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The importance of forensic storage support: DNA quality from 11 years old saliva on FTA cards

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Abstract:	Storage conditions influence the integrity of the recoverable DNA from forensic evidence in terms of yield and quality. FTA cards are widely used in the forensic practice as their chemically-treated matrix provides protection from the moment of collection to the point of analysis with current STR typing technology. In this study we assess the recoverability and the integrity of DNA from eleven years old saliva on FTA cards using a forensic quantitative real-time polymerase chain reaction (qPCR) commercial assay. The quality after long-term storage was investigated in order to evaluate if the FTA device could assure enough stability over time, applying some internally validated quality criteria of the STR profile. Furthermore, we used a 3D interpolation model to combine the quantitative and qualitative data from qPCR to calculate the Minimum Optimal DNA Input (MODI) to add to the downstream PCR reaction based on the quantitative and qualitative data of a sample. According to our results, when saliva sample is properly transferred onto FTA cards and then correctly stored according to the manufacturer's instructions, it's possible to recover sufficient amounts of DNA for human identification even after more than a decade of storage at ambient temperature. Degradation affected the quality of results especially when the Degradation Index exceeds the value of 2.12, requiring modifications of the standard internal workflow to improve the genotyping quality. Above this value, the application of a "corrective factor" to the PCR normalization process was necessary in order to adjust the recommended manufacturer's PCR DNA input taking into account the degradation level. Our results demonstrated the importance to consider in predictive terms the parameters obtained with the real-time quantification assay, both in terms of quantity (DNA concentration) and of quality (DI, Inhibition). Informatics predictive tools including qPCR data together with the variables of storage duration and conditions should be developed in order to optimize the DNA analysis process.

Author Comments:	
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The importance of forensic storage support: DNA quality from 11 years old saliva on FTA cards

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Abstract

Storage conditions influence the integrity of the recoverable DNA from forensic evidence in terms of yield and quality. FTA cards are widely used in the forensic practice as their chemically-treated matrix provides protection from the moment of collection to the point of analysis with current STR typing technology. In this study we assess the recoverability and the integrity of DNA from eleven years old saliva on FTA cards using a forensic quantitative real-time polymerase chain reaction (qPCR) commercial assay.

The quality after long-term storage was investigated in order to evaluate if the FTA device could assure enough stability over time, applying some internally validated quality criteria of the STR profile.

Furthermore, we used a 3D interpolation model to combine the quantitative and qualitative data from qPCR to calculate the Minimum Optimal DNA Input (MODI) to add to the downstream PCR reaction based on the quantitative and qualitative data of a sample.

According to our results, when saliva sample is properly transferred onto FTA cards and then correctly stored according to the manufacturer's instructions, it's possible to recover sufficient amounts of DNA for human identification even after more than a decade of storage at ambient temperature.

Degradation affected the quality of results especially when the Degradation Index exceeds the value of 2.12, requiring modifications of the standard internal workflow to improve the genotyping quality. Above this value, the application of a "corrective factor" to the PCR normalization process was necessary in order to adjust the recommended manufacturer's PCR DNA input taking into account the degradation level.

Our results demonstrated the importance to consider in predictive terms the parameters obtained with the real-time quantification assay, both in terms of quantity (DNA concentration) and of quality (DI, Inhibition).

Informatics predictive tools including qPCR data together with the variables of storage duration and conditions should be developed in order to optimize the DNA analysis process.

Keywords: STR typing, FTA cards, degradation, DNA quantification

Introduction

The medium and long-term storage of biological samples is a real challenging task in the forensic genetics practice. From the moment when a specimen assumes a forensic relevance, the operator should make important choices to preserve it in the best way, in order to maximize the chance to retrieve potentially useful results and facilitate prospective and retrospective analyses.

The first step of this decision process concerns the identification of the most appropriate method for evidence sampling and storage before performing laboratory analysis.

Storage conditions can influence the integrity of the recoverable DNA in terms of yield and quality. The exposure to environmental (light, humidity, elevated temperatures) and microbial factors affect the rate of physical, chemical and biochemical DNA degradation. Working with fragmented DNA has several negative implications for forensic DNA profiling using currently analytical techniques that are principally based on the determination of the size or the sequence of a template [1,2].

Controlling these DNA “decay” factors could maximize the chance to obtain informative genomic profiles especially when the biological evidence is not immediately processed or it needs to be re-analyzed after a more or less long period of time.

Freezing is actually the most common method for storing casework evidence, reference samples and the corresponding DNA extracts. However, it could be non-practical and expensive especially for extended storage periods. Additionally, it’s well known that repeated freeze and thaw cycles could damage the sample’s integrity [3].

In recent years, new solutions for room temperature storage of biological samples have been developed especially in the biobanking field.

Some of these, such as Whatman FTA cards have already been widely adopted in the forensic routine for ambient temperature archiving of single-source reference samples from living individuals [4] as well as for post-mortem DNA sampling [5,6].

FTA cards contain a chemically-treated matrix which stabilize DNA from various tissue sources immediately after sample deposition, providing protection from the moment of collection to the point of analysis with current STR typing technology, which could be also several years after collection. This storage medium is simple, flexible and the problem of refrigeration is avoided. Moreover, according to the manufacturer, even old blood and buccal cells can yield full STR profiles [7].

However, in our knowledge, only a study on old post-mortem bloodstained FTA cards demonstrates that DNA recovered from this substrate is rather stable over a long period of time and it’s then suitable for human identification purposes with current STR typing methods, even if other molecular applications requiring longer PCR amplicons (> 400 bp) are not recommended due to the time dependent fragmentation [8].

The first intent of our study was to verify the integrity of DNA from buccal cells of living subjects stored on FTA cards for more than 10 years, in terms of amount and quality of recoverable DNA through a quantitative real-time polymerase chain reaction (qPCR) assay using a commercial forensic quantitation kit.

In addition, we assess the capability of this collection device to ensure the maintenance of DNA integrity in the long term period verifying if we could recover complete high quality STR profiles that fit the internal quality acceptance criteria normally applied to “fresh” single-source reference samples. With this purpose,

the following STR profile quality parameters were investigated: average peak heights, number of alleles detected (allelic drop-out), peak height ratio (PHR), intra-color and inter-locus (profile) balance.

Currently, several quantitative PCR (qPCR) assays are available in forensics allowing the estimation of useful information on a sample in one go (DNA quantity, level of degradation and inhibition, presence of a male component). However, as underlined recently, those precious data are not fully exploited in the subsequent analysis due to the lack of a practical strategy to combine them in the best way [9].

Therefore, we try to assess if the qPCR derived quantitative and qualitative data really reflect the downstream STR typing success and then if they could be used as indicators to optimize the downstream forensic STR typing process for this kind of samples. In particular we focused on the qualitative indicator "degradation" derived from qPCR analysis to adjust the sample downstream STR typing workflow for those samples.

Finally, following Hedell et al. [9], we used a 3D interpolation to combine the quantitative and qualitative data from qPCR to calculate the Minimum Optimal DNA Input (MODI) to add to the downstream PCR reaction. In order to do that, we lean on a quality pass which is settled on the basis of the achievement of established thresholds of quality parameters of the final STR profile.

The construction of decision maps based on qPCR results could be a valid tool for the forensic analyst. This offers the possibility to quickly predict and choose the optimal analysis workflow for a specific sample from the moment of collection to that of profile interpretation, minimizing lab costs, sample consuming and maximizing the information recovery. In this study we show that also the variables of "storage time" and "storage device" should be considered in future predictive models as they affect the preservation and then the quality of DNA over time.

Materials and methods

Sample collection

Buccal cells were collected directly onto Indicating FTA Mini Cards (GE Healthcare) during the year 2008 from 179 anonymous living donors. After sample deposition the cards were allowed to dry and then stored at ambient temperature (~ 23 °C) inside multi-barrier pouches with desiccant protected from UV light for eleven years until testing.

DNA extraction and quantification

FTA cards were pierced with an Harris Uni-core Punch 3.0 mm for manual punching (GE Healthcare) and two punches of 3.0 mm diameter for each card were removed and used as input material for DNA extraction using ReadyAmp Genomic DNA Purification System (Promega).

All samples were quantified performing quantitative PCR (qPCR) with the PowerQuant System (Promega) on a 7500 Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA). DNA standards, negative controls and DNA extracts were quantified in duplicate. Each quantitative reaction contained 1µL of

PowerQuant 20X Primer/Probe/IPC mix, 10 µL of PoweQuant 2X Master Mix, 7 µL of Water Amplification Grade and 2 µL of template DNA for a total reaction volume of 20 µL.

Quantification data were analyzed with the HID Real-Time PCR Analysis Software v1.2 and with the macro-based PowerQuant System Analysis Tool v1 (Promega). A R^2 value of ≥ 0.99 on the standard curve was accepted.

The results of the two replicates for each sample were averaged and only this final value was considered in data interpretation.

DNA quality of each FTA sample was assessed in terms of level of degradation and inhibition. The calculation of a Degradation Index (DI) was performed as the ratio between the concentration values of two probes included in the qPCR kit, the Autosomal DNA target (84 bp) and the Degradation target (294 bp). The higher the DI value the greater the entity of DNA degradation. Samples were assigned to four arbitrary categories of degradation based on their DI according to manufacturer's recommendations [10] : 1) 0 < DI < 2 no degradation 2) 2≤ DI < 4 mildly degraded 3) 4 ≤ DI < 10 degraded 4) DI ≥10 severely degraded.

The presence of inhibition was assessed controlling the amplification performance of an Internal Positive Control (IPC) in the qPCR assay. Inhibitors present in a sample cause lower amplification success of the IPC, with an upward shift in the cycle threshold (Ct) value. According to the manufacturer, an IPC shift threshold of 0.3 was applied [11]. A sample was flagged as inhibited if it shows an IPC shift ≥ 0.3.

STR analysis

DNA profiling was performed for all samples with the AmpFLSTR Identifiler Plus kit (Applied Biosystems, Foster city, USA). Normalization of PCR DNA input was calculated considering the quantitation value of the 84 bp PowerQuant System probe (Autosomal Target), according to the manufacturer's recommendations [11].

Identifiler Plus multiplex assay amplifies 15 autosomal STR loci plus the sex determining marker Amelogenin, and it's widely used in the forensic practice. Following the kit user's manual [12], the optimal amount of DNA is 1.0 ng in a maximum input volume of 10 µL for 28 PCR cycles and 0.5 ng in a maximum input volume of 10 µL for 29 PCR cycles. Samples with a DNA concentration < 1 ng/µL were normalized to 0.5 ng total DNA input with a 29-PCR-cycle protocol whereas samples with a DNA concentration ≥ 1 ng/µl were normalized to 1 ng input with the standard 28-PCR-cyle protocol. Two amplification replicates under the same conditions were performed for each sample.

PCR reaction setup and thermal cycling were performed according to the manufacturer [12], except for the final reaction volume which was reduced to 12.5 µL instead of 25 µL.

PCRs were performed on a Veriti 96-well thermal cycler with the following recommended cycling parameters: 95 °C for 11 min followed by 28/29 cycles of 94 °C for 20 sec, 59 °C for 3 min and a final extension at 60 °C for 10 min.

PCR products were separated and detected by capillary electrophoresis on an Applied Biosystems 3130 Genetic Analyzer using POP-4 polymer and run along with GeneScan LIZ-500 dye size standard. The injection condition was15 kV/5 s. Alleles were called using GeneMapper ID-X ver. 1.5 software.

The following thresholds were used for data interpretation: an analytical threshold (AT) of 50 relative fluorescent units (RFUs) and a stochastic threshold (ST) of 200 RFUs and 300 RFUs for 28 and 29 PCR amplification cycles respectively.

Data analysis

The quality of STR profiles was investigated by using the R package STR validator v.2.1 [13]. The following quality parameters and relative thresholds were applied:

- **Rate of drop-out**=0
- **Average peak heights (APH) across the profile** ≥ 2000 RFU
- **Heterozygous balance (Peak Height Ratio, PHR)** ≥ 60%

Intra-locus peak height ratio (PHR) was calculated for each locus by dividing the peak height (in RFU) of the high molecular weight (HMW) allele by the peak height of the low molecular weight (LMW) allele and then multiplying this value by 100 to express the PHR as a percentage. This definition of heterozygous balance is currently preferred [14].

- **Average PHR across the profile** ≥ 80%
- **Intra-color balance (ICB)** ≥ 50%

This parameter indicates if the peak heights within a dye channel are well-balanced. Intra-color peak height balance was calculated by first averaging heterozygous peaks and dividing the homozygous peaks in half. Once normalized for diploidy, the lowest score for the locus labeled with a given dye was divided by the highest and the result expressed as a percentage.

- **Inter-locus balance (profile balance)** ≥ 60% with a standard deviation (SD) ≤ 20%

The peak balance was calculated globally across the profile with the *"Normalised*" option of the function *"Balance-calculate inter-locus balance"* in *STRvalidator* .

The quality criteria applied were internally validated previously for reference single-source samples from "fresh" FTA cards following the literature and the most recent international guidelines on autosomal STR typing [15].

These criteria were initially applied on "old" FTA Cards both for non-degraded as well as for samples showing some level of degradation.

If a sample didn't meet one of the aforementioned criteria and therefore it didn't pass the quality control, it was re-amplified adjusting the kit input DNA amount in order to improve its quality. A range of different DNA input amounts were tried in order to find the Minimum Optimal DNA Input (MODI) to add to the amplification reaction which allows to obtain a profile above the applied quality thresholds minimizing the sample consumption. This value was increased using a factor of +0.1 ng in order to find the MODI with more precision (e.g. a MODI was set to 0.6 ng instead of the standard 0.5 ng recommended for the 29-PCR-cycle protocol). To test if this new normalization works well, two novel amplification replicates were performed for that sample.

When the best MODI was found, it was selected as the value of choice at which effectively normalize that sample to perform amplification with the selected kit and PCR conditions.

Finally, we used a fitting tool available in the MATLAB software to perform a 3D interpolation of the corrective factor for normalization and the PCR volume, having as Cartesian pair of coordinates the Degradation Index (DI) and the quantity of DNA. Moreover, we used a MATLAB tool called Classification Learner to predict the quality pass on the basis of such pair of variables.

Results

The amount of DNA extracted from the 11 years old saliva samples on FTA cards ranged from 0,096 to 2,771 ng/µl with a mean of 0,520 ng/µL ± 0.42 SD.

The number of starting punches per card used for DNA extraction (2 x 3 mm diameter) was the same for all samples and it could provide sufficient DNA material for the STR analysis with the conditions implemented. However the variability between samples in terms of DNA concentration is high.

Several factors, such as an individual's epithelial cells number at the time of sampling and the amount of saliva deposited are responsible of sample-to-sample and likely of intra-sample variability.

Moreover, buccal samples have historically produced greater variability in DNA yield than blood samples on FTA cards, most likely due to the clumping of cells on the matrix [16].

Even it's difficult to standardize the method for the abovementioned reasons, the use of multiple discs per extraction, at least two, is recommended especially when the analysis is performed after several years after collection.

According to our results, when saliva sample is properly transferred onto FTA cards and then correctly stored according to the manufacturer's instructions, it's possible to recover sufficient amounts of DNA for human identification with the current CE-based STR typing technology even after more than a decade of storage at ambient temperature.

All samples have sufficient DNA available to obtain the optimal absolute DNA input amount of 0.5 ng and 1 ng (29 and 28 PCR cycles respectively) for the amplification with the STR typing kit used.

The quality after long-term was also investigated in order to evaluate if the conservation on a FTA device could assure enough stability over time. The DI represents a general indicator of a sample's integrity, where the higher is the value of the Degradation Index the greater is the entity of DNA degradation in the sample.

The samples tested showed DI values ranging from a minimum of 0.96 to a maximum of 6.28, with a mean of 1.98 ± 1.3 SD. Therefore, even if all samples were subjected to the same storage period and conditions, the degradation rate is different. Inter-sample comparison was not performed in this study, however it's possible that the degradation rate is also different within the same FTA deposition area.

Only a sample exhibited a DI < 1, probably due to a minor efficiency of the smaller probe (Autosomal Target) during the qPCR reaction. Degradation indices across FTA samples fall between the first and the third category (see Materials and Methods), even if most samples belong to the first one (no degradation). In

particular, 110 samples were attributed to the first category (no degradation) with DI values ranging from 0.96 to 1.99 (mean 1.5 ± 0.3 SD), 60 samples were mildly degraded with DI values ranging from 2.0 to 3.82 (mean 2.7 ± 0.6 SD) and finally 9 were degraded with DI values ranging from 4.02 to 6.28 (mean 5.2 ± 0.6 SD). None of the analyzed samples showed severe degradation (fourth category). This strengthens the evidence of a quite high stability of DNA at ambient temperature on this type of support. In addition, no inhibition was observed.

We then investigated if it's possible to recover high quality full STR profiles applying to "old" FTA samples the same internal quality thresholds normally applied to reference single-source "fresh" FTA samples.

All samples amplified gave a complete STR profile (no drop-out). However the optimal DNA input for PCR varied between samples and in some cases deviates from the standard value recommended by the STR kit's manufacturer, depending on the initial DNA concentration and mostly on the degradation rate.

In theory, under optimal conditions and without degradation, the limiting issue in STR typing success is the DNA concentration value.

The optimal DNA concentration for input into STR amplification reaction is commonly determined after qPCR on the basis of the qPCR small probe concentration.

We could suppose that for non-degraded samples, where the peak height is presumably not affected by fragment length, this could a realistic approximation. However, when a sample shows degradation of different entity, this evaluation couldn't be so accurate as smaller STRs behave differently than larger STRs that may drop out resulting in a partial or unbalanced profile.

Moreover, the manufacturer's STR kit manual used here suggests that "if the sample contains degraded or inhibited DNA, amplification of additional DNA may be beneficial" [17]. But how to establish this generic "additional DNA" in quantitative terms minimizing the sample waste is not specified.

In this study it was possible to follow the standardized internal workflow for "fresh" FTA saliva samples until the DI of old FTA cards reached the value of 2.12 for samples submitted to the 29 cycles protocol.

When the DI exceeds the 2.12 value, the standard internal process needs modifications to improve the genotyping quality. Above this value, the PCR normalization process required the application of a "corrective factor" to adjust the recommended manufacturer's PCR DNA input taking into account the degradation level. In all cases this value was obviously increased with respect to the standard.

Table 1 illustrates the results for FTA samples with a DNA concentration < 1 ng/µl for which a normalization to 0.5 ng and a 29-PCR-cycle protocol should be applied.

Among these, samples with a DI > 2.12 required a corrective factor of +0.1-0.4 with respect to the to the kit's standard normalization (0.5 ng). Samples of the third degradation category required an higher corrective factor in the range of +0.4-0.8.

Samples with a DI > 4.82 couldn't achieve all the established thresholds of the quality indices even with a corrective factor of + 0.8.

It's noteworthy that even with this correction which brought the MODI to 1.3 ng (we decided to not test higher corrective factors) it was not possible to reach the thresholds of profile quality. Reasonably, new thresholds of profile acceptability should be defined for old FTA samples with a DI above this cut-off value.

For samples with a DNA concentration > 1 ng/µl submitted to the 28 cycles protocol, it was possible to follow the standardized workflow until the DI reached the value of 2.38 (Table 2). Above this DI value, the

corrective factor for normalization ranged from + 0.1 to +0.6 with respect to the standard (1 ng). No sample submitted to the 28-PCR-cycle protocol belongs to the third category of degradation. Applying the corrective factor all samples in this group passed the quality check with the applied criteria, presumably due to a low level of degradation.

The average of peak heights (APH) decreases passing from non-degraded to degraded samples.

The balance calculations showed that the intra-color peak height balances for each dye channel also decreased when the degradation level of samples increases. Even the overall profile balance was affected by degradation and the mean value for samples belonging to the third degradation category falls below the internal quality threshold of 60% (Table 1).

With the purpose to give the forensic scientist a practical "predictive" tool, we interpolated the qPCR data of all samples analyzed, in order to predict the right value of the normalization factor required for that sample as well as the final PCR input volume.

The following input variables were introduced in a first raw model: body fluid, collection device, time of storage, storage temperature, DNA extraction method, quantification method, DNA concentration, degradation index, presence of inhibition, STR typing kit. .

With the purpose to understand if any sort of dependence between the triplets of variables (quantity, DI/Inhibition, PCR Volume), and (Quantity, DI/Inhibition, normalization factor) exists, we interpolated the data which have passed the quality control. Supplementary Figures 1-4 show the raw data together with the interpolating surfaces.

It is possible to fit the data by using the following polynomial function:

$$f(x,y) = p_{0,0} + p_{10}x + p_{01}y + p_{20}x^2 + p_{11}xy + p_{02}y^2 + p_{30}x^3 + p_{21}x^2y + p_{12}y^2x + p_{03}y^3 \quad (1)$$

Supplementary Figure 5 provides a prediction of the quality-pass for a given pair of variables DI/Inhibition and quantity: the dots represent the data obtained from the experiments and their color denotes the quality-pass on the basis of the quality parameters established (red for bad quality and green for good quality). For any pair of DI/Inhibition and quantity chosen outside the available data, the colored region provides the prediction of the quality pass. In other words, if the selected point falls into the red region, it has not passed the quality control and vice-versa. Thanks to this predictive tool, the goodness of the data can be easily checked.

On the basis of the empirical data, we start to build a program able to extrapolate the piece of information closest to the one required by the user. For any pair of coordinates (Quantity, DI) it looks for the closest point already present among the empirical data and displays the related set of information (Supplementary Figure 6). This is a practical tool could give the forensic scientist an overview on what is already present among the data and a quick way for treating similarly the closest cases. Data of each sample could be collected, for instance, in different excel sheets so that it is easy for the program to access information of the desirable sample.

Furthermore, it is possible to provide a prediction of the expected value of the Normalization, the PCR

volume and the obtainable quality pass, respectively, from the interpolation strategy and a relatively simple machine learning tool (still starting from the knowledge of the variables Quantity and DI). A comparison between the predicted value and the one returned as the closest, among the data, to the pair given as input, allows the user to establish if the prediction is reliable and, possibly, what is the best trade-off to opt for. Also a visual check, obtained by drawing the new point on the forecast region, could be helpful in such direction.

Notably, if the quantity of DNA in a sample is relatively low together with a Degradation Index > 2, the probability to obtain a good STR profile in qualitative terms is hard, based on the parameters investigated herein.

Discussion

This study evaluates the performance in the long time period of a common forensic collection and storage system, in order to deepen the efficiency of sampling supports in preserving biological traces of forensic interest over time.

FTA cards are already widely used in the forensic practice and they have been recently proposed as the method of choice for DNA sampling even in extreme situations such as mass fatalities and within the mortuary [5,6].

As previously assessed for post-mortem blood [8], this study confirms that FTA card is a very stable sample carrier for the long-term, room-temperature archiving of saliva samples for more than a decade. It's possible to perform DNA analysis with FTA cards with three options: direct amplification, standard amplification with rinsing ("punch-in method") and extraction of DNA from the card. If the choice is to perform DNA extraction, five different methods are allowed [16]. Here we decided to perform DNA extraction with the same method usually applied to "fresh" FTA samples in our routine, which permits the recovery of single-stranded DNA and is simpler, quick and cheaper than other methods available.

Regarding the amount of recoverable DNA, all samples analyzed generated sufficient DNA yield to reach the recommended PCR input value for the STR typing kit used in this study, even if they displayed variability in terms of sample to sample concentration.

Consistent with prior observations [8], the quality of DNA in terms of integrity is substantially maintained over the years for fragments of ~ 300-350 bp, a dimension that is currently filled by the common STR typing kits.

During the last 5 years the STR multiplex amplification kits have been seriously improved in terms of sensitivity and robustness to PCR inhibitors, and it is now possible to generate full informative genotypic profiles from very low amounts of DNA template (~30 pg) [18]. In this study we evaluate the performance of a single typing kit (Identifiler Plus), which it's not part of the last generation autosomal STR amplification kit. However it's quite plausible that the quality results illustrated herein could be also achieved even with a less starting amount of DNA if using a new generation, more sensitive commercially available STR kit.

One of the main concern of a forensic analyst is to ensure the stability of forensic DNA source overtime. Few data on how different storage devices and conditions affect the DNA degradation kinetics for a specific biological material are currently available [19-20].

It’s fundamental to gain more knowledge on this in order to set up the analytical workflow in the best possible way.

Forensic degraded samples could be treated differently in order to maximize the amount of genotyping information and the entity of degradation should lead to the adoption of different strategies, for example using mini-STRs with amplicons below 220 bp or with the combined use of different commercial STR kits amplifying the same markers with a different set of primers.

Alternatively, a different genotyping method may be chosen such as mitochondrial testing, SNP typing, insertion/deletion polymorphisms (INDELS), or massively parallel sequencing.

Even if the degradation rate of our tested samples is quite restrained (maximum DI = 6.28) and full STR profiles were obtained in all cases, however this sample’s quality condition has interfered with the standard internal workflow requiring the application of a quantifiable “corrective factor” to normalize the PCR input amount (Supplementary Table 1).

Several commercial qPCR kits are currently available for the forensic use but information gained with this assay are underused as the analyst lack a concrete tool to combined together quantity and quality indicators in a useful way [21].

In a recent study, the use of decision maps based on qPCR indicators (DNA concentration and level of degradation) and the Bayesian decision theory are developed to guide the forensic scientist about the better analysis choice for the given circumstance [9].

Moreover, Hansson et al. [22] demonstrated that with knowledge of the degradation parameter the resulting characteristics of the DNA profiles can be predicted by simulation.

Here we provide a graphical representation of the raw data related to the normalization factor and the volume of PCR as a function of the pair qPCR variables quantity and DI, together with the interpolating surface. Moreover we trained the data-set with a MATLAB tool in order to predict, for a given pair of variables DI and quantity, the related quality pass. This allows the forensic scientist to extrapolate and hence to predict the MODI value for PCR and also to foresee the quality of the final STR profile for any new sample to analyze, even if the values of qPCR variables are not the equal of those already available in the program (closest point).

In this study only a single DNA extraction method, qPCR kit and STR typing kit per sample were examined. Furthermore, no inhibition was observed in our samples, therefore the only qualitative parameter analyzed was the degradation level. This is a limiting factor and surely more alternatives must be taken into account in order to choose the best possible forensic analysis procedure for a given sample.

However, our “raw” model represents a starting point for more sophisticated models including the greatest number of variables, including the impact of a specific workflow in terms of lab costs.

Predictive tools could aid the forensic expert in managing samples and to quickly find the optimal analytical strategy for that sample in order to reduce as much as possible investments of time, money, and the loss of precious samples optimizing the efficiency of DNA typing.

It’s reasonable that when the sample is low template (LT-DNA) and/or with low quality (degraded, inhibited or both) as expected in most forensic casework, the preferred strategy to apply should be more complex to predict and a lot of simulations are needed to construct a solid and reliable predictive model as the variables affecting the final genotype are countless.

Our results demonstrated the importance to consider in predictive terms the parameters obtained with the real-time DNA quantification assay, both in terms of quantity and of quality. For that purpose, the meaningful thresholds for quantitative and qualitative indices should be evaluated.
Furthermore, variables such as storage duration and conditions should be included.

Conclusion

This study demonstrated that when using the applied analytical workflow, FTA cards are suitable for robust and reliable DNA recovery from saliva samples even up to 11 years after collection.
It's known that storage time and conditions strongly affect DNA survival. In order to enhance the analysis, the rate of degradation should be deepen as it change the approach towards a sample.
Therefore, the forensic scientific community should provide more data on the capability of the currently accepted storage device and methods of conservation in preserving the quality of nucleic acids for forensic purposes over time, especially considering the possibility of further analyses after a long period after collection.
Informatics predictive tools including the variables affecting the quality and quantity of a sample as well as the data derived from qPCR should be developed in the future in order to optimize the DNA analysis process.

FOR ETHICAL STATEMENT:

Compliance with Ethical Standards

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Table

Table 1 Characteristics of FTA samples according to their degradation category for the 29-PCR-cycle protocol

Degradation Category	DI range	No. samples	Corrective factor	MODI	APH (RFU)	Mean PHR	Intracolor balance (mean) **	Profile balance (mean)
1	0.96-1.99	90	0	0.5 ng*	2958	86%	B 67,5% G 68,2% Y 69,1% R 71,2%	63,4%
2	2.03-3.82	50	+ 0-0.4	0.5-0.9 ng	2817	85%	B 63,5% G 60,3% Y 68% R 66,2%	62%
3	4.02-6.28	9	+0.4-0.8	0.9-1.3 ng	2334	85%	B 41% G 44,3% Y 49,7% R 59,5%	48,2%

DI = Degradation Index; MODI = Minimum Optimal DNA Input; APH = Average peak heights; PHR = Peak Height Ratio

* Standard normalization as indicated by the STR kit

** B=Blue, G=Green, Y=Yellow, R=Red

Table 2 Characteristics of FTA samples according to their degradation category for the 28-PCR-cycle protocol

Degradation Category	DI	No. samples	Corrective factor	MODI	APH (RFU)	PHR	Intracolor balance (mean) **	Profile balance (mean)
1	1.39-1.97	20	0	1 ng*	2460	89,3%	B 69,1% G 71,6% Y 69,3% R 65,8%	63,6%
2	2.00-3.82	10	+ 0-0.6	1.1-1.6 ng	2184	87%	B 56,9% G 66,1% Y 68,5% R 72,9%	61,7%

DI = Degradation Index; MODI = Minimum Optimal DNA Input; APH = Average peak heights; PHR = Peak Height Ratio

* Standard normalization as indicated by the STR kit

** B=Blue, G= Green, Y=Yellow, R=Red