as deletions and misassemblies and not false sequencing reads (Schmutz *et al.*, 2004).

Another example is the sequencing of genomes of extinct human (sub-) species. PCR-based NGS technologies enabled the generation of high-quality genomic data from Neanderthals and Denisovans (e.g. Meyer *et al.*, 2012; Prüfer *et al.*, 2014). This in turn made it possible to trace the genetic history and ancestry of *Homo sapiens* sapiens, Neanderthals and Denisovans (Disotell, 2012; Ahmed and Liang, 2013; Sánchez-Quinto and Lalueza-Fox, 2015; Slon *et al.*, 2018; Gokcumen, 2020; Sankararaman, 2020; Bergström *et al.*, 2021; Lalueza-Fox, 2021; Schaefer *et al.*, 2021; Zeberg *et al.*, 2024).

All this progress was only possible with high-quality genomic data obtained with PCR-based methods. The reaction conditions are even more critical than with a "standard PCR": The starting material for each individual sequence is a single DNA molecule. If thermal DNA damage were a significant factor, all these NGS/MPS methods would not work.

### Assessing error sources in PCR

Indeed quite a few studies exist on error sources caused by PCR; some of them will be mentioned now.

It became clear early on that the length of the amplicons is limited by incorrectly inserted nucleotides due to the lack of proof-read activity of the TaqPol. Mismatched bases incorporated in this way either lead to errors in the amplicons or prevent the polymerase from continuing its work. In the 1990s, alternative polymerases with proof-read activity became available. Used in PCR, they enabled the amplification of fragments of more than a dozen kBp – see e.g. Barnes (1994): his protocol generated clear, sharp amplicon bands in AGE up to 18 kbp. He discussed various sources of error, including heat-induced DNA damage. According to Barnes, at 95 °C and pH ~6.5, one apurinic site can be expected to be generated every 20–30 kb of single-stranded DNA per minute. However, he found no evidence for more frequent strand breaks during the denaturation intervals.

From the very beginning, PCR products have been cloned into vectors (e.g. Finney *et al.*, 2001). In the 1990s, many protocols relied on elution of the amplicons from excised gel slices by boiling. Frost and Guggenheim (1999) have determined the degree of DNA fragmentation under various conditions. They found that the extent of hydrolytic strand breaks depended strongly on the buffer. After 90 minutes boiling (!), the yield in pure water was zero and in TE buffer it was really low, but boiling in PCR buffer produced very good results (see Figure 2 in Frost and Guggenheim, 1999).

Pienaar *et al.* (2006) investigated errors caused by the polymerase as well as thermal DNA damage. They found that thermally induced damage is a relevant source of errors in PCR-generated amplicons. Three types can be distinguished: A + G depurination, oxidative damage of guanine to 8-oxoG, and cytosine deamination to uracil (see also refs. in Pienaar *et al.*, 2006). However, depurination does not lead to defective amplicons, as synthesis stops at depurinated sites. Therefore, depurination can only reduce the yield, while the other two processes produce point mutations. They found no evidence that heat-induced depolymerization is a relevant problem in PCR.

Orton *et al.* (2015) conducted a very thorough and comprehensive experiment. Their initial question was as follows: When genomes of RNA viruses are sequenced, for example during an epidemic or pandemic, mutations are mapped to track the emergence and development of new virus strains. To do this, it is important to distinguish real mutations from artefacts. To this end, the authors created the following experimental setup to distinguish errors produced by (a) transcription, (b) reverse transcription, (c) PCR, and (d) base calling:

First, the genome of an RNA virus (FMDV) was cloned into a plasmid vector. Then, four different experimental pipelines were run in parallel. (#1) The cloned FMDV fragment was excised from the vector and prepared by AGE. (#2) The cloned FMDV genome was diluted and subjected to PCR [19 cycles]. (#3) The same as (#2), but a higher dilution was prepared and the PCR was run for 39 cycles. (#4) The cloned FMDV genome was transcribed, the RNA synthesized was reverse transcribed and then subjected to a 39-cycle PCR. Last, all samples were analysed by Illumina NGS. Pipeline #4 displayed a total error rate (i.e. sum of errors from all sources mentioned) of less than 0.14%/site for 95% of the genome and a maximum of 0.66% for certain sites. There was no hint of significant problems caused by DNA fragmentation during PCR.

Potapov and Ong (2017) also employed NGS to examine sources of error in PCR. As expected, they found for TaqPol the well-known base substitutions, rare cases of single base deletions, and even rarer cases of single base insertions. Polymerases with proofread activity performed [some of them much] better. Two artefacts were described that had previously been underestimated: In rare cases, a template/strand switch occurs in palindromes, and also, rare cases of PCR-mediated recombination via template switching were observed. As far as damage caused by heat is concerned, the effects described above have been observed. There were no indications of noteworthy problems due to heat-induced strand breaks.

### Forensic DNA analysis: STR-PCR

In the review by Serpieri and Franchi (2024), the use of PCR in forensic genetics is explicitly mentioned. PCR methods have been used routinely for some decades to create individual, specific DNA profiles based on the analysis of short tandem repeats (STRs) (Schneider, 1997; Morling, 2009; Butler, 2011). Moreover, PCR analysis is also a necessary part of mitochondrial DNA (mtDNA) analyses (Holland and Parsons, 1999; Bär *et al.*, 2000; Parson and Dürr, 2007; Dumache *et al.*, 2016).

STRs are short repetitive sequence units, which can differ in the number of repeats between different individuals. To establish a DNA profile of an individual, these STRs are first amplified using PCR and then separated lengthwise by capillary electrophoresis (Butler, 2007). Alternatively, newer methods employ the massive parallel sequencing (MPS) technique using Bridge Amplification as already described before. MPS not only determines the length of the fragments but also detects the exact nucleotide sequence of the STRs (Alonso *et al.*, 2018). Nowadays, 16 different STRs are analyzed in Germany to create a DNA profile (Butler, 2005; Schneider and Martin, 2001).

The sequence of the individual DNA bases of a given STR and the flanking areas of these STRs are conserved sequences, with the exception of individual mutations (Kline *et al.*, 2011). If a "thermal degradation" during PCR as described by Serpieri and Franchi (2024) occurred, a reliable and consistent establishment of a DNA profile would not be possible.

A simple experimental approach shows that these assumptions by Serpieri and Franchi (2024) cannot be confirmed. Six different tissues (brain, heart, lung, liver, kidney, and muscle) were taken from each of three different people and analyzed using a commercially available STR kit (see methods section). The results for all three individuals showed identical, complete, and reproducible results in all STRs for

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

each individual, regardless of tissue used. In addition, for routine PCR and subsequent fragment length analysis, a positive control, commercial control DNA 2800 M (Promega, Mannheim, Germany), has been analyzed in our laboratory for 10 years. This consistently showed the same DNA profile, which matches the Promega reference DNA profile at all loci (James, 2011). It shows no random fragmentation during the PCR (Figure 4) over the entire time, regardless of the equipment used (various thermal cyclers and capillary electrophoresis systems) or applied methods (e.g. PCR and fragment length analysis and/or MPS). This reliability and reproducibility show that the assumptions of Serpieri and Franchi (2024) are not true. Obviously, neither the target fragments to be amplified themselves nor their primer binding sites are subject to randomized, chaotic fragmentation and polymerization during the denaturing phases of the PCR.

A major challenge in the field of forensic genetics is samples with degraded DNA. When analyzing these so-called *low-template DNA* traces, alleles may not be displayed (*allele drop-out*) and additional alleles may occur in the electropherogram that are not present in the DNA sample at all (*stutter* and *drop-in*). These phenomena are very well understood and the literature shows that these drop-outs and drop-ins are simply stochastic effects (Weusten and Herbergs, 2012; Timken *et al.*, 2014).

As early as 1996, Taberlet *et al.* (1996) showed in their study that reliable genotyping by PCR is possible even using just a few picograms of gDNA. In addition, Poetsch *et al.* (2016) demonstrated in

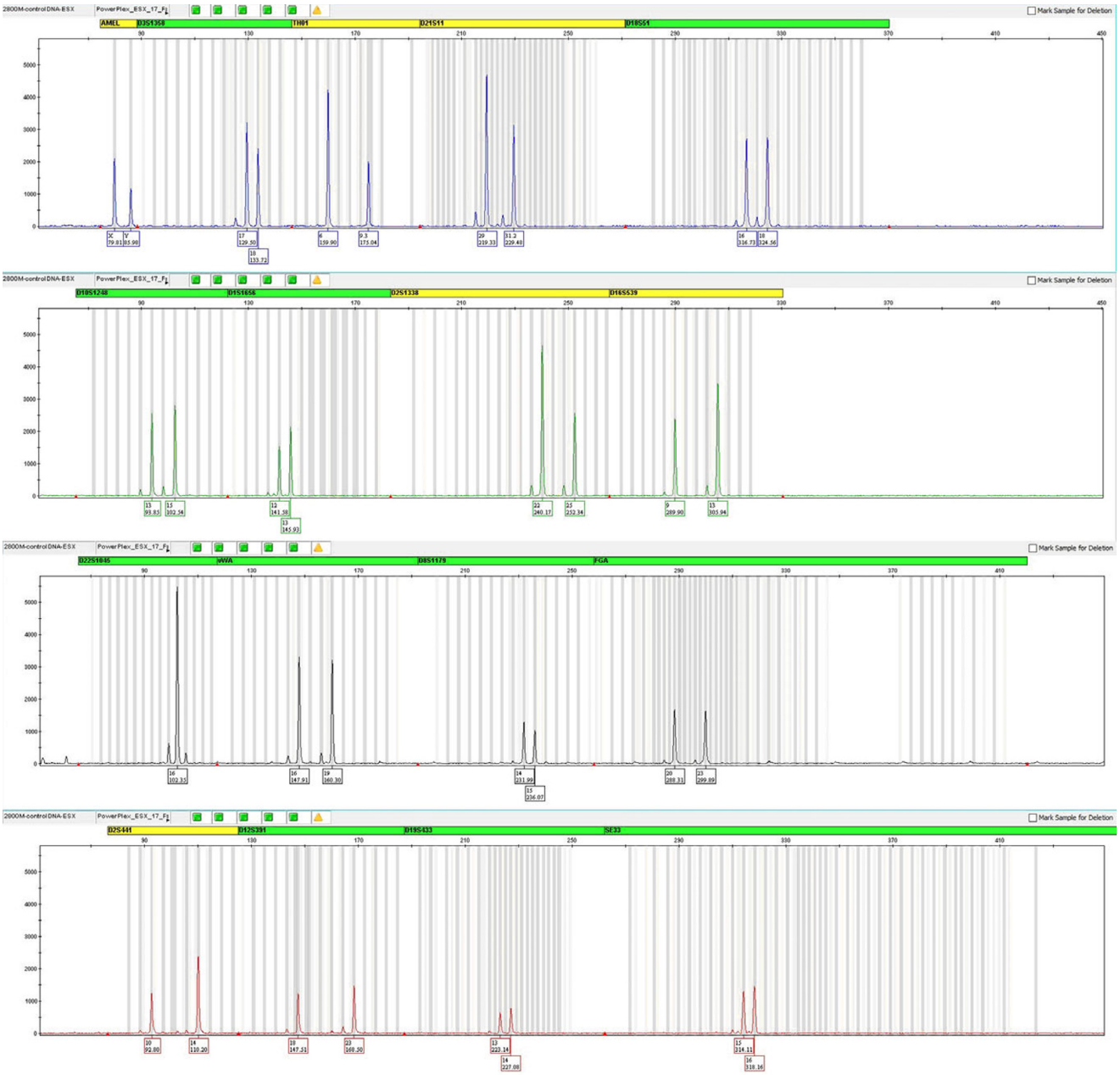


**Figure 4.** STR profile of control DNA 2800 M (Promega, Mannheim, Germany). The figure shows STR profile of control DNA 2800M (Promega, Mannheim, Germany) generated with PowerPlex® ESX kit (Promega, Mannheim, Germany). The electropherogram, i.e. the DNA profile matches the Promega reference DNA profile in all alleles (James 2011). It shows no random fragmentation during PCR and has been reproducible for many years.

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

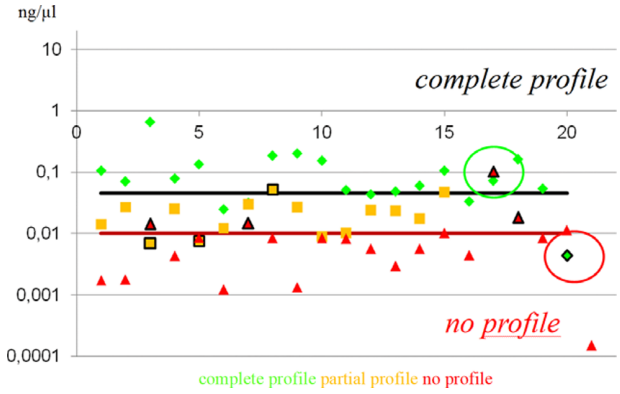



**Figure 5.** Kind of STR profiles obtained as a function of DNA concentration in a sample. The figure shows how many complete profiles (green diamonds), incomplete profiles (yellow squares) and no profiles (red triangles) could be determined from the samples depending on the DNA concentration. The horizontal lines indicate threshold values in order to be able to estimate the quality of the STR profile after quantification. From a concentration > 8 pg / µl (black line) a complete STR profile can be expected, > 3.4 pg / µl (red line) a partial STR profile and below that no STR profile. There were two outliers, marked with the circles (based on Poetsch et al. 2016).

a method comparison study that consistent and reliable DNA profiles could be amplified with commercial forensic genetics kits using the maximum input of 8 µl with samples of at least 8 pg/µl. This study also shows that partial STR results can be expected in analyses employing DNA concentrations between 3.4 pg/µl and 8 pg/µl (Figure 5) (Poetsch *et al.*, 2016). The mass of the genome of a single human cell is 6.51 pg for women and 6.41 pg for men (Piovesan *et al.*, 2019). It is therefore not surprising that newer methods in forensic genetics enable reliable and reproducible single cell STR analysis and that these studies also refute the statement by Serpieri and Franchi (2024), even for low concentration samples in the context of forensic genetics (Williamson *et al.*, 2018; Anslinger *et al.*, 2019; Meloni *et al.*, 2019; Watkins *et al.*, 2021).

In addition, the routinely used kits produce short STR amplicons to avoid drop-outs and drop-ins due to DNA degradation in the sampled trace DNA. The fragment lengths of analyzed STRs vary between ~100 bp to ~500 bp (PowerPlex® ESX Kit, Promega, Mannheim, Germany) and ~120 bp to ~450 bp (AmpFLSTR™ NGM SElect™, Thermo Fischer Scientific, Darmstadt, Germany). In the past, so-called miniplex kits were often used for degraded DNA samples. These amplify only very few loci, but have significantly shorter amplicon lengths. Examples are the PowerPlex® S5 Kit (Promega, Mannheim, Germany) with fragment lengths between ~80 bp and ~ 220 bp or the Investigator® Triplex AFS QS Kit (Qiagen, Hilden, Germany) with fragment lengths between ~100 bp and ~ 300 bp. However, some of these are no longer available, as the latest kits contain all loci (based on the loci used for the DNA database in Germany) with fragment lengths between ~80 bp - ~324 bp (PowerPlex® 18E System, Promega, Mannheim, Germany) and ~120 bp - ~350 bp (NGM Detect™, Thermo Fischer Scientific, Darmstadt, Germany). Contrary to the statements by Serpieri and Franchi (2024), it is therefore possible to generate reliable and reproducible STR results even despite poor DNA quality (Butler *et al.*, 2003; Coble and Butler, 2005; Wiegand *et al.*, 2006; Poetsch *et al.*, 2011).

In mtDNA analysis, usually the hypervariable sections HV1, HV2, and HV3 on the circular DNA molecules of the mitochondria are amplified using PCR and then analyzed using Sanger Sequencing (Sanger *et al.*, 1977; Chait *et al.*, 1988). New methods and platforms such as NGS/MPS (see section "Next Generation Sequencing (NGS) with Bridge Amplification PCR") enable the sequencing of the entire mitochondrial genome. Regardless of whether individual sections are analyzed or a whole genome analysis is performed, the detected sequence is displayed base by base. In a comparison of the results with the Cambridge Reference Sequence using an alignment for evaluation and assessment (Anderson *et al.*, 1981; Andrews *et al.*, 1999), differences are highlighted and the haplotype of a person is determined (Just *et al.*, 2004; Parson and Dürr, 2007; Strobl *et al.*, 2018). Since, as mentioned above (see section "Sanger Cycle Sequencing"), this is a linear amplification during cycle sequencing, any erroneous PCR artefacts as described by Serpieri and Franchi (2024) should appear in the electropherogram. However, in a small experimental analysis for this review, we could establish reproducible results without background signals (such as shown in Figure 2, data not shown). In three different tissue types (psoas muscle, brain, and heart muscle) from three different individuals, mtDNA regions HV I and HV II were amplified and sequenced by PCR. The resulting sequences were compared with the reference sequence and the respective haplogroup was determined independently using two different mtDNA databases/programs (https://haplogrep.i-med.ac.at/; https://empop.online/). An identical haplotype and consequently an identical haplogroup were determined for all three individuals, regardless of the tissue. We established haplogroup J1c, haplogroup T2f1, and haplogroup U5b1 for individuals 1, 2, and 3, respectively (Table 1). This small experiment also demonstrates the reliability and

**Table 1.** The table shows the results of the haplogroup determination for all three individuals

| | Detected mutations | | | |
|---|---|---|---|---|
| Person | Psoas muscle | Brain | Heart muscle | Resulting haplogroup |
| 1 | 73G 185R 228R 263G 295T 315.1c 16069 T 16126C 16181 M 16182 M 16183 M 16519Y | 73G 185R 228R 263G 295T 315.1c 16069 T 16126C 16181 M 16182 M 16183 M 16519Y | 73G 185R 228R 263G 295T 315.1c 16069 T 16126C 16181 M 16182 M 16183 M 16519Y | J1c |
| 2 | 73G 195C 263G 315.1c 16126C 16181 M 16182 M 16183 M 16189C 16294 T 16296Y 16519Y | 73G 195C 263G 315.1c 16126C 16181 M 16182 M 16183 M 16189C 16294 T 16296Y 16519Y | 73G 195C 263G 315.1c 16126C 16181 M 16182 M 16183 M 16189C 16294 T 16296Y 16519Y | T2f1 |
| 3 | 73G 150T 199C 263G 315.1c 16181 M 16182 M 16183 M 16192 T 16270 T 16519Y | 73G 150T 199C 263G 315.1c 16181 M 16182 M 16183 M 16192 T 16270 T 16519Y | 73G 150T 199C 263G 315.1c 16181 M 16182 M 16183 M 16192 T 16270 T 16519Y | U5b1 |

In all three individuals, identical deviations were found in the three different tissues for HV1 and HV2 compared to reference sequence and therefore also the identical haplogroup.

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

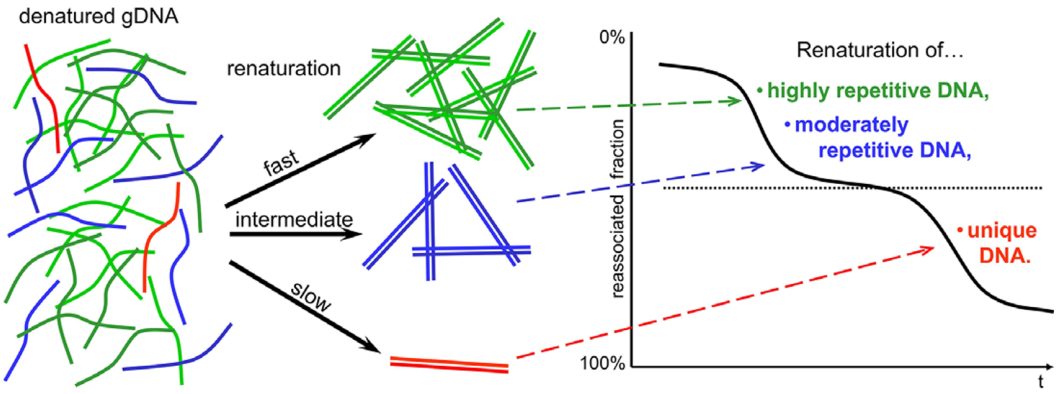



**Figure 6.** Simplified sketch of a $C_0t$ experiment. gDNA is denatured by heat (left) and then cooled down for denaturation (middle). There are fractions with a different number of copies (green, blue, red), which re-associate at different speeds (right). Reassociation is measured by measuring UV absorption.

reproducibility of this method, which is representative of PCR-based analyses routinely carried out in forensic genetics for decades, and accordingly contradicts the claims of Serpieri and Franchi (2024).

In summary, the statement by Serpieri and Franchi (2024) can therefore be refuted, also in the context of forensic genetics. In standard PCR protocols, there is no influential, random fragmentation of the DNA molecules during the relatively short heating steps. Accordingly, PCR products are not chaotic, randomized nucleotide sequences. This is not only shown by the small exemplary experiments in this review, but is above all confirmed by the countless existing literature, some of which is cited here, which on the one hand empirically confirms the reliability and reproducibility of PCR methods and, on the other hand, also clearly defines their limitations.

## Denaturation–renaturation and $C_0t$ analysis

Serpieri and Franchi (2024) apparently even question the mechanism of DNA denaturation and renaturation itself: they repeatedly use the term "*denaturation claim.*" Concerning renaturation/annealing they say "Caution should be applied to verify whether these annealing phenomena truly consist of an ordered reformation of a two-stranded material from the association of two one-stranded chains, or also involve the other much less ordered combined fragmentation-aggregation phenomena." This is somewhat surprising because already in the 1960s denaturation–renaturation studies have been performed to characterise different fractions of complex genomes (e.g. see Britten and colleagues – Waring and Britten, 1966; Britten and Kohne, 1968; Britten *et al.*, 1974). They discovered that the genomes of higher organisms contain repetitive elements in high copy numbers, as this fraction reassociates much faster than the rest after heat-induced denaturation (Figure 6). They could even distinguish a high-repetitive, a low-repetitive, and a unique fraction. During the renaturation process, highly repeated sequences renature rapidly: most strands find their complementary counterpart quite fast. The moderately repeated fraction will have an intermediate time of renaturation. Finally, unique sequences will have the slowest time of renaturation. This characteristic behavior of DNA reassociation can be visualized in the form a so called $C_0t$ *curve* (Figure 6).

In the post-genomics era, this has been confirmed by genomic sequence data (Martin, 1991; Jurka *et al.*, 2007; de Koning *et al.*, 2011; Lower *et al.*, 2019; Liehr, 2021). By now, elaborate mathematical models of the kinetics of the reassociation of DNA strands are available (e.g. Murugan 2002). Later, denaturation–renaturation kinetics were also used preparatively (instead of just analytically) to isolate defined fractions (e.g. of unique or low-copy sequences) from complex genomes (Peterson *et al.*, 2002; Yuan *et al.*, 2003; Lamoureux *et al.*, 2005; Rong *et al.*, 2013). All this is obviously only possible because the DNA survives the heat-induced denaturation without greater damage.

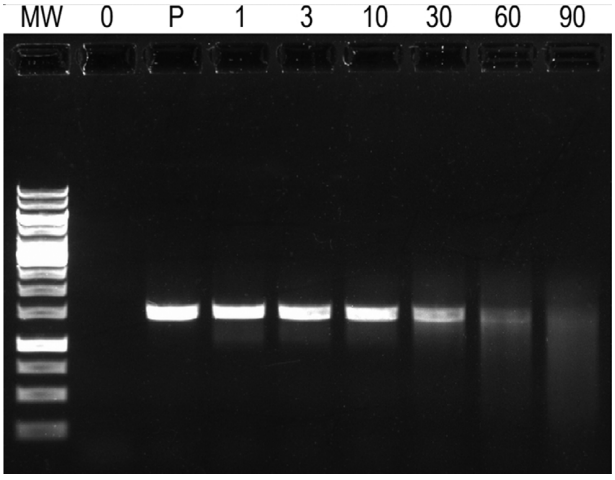



**Figure 7.** Electrophoretic analysis of denaturation-renaturation experiments of a PCR fragment. MW: molecular weight standard (o'gene ruler 1kb, Thermo Scientific).0: PCR-sample that has not been cycledP: PCR-sample 1, 3... 90: samples that were subjected to denaturation at 95°C for 1, 3, 10, 30, 60, and 90 minutes, respectively. After denaturation, all samples were incubated at 75°C for 20 minutes for renaturation. Sample volume = 10 µl, the complete samples were loaded on the gel. Faint bands at the bottom indicate residual primers.

## A simple experiment

At the end of this review, we would like to add some data from a simple experiment, namely a PCR reaction with subsequent denaturation phases, addressing the concern of thermal (in)stability of ssDNA. This experiment is carried out at the Westphalian University of Applied Sciences for students and also for pupils (a "pupils's lab program").

In this experiment, the cloned cDNA fragment from a plasmid was amplified (see Materials and methods section). Afterwards, the obtained fragment was denatured at 95 °C for different time intervals, followed by renaturation at 75 °C, and finally analysed by AGE. Figure 7 shows the result.

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

As expected, the sample that was not cycled showed no band. The input of 0.5 ng plasmid is not enough to be seen in AGE. The PCR produced a clear, sharp band. Lanes 1 … 90 demonstrate, that denaturation up to 10 minutes has virtually no effect. Only after 30 minutes first signs of degradation are visible. It takes, however, more than 1 hour of denaturation to introduce a break into all PCR fragments. Keep in mind that 30 minutes is more than the total denaturation time even of a PCR of 40 cycles.

## Loop-mediated isothermal amplification (LAMP): a modern alternative to PCR

LAMP is a variant of isothermal strand displacement amplification methods (developed by Notomi *et al.*, 2000. See also Kim and Easley, 2011, Fakruddin *et al.*, 2013). Without going into detail, it can be said that in all these cases, the temperature-dependent DNA denaturation is replaced by the strand displacement activity of an enzyme: typically, a DNA-dependent DNA polymerase with strand displacement activity like Bst polymerase is used. LAMP is a method for sequence-specific amplification, just like PCR. A special primer design ensures that loops are introduced at the ends of the amplicons. These loops harbor primer binding sites for the next DNA synthesis step.

Four to six primers (two inner primers, two outer primers, and optionally loop primers – see Nagamine *et al.*, 2002) are required for a single amplification target. For this reason, primer design is complicated and multiplexing is quite difficult. With standard PCR, however, multiplexing of over 100plex has been possible for more than a decade. (Li *et al.*, 2011). This is much more than could ever be achieved with LAMP. Furthermore, the cloning of LAMP products is tricky and the use of degenerate primers is very difficult. In addition, the exact quantification of targets using qPCR is now well established, whereas only approximate quantification is possible with LAMP (Soroka *et al.*, 2021; Rolando *et al.*, 2024). However, LAMP is fast and a laboratory is not essential for its application. For this reason, it is now routinely used in point-of-care diagnostics and on-site diagnostics (Jordan *et al.*, 2012, Nixon *et al.*, 2014).

## Coming back to the paper by Serpieri and Franchi

At this point, the question arises as to why Serpieri and Franchi (2024) come to such divergent views regarding the stability of the DNA and the reliability of the PCR. First of all, it is striking that the two authors mainly consider outdated literature from a time when, in fact, nothing was known about the molecular biology of DNA. Secondly, a citation bias is noticeable: as already mentioned, the authors ignore the $C_0t$ experiments. It is also surprising that the only citations that refer specifically to PCR parameters are from the early days of PCR, when those parameters had not yet been thoroughly tested. In doing so, they ignore the huge mountain of knowledge that has been acquired about PCR over the last 4 decades – a wealth of evidence, of which only a small selection is cited here. Thirdly, it is noteworthy that the authors did not undertake any experiments of their own on this topic – as shown in this review, even some very simple experiments are informative. Fourthly, some of the papers they cite are irrelevant for the question of whether DNA is thermoresistant enough for PCR – three examples:

- Zamenhof and Chargaff (1950) investigated the changes in the viscosity and thixotropy of DNA solutions after treatment of the DNA with acid, alkali, or heat and subsequent return to more physiological conditions. If you read this paper, you will notice that no real distinction is made between depolymerization (hydrolysis?) and denaturation. This is hardly surprising given the fact that virtually nothing was known about the DNA molecule at that time: the double helix structure of DNA was only published three years later by Watson and Crick (1953). The effects Zamenhof and Chargaff (1950) had observed can easily be explained without the assumption of extensive DNA damage. As soon as gDNA is released from cells during preparation, it breaks into pieces, no matter how carefully it is handled. (For this reason, the separation of complete chromosomes by pulsed-field electrophoresis is only possible if the cells are lysed in an agarose block so that they are never free in solution: see e.g. Finney, 2003: Unit 2.5B in Ausubel *et al.*, 2003.) Of course, all these gDNA fragments are different, so after denaturation, they will never find their original partner strands again during renaturation. Therefore, after the renaturation of gDNA, an undefined mixture of double strands with single-strand ends and cross-hybridized strands will result. Of course, this configuration has different mechanical properties than the gDNA originally prepared.
- The same reason applies to the altered behaviour of the gDNA during precipitation with ethanol (Goldstein and Stern, 1950).
- At the end of their paper, Serpieri and Franchi (2024) cite Williams (1952) who "sprayed 0.01% water solution of DNA prepared by the method by Schwander and Signer subjected to a process consisting of (1) freezing at −78 °C, (2) 60 °C heating for drying, and (3) rapid cooling." It is obvious that this treatment is far removed from the conditions during PCR.

## Conclusion: PCR is reliable

PCR is neither a magic remedy nor a miracle product. Of course, as with any technology, there are challenges and pitfalls with the PCR method, which are meanwhile known and well understood (e.g. see the sections "Background Information" and "Critical Parameters and Troubleshooting" in Coen *et al.* 2003: Unit 15 in Ausubel et al.; Lorenz, 2012).

- Just because of the sensitivity of the exponential PCR kinetics, contamination and carry-over is an extremely important issues (e.g. a case report by Wernike *et al.*, 2021).
- Template quality is also a crucial point (e.g. Lanoix *et al.*, 2012).
- Primer design is an essential aspect. This refers both to the primer sequences and the primer binding sites (to avoid secondary binding sites) (Dieffenbach *et al.*, 1993; Yuryev, 2007; Li and Brownley, 2010; Thornton and Basu, 2011; Ye *et al.*, 2012; Chuang *et al.*, 2013; Kumar and Chordia, 2015; Rodríguez *et al.*, 2015; Bustin *et al.*, 2020; Xie *et al.*, 2022).
- For qPCR, reference transcripts must be carefully selected (e.g. Lanoix *et al.*, 2012). The math behind it is tricky (e.g. Freeman *et al.*, 1999).
- It can happen that a PCR system works close to a certain "limit line," so that tiny parameter changes push the system over a tipping point (Schrick and Nitsche, 2015 give a case report).
- As mentioned above, TaqPol does not have proof-read activity, so it displays an increased error rate (McInerney *et al.*, 2014). The use of alternative polymerases with proofread activity cannot completely eliminate this problem, as the reliability of the cellular replication and DNA repair systems cannot be achieved with these either.

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

- As already mentioned, PCR-mediated recombination and template/strand switch may happen (Potapov and Ong, 2017).
- However, template fragmentation induced by heat is not a significant problem, as has now been explained in detail. Fragmentation, among other effects, will contribute to the fact that an *E*-parameter value of exactly 1.00 cannot be achieved. However, this effect is of no significance for shorter amplicons. So can the (tiny) fraction of broken strands "with probability very close to certainty" be the reason "that PCR products consist of chaotically randomized sequences of nucleotides"? (Serpieri and Franchi, 2024) The clear answer is "no," because (a) a strand break within a primer binding site simply prevents primer binding. (b) A strand break within the amplicon, i.e., between the two primer binding sites, will result in the molecule in question only being amplified linearly from now on. Therefore, in cases (a) and (b), molecules are produced that simply drop out of the PCR process. (c) A strand break outside the amplicon in the primary DNA template has no effect. This is basic PCR knowledge.

The PCR variants discussed here are only a part of the "PCR universe"; the same applies to the literature cited. It was not the purpose of this mini review to provide a comprehensive overview of PCR in the year 2025; instead, we only wanted to address the question of whether PCR is reliable. Our conclusion: If a PCR system is carefully designed and validated, it is absolutely reliable, taking into account the limitations mentioned above. Degradation of DNA due to high temperatures only happens to a noteworthy extent when exposure is prolonged. This is not the case in contemporary PCR protocols. In typical PCR protocols, the denaturation step lasts 5–30 seconds per cycle, which is not enough time to induce significant DNA hydrolysis. It turned out that DNA remains intact and amplifiable even after 30–40 cycles of PCR (e.g. Saiki *et al.*, 1988).

Over the last four decades, millions of PCR systems have been developed and billions of PCR reactions have been performed. The PCR method has a very successful history.

## Materials and methods

### *Forensic DNA typing*

The tissue samples were collected at the Institute of Legal Medicine in Essen during court-ordered autopsies (ethics vote number 15-6345-BO). DNA extraction was performed using DNA IQ Casework Pro Kit and Casework Extraction Kit in the Maxwell® RSC 48 instrument (Promega, Walldorf, Germany). Subsequently, DNA was quantified with PowerQuant™ kit (Promega) in a 7500 Fast Real-Time PCR system (ThermoFischer Scientific, Darmstadt, Germany). STR typing was done using PowerPlex® ESX 17 Kit and mtDNA sequencing was done with BigDye™ Terminator version 3.1 cycle sequencing kit and BigDye XTerminator™ purification kit. The last step of both methods – electrophoretic analysis – was performed on 3500 Genetic Analyzer instrument (ThermoFischer Scientific, Darmstadt, Germany) and results were analyzed using either GeneMapper HID software (ThermoFischer Scientific, Darmstadt, Germany) or Chromas software (Technelysium Pty Ltd, South Brisbane, Australia).

### *PCR*

A cDNA plasmid clone of *Homo sapiens* RNA binding fox-1 homolog 2 (RBFOX2), transcript variant 1, cDNA, (GeneBank Acc NM_001031695.4), cloned in pDONR211, served as PCR template. It was propagated in *E. coli* DH5α in standard medium (LB) at 37 °C overnight with aeration and shaking. Plasmid isolation was performed with a standard miniprep kit (Nucleospin Plasmid EasyPure Preparation Kit, Macherey & Nagel, https://www.mn-net.com/).

DNA yield and purity were determined by photometry (OD 260/280 nm). A Biometra thermal cycler (Biometra TRIO 48 Multi Block Thermal Cycler, purchased from Analytik Jena, Germany) was used for incubation of the samples at defined temperatures and PCR as well.

As primers, standard primers were used:

M13fw(−20, modified by one base): 5'-TGTAAAACGACGGCCAGT-3′ /.

M13rev(−27): 5'-GGAAACAGCTATGACCATG-3′.

They were purchased from eurofins (https://eurofinsgenomics.eu).

Low-eeo-agarose was used for AGE. Staining of DNA in agarose gels was performed with SYBR safe in 1:10,000 dilution as recommended by the manufacturer (*Thermo Scientific*: https://www.thermofisher.com/de/de/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/dna-stains/sybr-safe.html). The Thermo Scientific™ O'GeneRuler 1 kb DNA ladder (Fisher Scientific, https://www.fishersci.de/) was used as a molecular weight standard.

All PCR samples were identical; therefore, a master mix (100 µl) was prepared (Table 2) and then split. Table 3 shows the PCR profile.

After PCR, one sample was set aside for AGE; the others were pooled to remove $Mg^{2+}$, 0.5 M EDTA was added to a final concentration of 3 mM, which inhibits TaqPol. The mixture was then split again into 10 µl samples and subjected to denaturation at 95 °C for various time intervals. Immediately afterwards, the samples were incubated at 75 °C for 20 minutes to allow renaturation. (For denaturation and renaturation, the incubation function of the PCR cycler was used.)

**Table 2.** Ingredients for PCR (a master mix was prepared; given values refer to a single 10 µl sample)

| Ingredient | Final concentration or quantity |
|---|---|
| $H_2O$ (A.bidest) | ad 10 µl (= final vol.) |
| 10x PCR-buffer | 1x |
| $MgCl_2$ | 2 mM |
| 4 dNTPs | 200 µM each |
| Oligonucleotide primers M13fw & M13rev | 1 µM each |
| TaqPol | 0,75 U |
| Template (pre-diluted to 1 ng/µl) | 0,5 ng |

**Table 3.** PCR profile

| | T | T | Segment | Cycle profile |
|---|---|---|---|---|
| 1. | 3 min | 95 °C | Initial denaturation | Steps 2–4 = PCR-cycle; 18 iterations |
| 2. | 20 sec | 95 °C | Denaturation | |
| 3. | 30 sec | 60 °C* | Annealing | |
| 4. | 2 min | 72 °C | Elongation | |
| 5. | 15 min | 72 °C | Final DNA synthesis | |
| 6. | ∞ | 10 °C | Final storage | |

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

**Table 4.** Sequences of primers (AF, AR) and probe (AP) for detection of GTS 40–3-2 (amplicon length 74 bp)

| Name | Sequence | Length | [GC] | *Tm* |
|---|---|---|---|---|
| 40–3,2 AF | TTCATTCAAAATAAGATCATACATACAGGTT | 31 bp | 25,8% | 52–59 °C |
| 40–3–2 AR | GGCATTTGTAGGAGCCACCTT | 21 bp | 52,4% | 59 °C |
| 40–3–2 AP | 6-FAM 5'-CCTTTTCCATTTGGG–3'MGBNFQ | 15 bp | 47% | 55 °C |

**Table 5.** Composition of the PCR assay

| Component | Final concentration |
|---|---|
| *SolisFAST® Probe qPCR Mix* (5x) | 1× |
| Primer 40–3–2 AF | 400 nM |
| Primer 40–3–2 AR | 400 nM |
| Probe 40–3–2 AP | 150 nM |
| DNA (dilution) | – 4 µl per 20 µl qPCR sample – |
| $H_2O$ (PCR grade) | Ad 20 µl |

**Table 6.** qPCR cycle conditions. A StepOnePlus Real-Time PCR-System from *applied biosystems* was used

| Temperature | Time | Comment |
|---|---|---|
| 95 °C | 3 min | Initial activation (hot start) |
| 95 °C | 5 sec | Cycling: 40× (a two-step protocol) |
| 55 °C | 20 sec | |

1% (w/v) agarose gels were prepared for AGE. Electrophoresis buffer was 1× TAE (final concentration: 40 mM tris-acetate pH 8.0; 1 mM EDTA). After boiling in the microwave oven (900 W), the gel solution was cooled to approximately. 50 °C before adding SYBR Safe (final dilution 1:10,000, according to the manufacturer). Then the gel was poured. Electrophoresis was performed at a field strength of 5 V/cm at room temperature.

### *qPCR*

Soya beans were purchased from standard traders and ground into flour in a mixer. As reference, soy meal from genetically modified soya containing glyphosate-resistant EPSP synthetase (#ERM-BF410bp GTS 40–3-2) was obtained from the Joint Research Center, Directorate F - Health, Consumers and Reference Material of the European Commission.

gDNA was isolated from this meal with the *Genomic DNA from food kit* from Macherey-Nagel (#740945.50). Yield and purity were determined by photometry (OD 260/280 nm).

A hot-start TaqMan qPCR mix (*SolisFAST® Probe qPCR Mix with UNG* from Solis Biodyne, https://solisbiodyne.com; Cat# 28–21-0000S) was used. Table 4 shows the primers and the probe, Table 5 the ingredients of the PCR sample, and Table 6 the cycle conditions.

### Abbreviations

AGE agarose gel electrophoresis
bp base pairs
cDNA complementary DNA (reverse transcribed from RNA)
DNA deoxyribonucleic acid
gDNA genomic DNA
GMO genetically modified organism
HV I-III hypervariable regions I-III
kBp kilo base pairs
MPS massively parallel sequencing
mtDNA mitochondrial DNA
NGS next-generation sequencing
ORF open reading frame
PCR polymerase chain reaction
qPCR quantitative PCR
RT-PCR reverse transcription PCR
SMART switching mechanism at the 5′ end of RNA template
STR short tandem repeat
TaqPol thermostable DNA dependent DNA polymerase from *Thermus aquaticus*

**Data availability statement.** All relevant data are included in this publication or can be found in the cited literature.

**Acknowledgements.** We thank Prof. Dr. Katrin Grammann and Dennis Gluma for the qPCR data and Prof. Dr. Christian Hiepen for creating the illustrations of Figures 3, 6, and the graphical abstract.

**Author contribution.** Concept: A.B. / Manuscript: H.K. wrote the part about forensics, A.B. the rest/Experiments: STR profiling by H.K., DNA denaturation experiment by A.B., qPCR was planned by K.Grammann and performed by students of the Westphalian University of Appl. Sci., study course Molecular Biology (Bc) under supervision of D.Gluma.

**Financial support.** There has been no funding.

**Competing interests.** The authors declare none.

## References

**1000 Genomes Project Consortium *et al.*** (2015) A global reference for human genetic variation. *Nature* **526**(7571), 68–74. https://doi.org/10.1038/nature15393.

**Ahmed M and Liang P** (2013) Study of modern human evolution via comparative analysis with the Neanderthal genome. *Genomics & Informatics* **11**(4), 230–238. https://doi.org/10.5808/GI.2013.11.4.230.

**Al-Shuhaib MBS and Hashim HO** (2023) Mastering DNA chromatogram analysis in Sanger sequencing for reliable clinical analysis. *Journal of Genetic Engineering and Biotechnology* **21**(1), 115. https://doi.org/10.1186/s43141-023-00587-6.

**Alonso A *et al.*** (2018) Current state-of-art of STR sequencing in forensic genetics. *Electrophoresis* **39**(21), 2655–2668. https://doi.org/10.1002/elps.201800030.

**Anderson MW and Schrijver I** (2010) Next generation DNA sequencing and the future of genomic medicine. *Genes* **1**(1), 38–69. https://doi.org/10.3390/genes1010038.

**Anderson S *et al.*** (1981) Sequence and organization of the human mitochondrial genome. *Nature* **290**(5806):457–465. https://doi.org/10.1038/290457a0.

**Andrews RM *et al.*** (1999) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nature Genetics* **23**(2), 147. https://doi.org/10.1038/13779.

**Anslinger K *et al.*** (2019) Deconvolution of blood-blood mixtures using DEPArrayTM separated single cell STR profiling. *Rechtsmedizin* **29**, 30–40. https://doi.org/10.1007/s00194-018-0291-1.

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

**Bär W**, ***et al.*** (2000) DNA Commission of the International Society for forensic genetics: Guidelines for mitochondrial DNA typing. *International Journal of Legal Medicine* **113**(4), 193–196. https://doi.org/10.1007/s004140000149.

**Barnes WM** (1994) PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. *Proceedings of the National Academy of Sciences of the USA* **91**(6), 2216–2220. https://doi.org/10.1073/pnas.91.6.2216.

**Baross A *et al.*** (2004) Systematic recovery and analysis of full-ORF human cDNA clones. *Genome Research* **14**(10b), 2083–2092. https://doi.org/10.1101/gr.2473704.

**Bechtel S *et al.*** (2007) The full-ORF clone resource of the German cDNA consortium. *BMC Genomics* **8**, 399. https://doi.org/10.1186/1471-2164-8-399.

**Belsare S *et al.*** (2019) Evaluating the quality of the 1000 genomes project data. *BMC Genomics* **20**(1), 620. https://doi.org/10.1186/s12864-019-5957-x.

**Bergström A *et al.*** (2021) Origins of modern human ancestry. *Nature* **590**(7845), 229–237. https://doi.org/10.1038/s41586-021-03244-5.

**Beyer A *et al.*** (2003) Comparative sequence and expression analyses of four mammalian VPS4 genes. *Gene* **305**(1), 47–59. https://doi.org/10.1016/S0378-1119(02)01205-2.

**Blakesley RW** (1993) Cycle sequencing. In: Griffin HG, Griffin AM (eds.), *DNA Sequencing Protocols*. *Methods in Molecular Biology*™, vol **23**. Humana Press. https://doi.org/10.1385/0-89603-248-5:20.

**Blöchl E *et al.*** (1997) Pyrolobus fumarii, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113 degrees C. *Extremophiles* **1**, 14–21. https://doi.org/10.1007/s007920050010.

**Britten RJ and Kohne DE** (1968) Repeated sequences in DNA. *Science* **161**(841), 529–540. https://doi.org/10.1126/science.161.3841.529.

**Britten RJ *et al.*** (1974) Analysis of repeating DNA sequences by reassociation. *Nucleic Acids and Protein Synthesis Part E. Methods in Enzymology* **29**, 363–418. https://doi.org/10.1016/0076-6879(74)29033-5.

**Bustin SA *et al.*** (2009) The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55**(4):611–622. https://doi.org/10.1373/clinchem.2008.112797.

**Bustin SA *et al.*** (2020) Parameters for successful PCR primer design. *Methods in Molecular Biology* **2065**, 5–22. https://doi.org/10.1007/978-1-4939-9833-3_2.

**Bustin SA and Nolan T** (2004) Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *Journal of Biomolecular Techniques* **15**(3), 155–166.

**Butler JM *et al.*** (2003) The development of reduced size STR amplicons as tools for analyzing degraded DNA. *Journal of Forensic Sciences* **48**(5), 1054–1064.

**Butler JM** (2005) Constructing STR multiplex assays. *Methods in Molecular Biology* **297**, 53–66. https://doi.org/10.1385/1-59259-867-6:053.

**Butler JM** (2007) Short tandem repeat typing technologies used in human identity testing. *BioTechniques* **43**(4), ii–v. https://doi.org/10.2144/000112582.

**Butler JM** (2011) Forensic DNA testing. *Cold Spring Harbor Protocols* **12**, 1438–1450. https://doi.org/10.1101/pdb.top066928.

**Byrska-Bishop M *et al.*** (2022) High-coverage whole-genome sequencing of the expanded 1000 genomes project cohort including 602 trios. *Cell* **185**(18), 3426 e19–3440e.19. https://doi.org/10.1016/j.cell.2022.08.004.

**Cartolano M *et al.*** (2016) cDNA library enrichment of full length transcripts for SMRT long read sequencing. *PLoS One* **11**(6), e0157779. https://doi.org/10.1371/journal.pone.0157779.

**Chait E *et al.*** (1988) Battle of the DNA sequencers. *Nature* **333**(6172), 477–478. https://doi.org/10.1038/333477a0.

**Chan JFYip CC *et al.*** (2020) Improved molecular diagnosis of COVID-19 by the novel, highly sensitive and specific COVID-19-RdRp/Hel real-time reverse transcription-PCR assay validated in vitro and with clinical specimens. *Journal of Clinical Microbiology* **58**. https://doi.org/10.1128/jcm.00310-20.

**Chuang LY *et al.*** (2013) Specific primer design for the polymerase chain reaction. *Biotechnology Letters* **35**(10), 1541–1549. https://doi.org/10.1007/s10529-013-1249-8.

**Cline J *et al.*** (1996) PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Research* **24**(18), 3546–3551. https://doi.org/10.1093/nar/24.18.3546.

**Coble MD**, **Butler JM** (2005) Characterization of new miniSTR loci to aid analysis of degraded DNA. *Journal of Forensic Sciences* **50**(1), 43–53. https://doi.org/10.1520/JFS2004216

**Coen DM *et al.*** (2003) The polymerase chain reaction [chapter 15]. In Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (eds.), *Current Protocols in Molecular Biology*. John Wiley and Sons.

**Daniel R and Cowan D** (2000) Biomolecular stability and life at high temperatures. *Cellular and Molecular Life Sciences* **57**, 250–264. https://doi.org/10.1007/PL00000688.

**de Koning AP *et al.*** (2011) Repetitive elements may comprise over two-thirds of the human genome. *PLoS Genetics* **7**(12), e1002384. https://doi.org/10.1371/journal.pgen.1002384.

**Dieffenbach CW *et al.*** (1993) General concepts for PCR primer design. *PCR Methods and Appllications* **3**(3), S30–S37. https://doi.org/10.1101/gr.3.3.s30.

**Disotell TR** (2012) Archaic human genomics. *American Journal of Physical Anthropology* **149**(Suppl 55), 24–39. https://doi.org/10.1002/ajpa.22159.

**Dorak MT** (2006) *Real-Time PCR*. NY, USA: Taylor and Francis Group.

**Dumache R *et al.*** (2016) Molecular DNA analysis in forensic identification. *Clinical Labortory* **62**(1–2), 245–248. https://doi.org/10.7754/clin.lab.2015.150414.

**Dymond JS** (2013) Explanatory chapter: Quantitative PCR. *Methods in Enzymology* **529**, 279–289. https://doi.org/10.1016/B978-0-12-418687-3.00023-9.

**Finney M** (2003) Pulsed-field gel electrophoresis [Chapter 2, Unit 2.5B]. In Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (eds.), *Current Protocols in Molecular Biology*, Vol. **2003**. John Wiley and Sons.

**Finney M *et al.*** (2001) Molecular cloning of PCR products. [Chapter 15, Unit 15.4] in Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds.) *(2003) Current Protocols in Molecular Biology*. John Wiley and Sons.

**Lalueza-Fox C** (2021) Neanderthal assimilation? *Nature Ecology and Evolution* **5**(6), 711–712. https://doi.org/10.1038/s41559-021-01421-3.

**Fakruddin M *et al.*** (2013 )Nucleic acid amplification: Alternative methods of polymerase chain reaction. *Journal of Pharmacy and Bioallied Sciences* **5**(4), 245–252. https://doi.org/10.4103/0975-7406.120066

**Freeman WM *et al.*** (1999) Quantitative RT-PCR pitfalls and potential. *BioTechniques* **26**(1), 112-22–124-5. https://doi.org/10.2144/99261rv01.

**Freire-Paspuel B *et al.*** (2021a) Analytical and clinical comparison of Viasure (CerTest Biotec) and 2019-nCoV CDC (IDT) RT-qPCR kits for SARS-CoV2 diagnosis. *Virology* **553**, 154–156. https://doi.org/10.1016/j.virol.2020.10.010. Corrigendum in: Virology. 2021.

**Freire-Paspuel B *et al.*** (2021b) Corrigendum to „Analytical and clinical comparison of Viasure (CerTest Biotec) and 2019-nCoV CDC (IDT) RT-qPCR kits for SARS-CoV2 diagnosis" [Virology volume (2021) 154-156]. *Virology* **555**, 111. https://doi.org/10.1016/j.virol.2020.12.019. Erratum for: Virology 2021;553:154–156.

**Freire-Paspuel B *et al.*** (2022) Analytical sensitivity and clinical performance of "COVID-19 RT-PCR real TM FAST (CY5)" (ATGen, Uruguay) and "ECUGEN SARS-CoV-2 RT-qPCR" (UDLA-STARNEWCORP, Ecuador): High quality-low cost local SARS-CoV-2 tests for South America. *PLoS Neglected Tropical Diseases* **16**(4), e0010082. https://doi.org/10.1371/journal.pntd.0010082.

**Frost MR and Guggenheim JA** (1999) Prevention of depurination during elution facilitates the reamplification of DNA from differential display gels. *Nucleic Acids Research* **27**(15), e6. https://doi.org/10.1093/nar/27.15.e6.

**Grosjean H and Oshima T** (2007) How nucleic acids cope with high temperature. Chapter 4. In Gerday C, Glansdorff N (eds.), *Physiology and Biochemistry of Extremophiles*. https://doi.org/10.1128/9781555815813.ch4. Online ISBN: 9781683671657. Print ISBN: 9781555814229.

**Gokcumen O** (2020) Archaic hominin introgression into modern human genomes. *American Journal of Physical Anthropology* **171** (Suppl 70), 60–73. https://doi.org/10.1002/ajpa.23951.

**Goldstein G and Stern KG** (1950) Experiments on the sonic, thermal, and enzymic depolymerization of desoxyribosenucleic acid. *Journal of Polymer Science* **5**(6), 687–708.

**Gustafson CE *et al.*** (1993) Effect of heat denaturation of target DNA on the PCR amplification. *Gene* **123**(2), 241–244. https://doi.org/10.1016/0378-1119(93)90130-u.

**Hagemann-Jensen M *et al.*** (2022) Scalable single-cell RNA sequencing from full transcripts with smart-seq3xpress. *Nature Biotechnology* **40**(10), 1452–1457. https://doi.org/10.1038/s41587-022-01311-4.

**Heid CA *et al.*** (1996) Real-time quantitative PCR. *Genome Research* **6**(10), 986–994. https://doi.org/10.1101/gr.6.10.986.

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

**Higuchi R *et al.*** (1993) Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. *Nature Biotechnology* **11**(9), 1026–1030. https://doi.org/10.1038/nbt0993-1026.

**Holland MM and Parsons TJ** (1999) Mitochondrial DNA sequence analysis – validation and use for forensic casework. *Forensic Science Review* **11**(1), 21–50.

**James R** (2011) *New Control DNA for PowerPlex® Systems. Promega Corporation Web site*. https://www.promega.de/resources/profiles-in-dna/2011/new-control-dna-for-powerplex-systems/. Updated 2011. Accessed 20th March 2025.

**Jordan JA *et al.*** (2012) Evaluation of a manual DNA extraction protocol and an isothermal amplification assay for detecting HIV-1 DNA from dried blood spots for use in resource-limited settings. *Journal of Clinical Virology. The Official Publication of the Pan American Society for Clinical Virology* **54**(1), 11–14. https://doi.org/10.1016/j.jcv.2012.01.004.

**Jurka J *et al.*** (2007) Repetitive sequences in complex genomes: Structure and evolution. *Annual Review of Genomics and Human Genetics* **8**, 241–259. https://doi.org/10.1146/annurev.genom.8.080706.092416.

**Just RS *et al.*** (2004) Toward increased utility of mtDNA in forensic identifications. *Forensic Science International* **146**(Suppl), 147–159. https://doi.org/10.1016/j.forsciint.2004.09.045.

**Kampmann M and Stock D** (2004) Reverse gyrase has heat-protective DNA chaperone activity independent of supercoiling. *Nucleic Acids Research* **32**(12), 3537–3545. https://doi.org/10.1093/nar/gkh683.

**Kashefi K and Lovley DR** (2003) Extending the upper temperature limit for life. *Science* **301**, 934. https://doi.org/10.1126/science.1086823.

**Kim J and Easley CJ**. (2011) Isothermal DNA amplification in bioanalysis: Strategies and applications. *Bioanalysis* **2**, 227–239. https://doi.org/10.4155/bio.10.172.

**Kline MC *et al.*** (2011) STR sequence analysis for characterizing normal, variant, and null alleles. *Forensic Science International: Genetics* **5**(4), 329–332. https://doi.org/10.1016/j.fsigen.2010.09.005.

**Kubista M *et al.*** (2006) The real-time polymerase chain reaction. *Molecular Aspects of Medicine* **27**(2–3), 95–125. https://doi.org/10.1016/j.mam.2005.12.007.

**Kumar A and Chordia N** (2015) In silico PCR primer designing and validation. *Methods in Molecular Biology* **1275**, 143–151. https://doi.org/10.1007/978-1-4939-2365-6_10.

**Lamoureux D *et al.*** (2005) The efficacy of cot-based gene enrichment in wheat (*Triticum aestivum* L.). *Genome* **48**(6), 1120–1126. https://doi.org/10.1139/g05-080.

**Lanoix D *et al.*** (2012) Quantitative PCR pitfalls: The case of the human placenta. *Molecular Biotechnology* **52**(3), 234–243. https://doi.org/10.1007/s12033-012-9539-2. Erratum in: Mol Biotechnol. 2013 54(2):721–722.

**Lawrence Panchali MJ *et al.*** (2022) Accuracy of real-time polymerase chain reaction in COVID-19 patients. *Microbiology Spectrum* **10**(1), e0059121. https://doi.org/10.1128/spectrum.00591-21.

**Li K and Brownley A** (2010) Primer design for RT-PCR. *Methods in Molecular Biology* **630**, 271–299. https://doi.org/10.1007/978-1-60761-629-0_18.

**Li Y *et al.*** (2011) A universal multiplex PCR strategy for 100-plex amplification using a hydrophobically patterned microarray. *Lab on a Chip* **11**(21), 3609–3618. https://doi.org/10.1039/c1lc20526a. Epub 2011 Sep 9.

**Liehr T** (2021) Repetitive elements in humans. *International Journal of Molecular Sciences.* **22**(4), 2072. https://doi.org/10.3390/ijms22042072.

**Lorenz TC** (2012) Polymerase chain reaction: Basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments* (63), e3998. https://doi.org/10.3791/3998.

**Lower SE *et al.*** (2019) Special issue: Repetitive DNA sequences. *Genes (Basel)* **10**(11), 896. https://doi.org/10.3390/genes10110896.

**Mardis E and McCombie WR** (2017) Cycle-sequencing reactions. *Cold Spring Harbor Protocols* **2017**(7), pdb.prot094607.

**Margulies M *et al.*** (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**(7057), 376–380. https://doi.org/10.1038/nature03959. (Erratum in: Nature. 2006 441(7089):120. Ho, Chun He [corrected to Ho, Chun Heen].

**Marshall CJ and Santangelo TJ** (2020) Archaeal DNA repair mechanisms. *Biomolecules* **10**(11), 1472. https://doi.org/10.3390/biom10111472.

**Martin SL** (1991) LINEs. *Current Opinion in Genetics and Development* **1**(4), 505–508. https://doi.org/10.1016/s0959-437x(05)80199-6.

**McInerney P, *et al.*** (2014) Error rate comparison during polymerase chain reaction by DNA polymerase. *Molecular Biology International* **2014**, 287430. https://doi.org/10.1155/2014/287430.

**Meloni V *et al.*** (2019) Optimization of STR amplification down to single cell after DEPArrayTM isolation. *Forensic Science International: Genetics Supplement Series* **7**(1), 711–713. https://doi.org/10.1016/j.fsigss.2019.10.147.

**Meyer M *et al.*** (2012) A high-coverage genome sequence from an archaic Denisovan individual. *Science* **338**(6104), 222–226. https://doi.org/10.1126/science.1224344.

**Mitra RD *et al.*** (2003) Fluorescent in situ sequencing on polymerase colonies. *Analytical Biochemistry* **320**(1), 55–65. https://doi.org/10.1016/s0003-2697(03)00291-4. Erratum in: *Anal Biochem* (2004) **328**(2), 245.

**Morling N** (2009) PCR in forensic genetics. *Biochemical Society Transactions* **37**(Pt 2), 438–440. https://doi.org/10.1042/BST0370438.

**Murugan R** (2002) Revised theory on DNA renaturation kinetics and its experimental verification. *Biochemical and Biophysical Research Communications* **293**(2), 870–873. https://doi.org/10.1016/S0006-291X(02)00305-4.

**Murray V** (1989) Improved double-stranded DNA sequencing using linear polymerase chain reaction. *Nucleic Acids Research* **17**, 8889. https://doi.org/10.1093/nar/17.21.8889.

**Nagamine K *et al.*** (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes* **16**(3), 223–229. https://doi.org/10.1006/mcpr.2002.0415.

**Nagura-Ikeda M *et al.*** (2020) Clinical evaluation of self-collected saliva by quantitative reverse transcription-PCR (RT-qPCR), direct RT-qPCR, reverse transcription-loop-mediated isothermal amplification, and a rapid antigen test to diagnose COVID-19. *Journal of Clinical Microbiology* **58**, e01438–e01420. https://doi.org/10.1128/jcm.01438-20.

**Nixon G *et al.*** (2014) Comparative study of sensitivity, linearity, and resistance to inhibition of digital and nondigital polymerase chain reaction and loop mediated isothermal amplification assays for quantification of human cytomegalovirus. *Analytical Chemistry Journal* **86**(9), 4387–4394. https://doi.org/10.1021/ac500208w.

**Notomi T *et al.*** (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* **28**(12), E63. https://doi.org/10.1093/nar/28.12.e63.

**Orton RJ *et al.*** (2015) Distinguishing low frequency mutations from RT-PCR and sequence errors in viral deep sequencing data. *BMC Genomics* **16**(1), 229. https://doi.org/10.1186/s12864-015-1456-x.

**Parson W and Dür A** (2007) EMPOP–A forensic mtDNA database. *Forensic Science International: Genetics* **1**(2), 88–92. https://doi.org/10.1016/j.fsigen.2007.01.018.

**Peterson DG *et al.*** (2002) Efficient capture of unique sequences from eukaryotic genomes. *Trends in Genetics* **18**(11), 547–550. https://doi.org/10.1016/S0168-9525(02)02764-6.

**Pienaar E *et al.*** (2006) A quantitative model of error accumulation during PCR amplification. *Computational Biology and Chemistry*. **30**(2):102–111. https://doi.org/10.1016/j.compbiolchem.2005.11.002.

**Piovesan A *et al.*** (2019) On the length, weight and GC content of the human genome. *BMC Research Notes* **12**(1), 106, 30813969. https://doi.org/10.1186/s13104-019-4137-z.

**Poetsch M *et al.*** (2011) Powerplex® ES versus Powerplex® S5—Casework testing of the new screening kit. *Forensic Science International: Genetics* **5**(1), 57–63. https://doi.org/10.1016/j.fsigen.2009.11.002.

**Poetsch M *et al.*** (2016) Does zero really mean nothing?-first experiences with the new PowerQuant(TM) system in comparison to established real-time quantification kits. *International Journal of Legal Medicine* **130**(4), 935–940. https://doi.org/10.1007/s00414-016-1352-1.

**Potapov V and Ong JL** (2017) Examining sources of error in PCR by single-molecule sequencing. *PLoS One* **12**(1), e0169774. https://doi.org/10.1371/journal.pone.0169774.

**Prüfer K *et al.*** (2014) The complete genome sequence of a Neanderthal from the Altai Mountains. *Nature* **505**(7481), 43–49. https://doi.org/10.1038/nature12886.

**Rodríguez A *et al.*** (2015) Design of primers and probes for quantitative real-time PCR methods. *Methods in Molecular Biology* **1275**, 31–56. https://doi.org/10.1007/978-1-4939-2365-6_3.

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

**Rolando JC *et al.*** (2024) The present and future landscapes of molecular diagnostics. *Annual Review of Analytical Chemistry (Palo Alto, CAL)*. **17**(1), 459–474. https://doi.org/10.1146/annurev-anchem-061622-015112.

**Rong J *et al.*** (2013) Cot-based sampling of genomes for polymorphic low-copy DNA. *Molecular Breeding* **32**, 977–980. https://doi.org/10.1007/s11032-013-9964-3.

**Saiki RK, *et al.*** (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**(4839), 487–491. https://doi.org/10.1126/science.2448875.

**Salamanca-Neita LH *et al.*** (2022) Comparison of four real-time polymerase chain reaction assays for the detection of SARS-CoV-2 in respiratory samples from Tunja, Boyacá, Colombia. *Tropical Medicine and Infectious Disease* **7**(9), 240. https://doi.org/10.3390/tropicalmed7090240.

**Sánchez-Quinto F and Lalueza-Fox C** (2015) Almost 20 years of Neanderthal palaeogenetics: Adaptation, admixture, diversity, demography and extinction. *Philosophical Transactions of the Royal Society B: Biological Sciences* **370**(1660), 20130374. https://doi.org/10.1098/rstb.2013.0374.

**Sanger F *et al.*** (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**(12), 5463–5467. https://doi.org/10.1073/pnas.74.12.5463.

**Sankararaman S** (2020) Methods for detecting introgressed archaic sequences. *Current Opinion in Genetics and Development*. **62**, 85–90. https://doi.org/10.1016/j.gde.2020.05.026.

**Schaefer NK *et al.*** (2021) An ancestral recombination graph of human, Neanderthal, and Denisovan genomes. *Science Advances* **7**(29), eabc0776. https://doi.org/10.1126/sciadv.abc0776.

**Schmutz J, *et al*** (2004) Quality assessment of the human genome sequence. *Nature* **429**(6990), 365–368. https://doi.org/10.1038/nature02390.

**Schneider PM** (1997) Basic issues in forensic DNA typing. *Forensic Science International* **88**(1), 17–22. https://doi.org/10.1016/s0379-0738(97)00079-0.

**Schneider PM and Martin PD** (2001) Criminal DNA databases: The European situation. *Forensic Science International* **119**(2), 232–238. https://doi.org/10.1016/s0379-0738(00)00435-7.

**Schrick L and Nitsche A** (2015) Pitfalls in PCR troubleshooting: Expect the unexpected? *Biomolecular Detection and Quantification* **6**, 1–3. https://doi.org/10.1016/j.bdq.2015.11.001.

**Serpieri R and Franchi F** (2024) Resilience of DNA chains to molecular fracture after PCR heating cycles and implications on PCR reliability. *Quarterly Reviews of Biophysics* **57**, e8. https://doi.org/10.1017/S0033583524000064.

**Slon V *et al.*** (2018) The genome of the offspring of a Neanderthal mother and a Denisovan father. *Nature* **561**(7721), 113–116. https://doi.org/10.1038/s41586-018-0455-x.

**Soroka M, Wasowicz B, Rymaszewska A** (2021) Loop-mediated isothermal amplification (LAMP): The better sibling of PCR? *Cells* **10**(8), 1931. https://doi.org/10.3390/cells10081931.

**Strobl C *et al.*** (2018) Evaluation of the precision ID whole MtDNA genome panel for forensic analyses. *Forensic Science International: Genetics* **35**, 21–25. https://doi.org/10.1016/j.fsigen.2018.03.013.

**Sudmant PH *et al.*** (2015) An integrated map of structural variation in 2,504 human genomes. *Nature* **526**(7571), 75–81. https://doi.org/10.1038/nature15394.

**Svec D *et al.*** (2015) How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. *Biomolecular Detection and Quantification* **3**, 9–16. https://doi.org/10.1016/j.bdq.2015.01.005.

**Taberlet P, *et al.*** (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research* **24**(16), 3189–3194. https://doi.org/10.1093/nar/24.16.3189.

**Takai K *et al.*** (2008) Cell proliferation at 122 degrees C and isotopically heavy CH4 production by a hyperthermophilic methanogen under high-pressure cultivation. *Proceedings of the National Academy of Sciences* **105**(31), 10949–10954. https://doi.org/10.1073/pnas.0712334105.

**Tellinghuisen J** (2021) Estimating real-time qPCR amplification efficiency from single-reaction data. *Life (Basel)* **11**(7), 693. https://doi.org/10.3390/life11070693.

**Thornton B, Basu C** (2011) Real-time PCR (qPCR) primer design using free online software. *Biochemistry and Molecular Biology Education* **39**(2): 145–154. https://doi.org/10.1002/bmb.20461.

**Timken MD *et al.*** (2014) Stochastic sampling effects in STR typing: Implications for analysis and interpretation. *Forensic Science International: Genetics* **11**, 195–204. https://doi.org/10.1016/j.fsigen.2014.03.015.

**Tindall KR, Kunkel TA** (1988) Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase. *Biochemistry* **27**(16), 6008–6013. https://doi.org/10.1021/bi00416a027.

**VanGuilder HD *et al.*** (2008) Twenty-five years of quantitative PCR for gene expression analysis. *BioTechniques* **44**(5), 619–626. https://doi.org/10.2144/000112776.

**Valasek MA, Repa JJ** (2005) The power of real-time PCR. *Advances in Physiology Education* **29**(3), 151–159. https://doi.org/10.1152/advan.00019.2005.

**Voelkerding KV *et al.*** (2009) Next-generation sequencing: From basic research to diagnostics. *Clinical Chemistry* **55**(4), 641–658. https://doi.org/10.1373/clinchem.2008.112789.

**Waring M, Britten RJ** (1966) Nucleotide sequence repetition: A rapidly reassociating fraction of mouse DNA. *Science* **154**(3750), 791–794. https://doi.org/10.1126/science.154.3750.791.

**Watkins DRL *et al.*** (2021) Revisiting single cell analysis in forensic science. *Scientific Reports* **11**(1), 7054. https://doi.org/10.1038/s41598-021-86271-6.

**Watson JD and Crick FH** (1953) Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* **171**(4356), 737–738. https://doi.org/10.1038/171737a0.

**Wellenreuther R *et al.*** (2004) SMART amplification combined with cDNA size fractionation in order to obtain large full-length clones. *BMC Genomics* **5**(1), 36. https://doi.org/10.1186/1471-2164-5-36.

**Wernike K *et al.*** (2021) Pitfalls in SARS-CoV-2 PCR diagnostics. *Transbound and Emerging Diseases* **68**(2), 253–257. https://doi.org/10.1111/tbed.13684.

**Weusten J and Herbergs J** (2012) A stochastic model of the processes in PCR based amplification of STR DNA in forensic applications. *Forensic Science International: Genetics* **6**(1), 17–25. https://doi.org/10.1016/j.fsigen.2011.01.003.

**Williams RC** (1952) Electron microscopy of sodium desoxyribonucleate by use of a new freeze-drying method. *Biochimica et Biophysica Acta* **9**, 237–239. https://doi.org/10.1016/0006-3002(52)90157-1.

**Williamson VR *et al.*** (2018) Enhanced DNA mixture deconvolution of sexual offense samples using the DEPArray™ system. *Forensic Science International: Genetics* **34**, 265–276. https://doi.org/10.1016/j.fsigen.2018.03.001.

**Wiegand P *et al.*** (2006) Short amplicon STR multiplex for stain typing. *International Journal of Legal Medicine* **120**(3), 160–164. https://doi.org/10.1007/s00414-005-0048-8.

**Wiemann S *et al.*** (2003) The German cDNA network: cDNAs, functional genomics and proteomics. *Journal of Structural and Functional Genomics* **4**(2–3), 87–96. https://doi.org/10.1023/a:1026148428520.

**Wiemann S *et al.*** (2001) Toward a catalog of human genes and proteins: Sequencing and analysis of 500 novel complete protein coding human cDNAs. *Genome Research* **11**(3), 422–435. https://doi.org/10.1101/gr.gr1547r.

**Winichakoon P *et al.*** (2020) Negative nasopharyngeal and oropharyngeal swab does not rule out COVID-19. *Journal of Clinical Microbiology* **58**. https://doi.org/10.1128/jcm.00297-20.

**Xie NG *et al.*** (2022) Designing highly multiplex PCR primer sets with simulated annealing design using dimer likelihood estimation (SADDLE). *Nature Communications* **13**(1), 1881. https://doi.org/10.1038/s41467-022-29500-4.

**Yang Y *et al.*** (2020) Laboratory diagnosis and monitoring the viral shedding of SARS-CoV-2 infection. *The Innovation* **1**, 100061. https://doi.org/10.1016/j.xinn.2020.100061.

**Ye J *et al.*** (2012) Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134. https://doi.org/10.1186/1471-2105-13-134.

**Yuan Y *et al.*** (2003) High-cot sequence analysis of the maize genome. *The Plant Journal* **34**(2), 249–255. https://doi.org/10.1046/j.1365-313X.2003.01716.x.

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press

**Yuryev A** (ed.) (2007) *PCR Primer Design (Part of the Book Series: Methods in Molecular Biology – MIMB, Volume 402)*. Humana press. eBook ISBN978-1-59745-528-2/Hardcover ISBN978-1-58829-725-9/Softcover ISBN978-1-61737-743-3.

**Zamenhof S *et al.*** (1953) Studies on the chemistry of the transforming activity: I. *Resistance to Physical and Chemical Agents. The Journal of Experimental Medicine* **98**(4), 373–397.

**Zamenhof S and Chargaff E** (1950) Studies on the diversity and the native state of desoxypentose nucleic acids. *Journal of Biological Chemistry* **186**(1), 207–219.

**Zeberg H *et al.*** (2024) The genetic changes that shaped Neandertals, Denisovans, and modern humans. *Cell* **187**(5), 1047–1058. https://doi.org/10.1016/j.cell.2023.12.029.

**Zhu YY *et al.*** (2001) Reverse transcriptase template switching: A SMARTTM approach for full-length cDNA library construction. *BioTechniques* **30**(4), 892–897. https://doi.org/10.2144/01304pf02.

https://doi.org/10.1017/S0033583525100024 Published online by Cambridge University Press