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Room temperature DNA preservation of soft tissue for rapid DNA extraction: An addition to the disaster victim identification investigators toolkit?

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Abstract

In mass fatality incidents, for example following a vehicle accident or terrorist event, severe fragmentation of bodies may occur, making identification by the use of traditional techniques such as fingerprinting or odontology difficult. In such situations DNA profiling can be employed for individualisation and re-association of fragmented remains. As at times disrupted soft tissue may be the predominate tissue type requiring identification and re-association. We have investigated the use of two buffer solutions for preservation of soft tissue samples that may be collected during such investigations, when buccal cells, blood samples or teeth or bone may not be available. Both buffer solutions have shown sufficient DNA preservation over a 12-month period of storage at room temperature to allow for DNA profiling to be successfully performed when 5–1000 mg muscle tissue was stored in each solution.

Keywords: DNA; Preservation; Tissue storage; STR profiling; Disaster victim identification; Mass fatality incident

1. Introduction

DNA profiling is a well-established scientific technique that is used throughout the world for paternity testing, criminal intelligence and individual identification. One of the outstanding qualities of this technique comes from the fact that DNA profiling can be performed on any biological sample, providing complete DNA degradation has not occurred. Every year a number of disasters occur throughout the world, claiming the lives of thousands of individuals. These disasters can occur due to many reasons but are broadly classified as environmental, medical, industrial, vehicle, or terrorist-related; the latter of which one must be prepared for the use of chemical, biological or radiological components. No matter what the cause, there is a legal and humanitarian requirement for the individual identification of victims and, in the case of body disruption, the re-association of remains both to aid the grieving process of friends and relatives of the deceased and depending on the cause, for legal investigation of the incident.

Disasters can result in the death of any number of individuals. Mukaida et al. reported the DNA identification of two individuals who perished in a military aircraft training incident that occurred over the sea. This accident resulted in the death of a small number of persons, however, due to its nature, a high degree of body fragmentation occurred resulting in the recovery of 33 body parts after 2 days of searching [1]. In contrast to this report, the Indian Ocean Tsunami that occurred on 26th December 2004 resulted in the death of over 200,000 individuals, affecting more than 10 countries in the immediate area [2]. In this case a massive number of bodies were recovered, showing little or no fragmentation, but extensive decomposition and putrefaction. This had occurred due to the tropical climate of the area, and presented a different challenge to disaster victim identification (DVI) teams [3].

There are many methods available for identification of the dead. For DVI there are four primary identification criteria that can be used as evidence for individual identification: odontology, fingerprinting, DNA and observation of unique characteristics, such as numbered surgical prostheses. Of these

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techniques, DNA profiling can prove useful in allowing identification and re-association of fragmented, burnt or decomposed corpses that would be very difficult or impossible using traditional techniques. Successful DNA profiling is, however, dependent upon the collection and preservation of suitable biological material from the deceased and the availability of reference samples to which this DNA profile can be compared. Samples collected for DNA identification are usually stored at −20 °C to halt the degradation processes that begin after death.

An alternative method of sample preservation that could potentially aid DNA-DVI is investigated in this report. We have investigated two buffer solutions for their ability to preserve soft tissue samples at room temperature preservation over a 52-week period.

2. Materials and methods

2.1. Sample collection

Local ethical committee permission was granted for the collection of limbs from adult amputation patients at the University Hospitals of Leicester NHS Trust, Leicester, UK (LREC, 06/Q2501/17). Written informed consent was obtained from patients undergoing amputation for the donation of their limb to the Forensic Pathology Unit, University of Leicester, UK for DNA identification research. Two lower limbs were collected for use in this project. Both limbs were amputated due to chronic lower leg ischemia caused by diabetes. Immediately after amputation the limbs were taken to the Forensic Pathology Unit, and viable muscle tissue was dissected from the limb. Muscle was chosen as it is a dominant soft tissue type throughout the body and is often present and easily identifiable in fragmented body parts.

2.2. Preservation methods

Two methods of room temperature storage were identified by literature review: lysis storage and transportation (LST) buffer consisting of 100 mM Tris–HCl pH 7.6, 0.5 M KCl, 4.5% Nonidet P40, 4.5% Tween 20 and 1% sodium azide [4,5] and the Oragene™ DNA self-collection kit (DNA Genotek, Ottawa, ON, Canada).

2.3. Experimental design

Pieces of muscle were removed on receipt of each of two limbs and placed into the solutions to assess the amount required to be sampled in each buffer and for how long DNA is preserved at room temperature. Samples weighing 1000, 500, 250, 100, 50, 25, 10 and 5 mg were placed into Oragene™ collection pots. Samples weighing 1000, 500, 250, 100 and 50 mg were placed into 5 ml LST buffer, and finally, samples weighing 100, 50, 25, 10 and 5 mg were placed into 1 ml LST buffer. DNA extraction was performed on each sample after 1, 2, 4, 12, 36 and 52 weeks.

2.4. DNA extraction

Incubation at 50 °C for 3 h was performed on all Oragene™ preserved samples in accordance with manufacturer's advice. The Oragene™ purification protocol involves the addition of 1/25th Oragene™ purifier solution to the sample and incubation on ice for 10 min. This is then followed by a series of centrifugation and wash steps to finally recover the DNA in pellet form which requires re-hydration in a chosen volume of buffer or water. This purification technique, recommended by the manufacturer was designed for use with saliva samples, and was found to be insufficient for purification of DNA from muscle samples, as undigested tissue was present in the sample after three repetition steps of the purification. The recommended protocol was abandoned in favour of the Qiagen DNA mini kit (Qiagen, West Sussex, UK) which is designed to recover DNA from a number of different body fluids and tissues by inclusion of additional digestion stages depending on which protocol is chosen: 100 μl aliquots were removed from both Oragene™ and LST preservation buffers for DNA extraction using the Qiagen DNA mini kit—blood/body fluids protocol as per manufacturer's instruction. DNA was eluted in 100 μl buffer AE (Qiagen).

2.5. DNA quantification

DNA quantification was carried out on 1 μl of each extracted sample in duplicate using the Quantifiler Human DNA Quantification kit (Applied Biosystems) in a total reaction volume of 12.5 μl. Thermal cycling was carried out on a 7500 Real-Time PCR System (Applied Biosystems) according to manufacturer's instructions.

2.6. DNA profiling

Profiling was carried out on DNA from all extracted samples using the AmpF*l*STR® SGM Plus® PCR Amplification kit (Applied Biosystems, Foster City, CA) in a final reaction volume of 12.5 μl, and 1 ng template DNA was added to each reaction whenever possible. Initial DNA profiling was carried out using 28 amplification samples. Samples for which a partial or failed DNA profile was observed after 28 cycles were re-amplified for 34 PCR cycles. PCR products of week 1, 2, 4, 12 and 36 extracts were separated and visualised on an ABI PRISM® 377 DNA Sequencer (Applied Biosystems). Fragment sizing was carried out using GeneScan® software version 2.1 (Applied Biosystems), and allele designation was carried out using Genotyper® software version 3.7 (Applied Biosystems). PCR products of week 52 extracts were separated and visualised on an Applied Biosystems 3130 Genetic Analyser, and were analysed using GeneMapper ID software version 3.2 (Applied Biosystems).

3. Results

DNA extraction was carried out on 100 μl aliquots of each preservation buffer. The results of DNA quantification of

Table 1
Average result of real-time PCR quantification performed in duplicate for all samples extracted during a 52-week period

Preservative	Muscle A (mg)	Week number						Muscle B (mg)	Week number					
		1	2	4	12	36	52		1	2	4	12	36	52
Oragene™	1000	33.500	60.430	33.500	97.100	111.550	68.950	1000	79.610	76.260	79.610	112.760	303.360	139.700
	500	46.110	52.840	46.110	65.730	67.270	61.670	500	27.830	52.600	27.830	137.930	161.510	61.570
	250	15.140	19.850	15.140	40.380	42.770	21.790	250	9.770	10.590	9.770	15.640	42.710	14.260
	100	1.890	6.770	1.890	4.000	9.090	5.670	100	1.630	1.850	1.630	2.770	6.460	2.040
	50	0.798	1.150	0.798	0.912	1.410	1.790	50	0.297	0.732	0.297	2.540	6.120	1.970
	25	0.233	0.523	0.233	0.626	3.260	0.458	25	0.630	0.877	0.630	1.160	2.010	0.609
	10	0.143	0.127	0.143	0.230	0.233	0.009**	10	0.070*	0.053	0.070	0.239	0.062	0.006**
	5	0.039	0.032*	0.039	0.035*	0.012**	0.005*	5	0.156	0.171	0.156	0.352	0.111*	0.014**
5 ml LST	1000	0.093	0.179	0.093	1.580	0.948	0.047	1000	0.059	0.278	0.059	9.490	64.280	3.530
	500	0.185	0.248	0.185	0.512	3.290	0.289	500	0.253	0.276	0.253	1.340	6.740	0.983
	250	0.129	0.131	0.129	0.219	0.371	0.101*	250	0.239	0.182	0.249	0.376	1.550	0.154
	100	0.096	0.060	0.096	0.059	0.190	0.007*	100	0.072	0.061	0.072	0.117	0.330	0.105
	50	0.029	0.031	0.029	0.068	0.113	0.027*	50	0.035	0.014	0.035	0.062	0.128	0.022**
1 ml LST	100	0.270	0.302	0.270	0.648	0.766	2.680*	100	0.432	0.264	0.432	3.190	14.690	12.880
	50	0.108	0.134	0.108	0.320	0.220	0.628	50	0.153	0.172	0.153	0.674	4.250	2.150
	25	0.089	0.100	0.089	0.145	0.239	0.218	25	0.112	0.089	0.112	0.083	1.580	0.855
	10	0.021	0.021*	0.021	0.070	0.052	0.072	10	0.035	0.048	0.035	0.097	0.275	0.105
	5	0.000***	0.009*	0.010**	0.008*	2.630	0.025*	5	0.014*	0.018*	0.014*	0.035*	0.157*	0.036

All results are expressed as the concentration of DNA in nanograms per microlitre. Results followed by (*) indicate samples from which a partial DNA profile was observed, and (**) for which DNA profiling failed after amplification at 28 cycles. A full DNA profile was obtained when all partial and failed samples were re-amplified for 34 cycles, with the exception of 5 mg muscle A stored in Oragene™ solution for 1 week (***), for which no DNA profile could be generated.

samples stored at room temperature in both Oragene™ collection pots and LST buffer are given in Table 1. The average temperature of the room in which samples were stored was 24.2 °C with a minimum temperature of 16 °C and maximum temperature of 30.5 °C during the 52-week period. The quantity of DNA recovered after each extraction remains consistent for extractions performed up to 12 weeks after this study was initiated. The quantity of DNA recovered shows an increase in yield at weeks 36 and 52. These results may be explained by the decreased volume in which samples were stored as repeated sampling from the same container resulted in the total buffer volume being reduced by 100 μl following sampling at each time point. The Ct values for all quantified samples were in the expected range (20–35) indicating that no PCR inhibition occurred during template amplification. The results of DNA quantification were analysed using an ANOVA. There was no significant difference in the yield of DNA for either muscle A ($p > 0.2$) or muscle B ($p > 0.5$) for any sampling time point. The DNA yield from solutions containing muscle A and B was analysed using a paired t test, assuming no variance. There was no significant difference observed for the total quantity of DNA recovered from muscle A or B.

As there was no significant difference in the yield of DNA obtained between samples extracted from muscle A or B, or

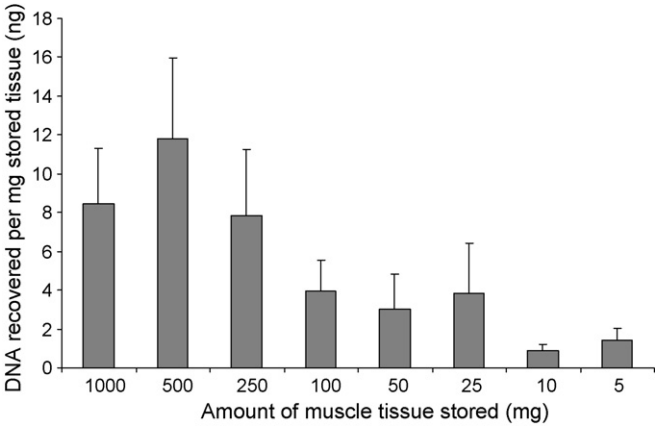

Fig. 1. Bar chart to illustrate the quantity of DNA recovered from muscle tissue stored in Oragene™ solution. Each bar represents the average quantity of DNA recovered from a 100 μl aliquot of Oragene™ solution over the six time periods sampled. The concentration of DNA was normalised by dividing the total quantity of DNA recovered (ng) by the amount of tissue stored (mg) in each sample in order to compare the efficiency of each extraction. The error bars indicate the 95% confidence interval for each data set.

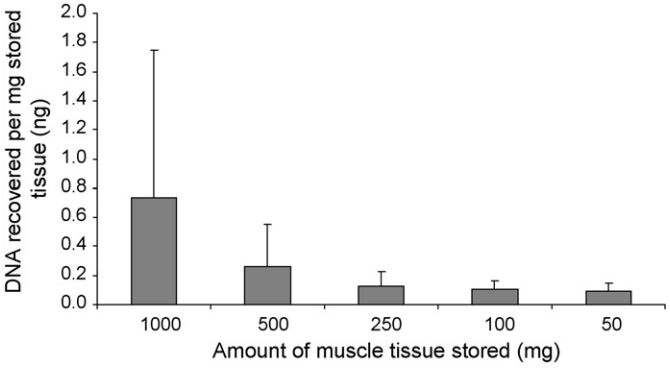

Fig. 2. Bar chart to illustrate the quantity of DNA recovered from muscle tissue stored in 5 ml LST buffer. Each bar represents the average quantity of DNA recovered from a 100 μl aliquot of Oragene™ solution over the 6 time periods sampled. The concentration of DNA was normalised by dividing the total quantity of DNA recovered (ng) by the amount of tissue stored (mg) in each sample in order to compare the efficiency of each extraction. The error bars indicate the 95% confidence interval for each data set.

between the time point at which sampling was carried out, the results were complied to assess the efficiency of DNA extraction for each mass of tissue stored in each preservative solution. Fig. 1 shows the average yield of DNA obtained after extraction was performed on 100 μl aliquots of preservative buffer for each mass of tissue stored in Oragene™ solution. These data have been normalised by dividing the total DNA yield by the amount of tissue in mg to allow each tissue mass to be directly compared and presented on a single chart. Similarly, Fig. 2 shows an evaluation of the DNA recovery efficiency for muscle tissue stored in 5 ml LST buffer, and Fig. 3 for tissue stored in 1 ml LST buffer. The results presented in Fig. 1 suggest that storing 500 mg tissue in Oragene™ collection pots results in an optimal DNA recovery ratio, and Fig. 3 suggests that the greatest DNA recovery efficiency for samples stored in 1 ml LST buffer is achieved when 5 mg tissue is stored. The results presented in Fig. 3 suggest that 100 mg tissue should be stored in 5 ml LST buffer to provide a maximum DNA return per mg tissue stored. The total yield of DNA recovered by extraction of 100 μl aliquots taken from containers holding 5 ml LST buffer and tissue samples, however, shows a significantly reduced total yield compared with samples stored in Oragene™ pots and 1 ml LST buffer.

DNA profiling was carried out on 1 ng of template, or reduced amounts when DNA concentration was below 0.2 ng/μl, as 5 μl template was used for each reaction in a total volume of 12.5 μl. A full DNA profile was obtained for the vast majority of samples. A partial DNA profile, where allele and/or locus dropout seemed apparent was observed in 8.8% of amplifications, and amplification failure was observed in 2.8% of all samples, as indicated by asterisks in Table 1. All samples showing drop-out or failure were re-amplified using 34 PCR cycles. This resulted in full profile generation for all samples with a single exception. No DNA profile could be produced when attempting to amplify material recovered from the 100 μl aliquot taken from muscle A stored in Oragene™ preservative for 1 week. Additionally, this was the only sample to be undetected during DNA quantification. It is hypothesised that this is due to the very small tissue fragment adhering to the lid portion of the Oragene™ collection pot, preventing it from being in contact with the preservative solution during the first week of storage. Care was taken after this occurrence to ensure that tissue samples were present in the preservative solution, not adhering to the lid portion of Oragene™ collection points for all samples.

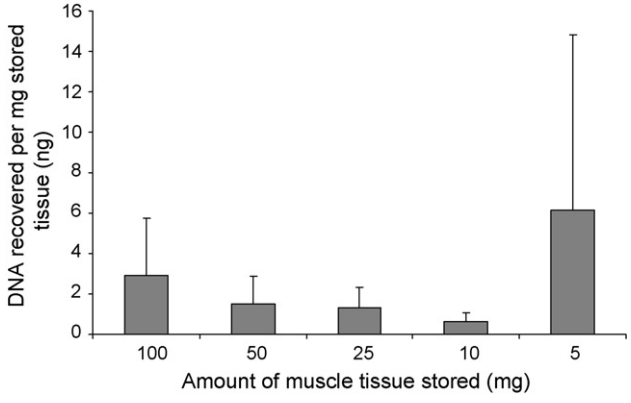

Fig. 3. Bar chart to illustrate the quantity of DNA recovered from muscle tissue stored in 1 ml LST buffer. Each bar represents the average quantity of DNA recovered from a 100 μl aliquot of Oragene™ solution over the six time periods sampled. The concentration of DNA was normalised by dividing the total quantity of DNA recovered (ng) by the amount of tissue stored (mg) in each sample in order to compare the efficiency of each extraction. The error bars indicate the 95% confidence interval for each data set.

Of the samples that showed partial profiles following the first amplification, drop-out can be explained by the addition of inadequate template for the majority of samples, these profiles showed electropherograms typical of this cause, with low average peak height observed across all amplified loci. This observation was supported by the quantification data, with low (<0.04 ng/μl) quantities being recorded for these samples. Of the exception to this explanation, it is not known why a full DNA profile could not be produced when DNA extracted for 10 mg muscle tissue B was stored in Oragene™ buffer for 1 week as DNA quantification indicated that a concentration of 0.07 ng/μl DNA was recovered. The results of 34-cycle amplification produced a profile typical of addition of too much DNA template, with pull-up peaks observed due to peak heights exceeding 6000 RFUs in the electropherogram. This may be explained by a human error made during the first amplification attempt using 28 PCR cycles.

Partial profiles were also generated for tissue samples stored for 36 and 52 weeks when DNA quantification indicated that sufficient template was entered into each reaction for full profile generation after 28 PCR cycles. Although a full profile was generated after re-amplification using 34 PCR cycles, the electropherogram image demonstrated a pattern of amplification typical of degraded template, with lower peak heights observed for the longer loci in the SGM Plus amplification kit, such as D18S51 and FGA. These results suggest that the quality of DNA recovered from muscle tissue stored in both Oragene™ and LST preservative buffers may begin to diminish after 6 months at room temperature.

4. Discussion

A number of samples can be taken from the body for the purpose of DNA identification. In the majority of forensic cases whole and non-disrupted cadavers will be examined at autopsy. In such cases buccal swabs, liquid blood samples or blood spots on filter paper can collected for DNA profiling (depending upon where one practices in the world) due to the ease of laboratory processing of these sample types. In the United Kingdom (UK), even with whole bodies, deep muscle samples may also be collected.

In situations where buccal cells and/or blood are not available, such as during the examination of highly fragmented remains, an alternative biological sample must be collected. In the UK, recent incidents involving severe body disruption have led to the decision for scene recovery of those body parts greater than 5 cm^3. Identification and re-association use this same tissue size. Thus in the case of fragmented bodies the predominantly available tissue type will be soft tissue rather

than bone or teeth, and as muscle is often present, this investigation has focused on muscle tissue as an alternative source of DNA. It is widely acknowledged that DNA preservation in bone and teeth is superior to soft tissues, especially when putrefaction has occurred [6–8], and in such circumstances bone or teeth samples may contain the only surviving DNA molecules. The processing of hard tissue is, however, extremely time-consuming and labour-intensive, requiring de-fleshing, cleaning, drying, cutting, grinding and de-calcification before DNA extraction can be carried out. Comparatively, the downstream processing of soft tissues will require a simple cutting and maceration step before DNA extraction is performed.

Similarly, under routine circumstances, sample collection for DNA profiling will take place during the autopsy examination, within a permanent mortuary facility. Samples can then be refrigerated or frozen to preserve the DNA. By cooling the samples many of the factors that contribute to DNA degradation, such as the action of endogenous enzymes and microbial/bacterial decomposition are slowed, or even halted at very low temperatures (−70 to −80 °C). In certain situations, including mass fatality incidents, depending on the location and number of victims, immediate refrigeration of samples may not be possible. Alternative methods of DNA preservation have been suggested for use in such cases. A low-cost LST buffer was developed for transportation and storage of field collected specimens, without refrigeration [4,5]. This buffer contains a combination of chemicals designed to lyse cells, inactivate nucleases, prevent microbial growth and preserve the DNA. Original tests found LST buffer was effective for preservation of DNA for up to 8 weeks at room temperature from both blood and tissue samples [4]. A subsequent publication, using clinical biopsy samples, directly compared the preservation capacity of LST buffer with snap-freezing and storage at −75 °C [5]. The findings of this investigation concluded that snap-freezing and storage at −75 °C was more effective at DNA preservation but also concluded that LST buffer is a suitable, cost-effective alternative for short-term (up to 4 weeks) storage of tissue samples [5]. The use of LST buffer in DVI projects is not directly suggested by the authors of either publication. We, however, feel that there is a role for such preservative solutions within this arena.

The use of a similar buffer is suggested by Fregeau et al. for the purpose of DVI [9]. This alternative buffer, GenoFix™ (DNA Genotek), is an alcohol-based tissue fixative designed for room temperature storage of tissue samples. Tests carried out on smooth muscle biopsies showed that GenoFix™ was effective for DNA preservation after 1 year storage at room temperature and 3.5 years storage at −20 °C [9]. A letter to the authors of this GenoFix™ publication commented favourably on the original findings of Fregeau et al. and additionally suggested that RNA will be equally preserved by this fixative solution [10]. Unfortunately, GenoFix™ solution was not available at the time our research was initiated. An alternative solution, Oragene™ DNA self-collection kit (DNA Genotek), designed for collection and room temperature preservation of DNA from saliva samples was available and was thus investigated as an alternative.

We have examined the ability of both Oragene™ solution and LST buffer to preserve DNA present in fresh muscle tissue over a 12-month time period at room temperature. Results of these tests have shown that it is possible for full DNA profiles to be produced by the use of standard DNA extraction and amplification procedures over this time period. Consideration of the quantification data suggests that the preservative solution contained within the Oragene™ collection pots is superior to LST buffer in recovery of high DNA yield, especially when compared with DNA yield of muscle tissue stored in 5 ml LST buffer, from which very low comparative yields were obtained. The results of DNA profiling carried out on all extracted samples does, however, suggest that the quality of DNA recovered from tissue stored in LST buffer is not significantly reduced compared with that recovered from samples stored in Oragene™ collection pots. The yield of DNA per mg of tissue stored was vastly greater when samples were stored in 1 ml rather than to 5 ml LST buffer, as illustrated in Figs. 2 and 3. These results may suggest that LST buffer is better suited to the preservation of small (<100 mg) amounts of tissue. Due to the limited data set presented in this initial article, further investigation of this issue should be carried out before a conclusion can be drawn on the optimal volume of LST buffer required for sample preservation.

This experiment was designed to replicate a situation whereby multiple samples may need to be recovered from a single sample collected for personal identification of an individual who has lost their life during a mass fatality incident, for this reason multiple samples were not set up to provide a previously un-sampled specimen for each time point. The total volume of preservative solution of both LST buffer and Oragene™ was designed to be in excess of the volume required for sampling over a 1-year sampling period, totalling 600 μl. This resulted in a gradual reduction in the total volume of preservative solution held within each container during the 52 weeks for which sampling was undertaken. The increase in DNA yield after 36 and 52 weeks of tissue storage is most likely attributable to a reduction in the remaining tissue to preservative solution ratio due to the removal of 100 μl at each time point. The results of DNA profiling over the full 52-week sampling period do, however, suggest that multiple samplings from a single-collected sample do not adversely affect the outcome to an extent that DNA quantity or quality slips below a standard required for use with current downstream processing, and that individualisation of samples by DNA profiling can still be achieved.

Finally, an additional benefit of using room temperature storage buffers is that DNA extraction can be performed directly on aliquots of the solution, i.e. no additional processing of tissue is required. With samples stored at −20 °C before extraction can be undertaken the sample must first be defrosted, removed from the container, dissected, weighed, macerated and then digested for 1–3 h (or overnight, in many cases). The use of a preservation solution removes this process entirely as DNA can be extracted from small aliquots of untreated buffer, using shorter DNA extraction protocols. The ability to extract directly from aliquots of the storage buffer will also allow for the

automation of the entire extraction process by use of robotic platforms such as the Qiagen Biorobot (Qiagen, West Sussex, UK). This would allow for an increase in sample throughput, and could allow for more rapid DNA profiling to be achieved than is possible with current protocols.

5. Recommendations

We have demonstrated that both Oragene™ and LST buffers are suitable for preservation of muscle tissue for up to 12 months at room temperature, and as such could be used as an alternative to freezing of samples when refrigeration is not immediately available or where transportation of samples from one country to another may be required. The use of preservation solutions will also benefit the downstream processing of biological samples by removing the requirement for further manipulation of solid tissue. The fact that DNA extraction can be performed on an aliquot of either buffer solution, without further processing could also allow for automation of DNA extraction for high throughput processing of numerous soft tissue samples, if required. Another observation is that less material need be collected from corpses than current guidelines suggest [11]. Our findings show that a full SGM Plus STR profile can be obtained from as little as 5 mg muscle tissue, preserved in both Oragene™ and LST buffers for up to 52 weeks. In practice, an amount of tissue weighing between 25 and 500 mg should ideally be collected for identification purposes to ensure adequate DNA quantities are available for multiple examinations.

This system thus allows for the collection of small pieces of muscle (or other soft tissue) for room temperature preservation of DNA identification samples with potentially increased throughput by automated systems. It is fully portable and is compatible with bar-coding management systems. The initial results of ongoing work shows that it is applicable to burnt remains and those showing changes of decomposition, two situations that may be encountered during a mass fatality investigation and will be addressed in a subsequent communication. This builds upon previously published work using similar preservation buffers which have been promoted for DVI field work and is especially applicable in an incident involving disrupted body parts where traditional DNA samples or teeth and bone may not be readily available for identification and fragment re-association. This system should therefore be considered as an additional method for sample storage during DVI work.

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