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Article

Title: Field-Based Detection of Biological Samples for Forensic Analysis: Established Techniques, Novel Tools, and Future Innovations

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Abstract

Field based forensic tests commonly provide information on the presence and identity of biological stains and can also support the identification of species. Such information can support downstream processing of forensic samples and generate rapid intelligence. These approaches have traditionally used chemical and immunological techniques to elicit the result but some are known to suffer from a lack of specificity and sensitivity. The last 10 years has seen the development of field-based genetic profiling systems, with specific focus on moving the mainstay of forensic genetic analysis, namely STR profiling, out of the laboratory and into the hands of the non-laboratory user. In doing so it is now possible for enforcement officers to generate a crime scene DNA profile which can then be matched to a reference or database profile. The introduction of these novel genetic platforms also allows for further development of new molecular assays aimed at answering the more traditional questions relating to body fluid identity and species detection. The current drive for field-based molecular tools is in response to the needs of the criminal justice system and enforcement agencies, and promises a step-change in how forensic evidence is processed. However, the adoption of such systems by the law enforcement community does not represent a new strategy in the way forensic science has integrated previous novel approaches. Nor do they automatically represent a threat to the quality control and assurance practices that are central to the field. This review examines the historical need and subsequent research and

developmental breakthroughs in field-based forensic analysis over the past two decades with particular focus on genetic methods. Emerging technologies from a range of scientific fields that have potential applications in forensic analysis at the crime scene are identified and associated issues that arise from the shift from laboratory into operational field use are discussed.

1. Introduction - Field Based Analysis vs Central Laboratory

The Star Trek Tricorder. Portable, light, small, rapid results, identifies multiple biological and genetic signals, and importantly it can be used by different groups regardless of their skill set; scientist, security officer, doctor, engineer. First introduced in the 1960's, this pop-culture device is perhaps one of the best known and clearly defined examples of what field-based instrumentation should do, albeit in a science fiction TV show. Fifty years later the device is almost a reality through the Qualcomm Tricorder XPrize, a 10 million USD competition to "stimulate innovation and integration of precision diagnostic technologies, helping consumers make their own reliable health diagnoses anywhere, anytime" [1]. Primarily designed for biomedical applications, such devices offer a tantalizing glimpse into what may become available to forensic science in a few years. Indeed, the field of forensic biology and forensic genetics has a history of deriving benefit from utilising the approaches and techniques initially pioneered in the health and medical arenas, PCR, DNA fingerprinting and capillary electrophoresis to name a few. However, the development of novel technology and its application in forensic casework is not a binary process; absent/present, can't use/can use, not ready/ready. Technology is continually developed, introducing new benefits but also presenting difficult decisions to make regarding when and what to use. The latter is compounded in the face of a highly regulated field that proceeds with caution and requires some form of standardisation and consensus in the forensic stakeholder community. In some instances novel technology represents such a seismic shift that it promises to change the way forensic laboratories operate.

The centralised laboratory is currently the standard in forensic investigation. Under this model, a single large laboratory representing a defined geographical range is responsible for providing forensic analysis

to all Law Enforcement Agencies within its bounds. The adoption of this model began in the United States in the 1920-1930s due to logistical, quality control, legal and cost issues [2, 3]. This development also coincided with the appearance of dedicated forensic pathology courses offered by US higher education institutions to train medical students in this emerging field, as well as the invention of the UV spectrophotometer which remains a standard tool in forensic chemistry to this day [2, 3]. Criminal activity is typically spread over a relatively local geographic range and historically, investigation of criminal activity was limited to the collection of eyewitness testimony and physical evidence from this area [4]. Individuals performing these activities were predominantly law enforcement officers with little or no scientific background. Consequently, as scientific techniques advanced, analysis became more complex requiring full time professional forensic analysts to carry out, document, and report their findings [5]. Housing such expertise in a small number of centralised laboratories was the logical solution. This division between crime scene and laboratory practitioner remains to this day [6]. However, in the last two decades there has been a slow shift towards introducing forensic roles into the police forces themselves to bring collaboration between police officers and forensic scientists closer together [7]. Events such as the closure of the United Kingdom (UK) Forensic Science Service in 2010 [8], decreasing budgets [9] and the high cost incurred from private sector providers [10] make the argument for adopting in-house processes more compelling. Indeed, this structure is already routinely practiced in the US. While increasingly common, the cost/benefit of developing an in-house local laboratory process vs a centralised laboratory is complicated as groups struggle with the balance of low cost, high quality assurance (QA), and ethical arguments about scientific independence [10].

As technology develops and applications become more user friendly, safer, and requires less expertise and training, they inevitably transfer into the field. The handover of this technology to non-laboratory specialists represents further monetary saving as staffing of specialist practitioners remains the highest cost of a forensic investigation [11]. Techniques such as the development of traditional fingerprinting, chemical tests to identify body fluids and the presumptive detection of drugs of abuse were pioneered in research laboratories but are now commonly used by enforcement officers and crime scene examiners [4]. Despite the drip feed of applications into field-based operational use, certain approaches have been slow to develop and be taken up, specifically those that are DNA-based approaches. There are a number

of reasons for the slow development and adoption of this technology. Conventional PCR and size separation of the resulting DNA fragments requires large, bench-mounted instruments, preventing their on-site use [12]. Much of the equipment still requires specialist training to operate, limiting the potential user-base. Forensic casework may rely on limited biological input and loss of that material through additional processing methods may severely affect the investigation [13]. There is also the raw cost of equipment, maintenance, reagents, and storage space which has become more constrictive in recent years as forensic budgets have been reduced [4, 9]. Fully equipped forensic laboratory setups are also unfeasible in remote areas or developing nations that require these diagnostic tests but lack the resources necessary. These factors have presented a need for cheaper, more convenient field-based forensic assays and have acted as drivers of development for new technologies and techniques [14]. Indeed, there is little argument that the development and practical application of a cost effective, robust and quality-controlled on-site test would greatly benefit the criminal justice system.

2. Established Field-Based Systems

One of the oldest forms of biological forensic analysis is in the identification of body fluids such as blood, saliva, and semen left behind at a crime scene [13, 15]. Table 1 provides an overview of the most common established forensic tests used to detect and identify biological material. A more thorough overview of the mechanisms of standard body fluid detection and identification methods is available in the review from Virkler and Lednev (2009) [13].

2.1. Chemical Testing

One of the earliest forensic tests for blood was the luminol chemiluminescence reaction, first being used over 50 years ago. This technique remains invaluable in the detection of latent blood, which can aid other forensic techniques such as blood spatter analysis. The test is cheap, works quickly, and can be applied to surfaces and stains without requiring pre-treatment. One of the most widely used commercial luminol formulations is BlueStar® [16]. This formulation has a greater sensitivity and longer-lasting luminescence than that of luminol [17], and is more resistant to the luminescence-reducing effects of

certain antioxidants than other formulations [18]. However, a qualitative study on the reliability of BlueStar found that certain compounds such as oil-based paints, certain vegetables, and metallic ions such as copper and ion could cause false positive results [19]. Another feature of BlueStar that has helped to ensure its continued use is that it is non-destructive to the DNA contained within the sample, allowing for the rapid identification of blood stains that can then be processed by DNA analysis. Other chemical tests for blood include the Kastle-Meyers (KM) test and tetramethylbenzidine (TMB). Similarly to luminol, they can be directly applied to suspected bloodstains at the crime scene and a positive result is clearly displayed as a colour change of the stain (pink for KM, blue-green for TMB). The KM test is widely used due to its comparatively high specificity and greater resistance to inhibition compared to other presumptive chemical tests for blood [20]. Conversely, TMB offers greater sensitivity than KM, but has largely fallen out of use due to poor specificity and concerns about safety as TMB may be carcinogenic [21]. Chemical tests for saliva and semen also exist. A standard chemical test for the detection of saliva is the Phadebas® Press Test, which detects and identifies latent saliva stains, which is a difficult body fluid to identify conventionally due to poor fluorescence under UV light [22]. Although the test is simple, cheap, and rapid there can be issues with reproducibility due to temperature variation at the scene, causing changes in evaporation of the sample [22]. The acid phosphatase (AP) test for semen has been in routine use as early as the 1940s [23], but due to its relatively high false-positive rate [24] it is unable to be used to as a confirmatory test and has recently been sidelined in favour of immunoassay approaches [25]. A considerable advantage of chemical tests is their low per-test cost, but this is often at the expense of specificity and sensitivity. This is an important point to consider when assessing the utility of field-based approaches, in that it is acceptable for sensitivity and specificity to be low if understood by the user, appropriately caveated in casework management and followed up with a more robust diagnostic test. Indeed while it is the goal to have confirmatory and diagnostic approaches in the field it is likely to be considered secondary to other requirements. A shared aspect of these tests is their singleplex activity, being able to detect only one body fluid. In practical usage, this becomes a potential issue, as forensically important stains may contain a mixture of body fluids [13]. This requires an expert in field-based testing to make an informed decision about which test should be used based on relative likelihoods of certain biological components being present. This

highlights a market niche for tests with multiplex activity that can identify multiple biomarkers at once, dispensing with the need for expert intuition. While these described tests are applicable at a crime scene, chemical approaches are also commonly applied in a centralised laboratory if the evidence is transferable from the crime scene.

Despite shared issues surrounding specificity, sensitivity, and tolerance to inhibitors, chemical tests have lengthy shelf-lives, enabling to be used in decentralised environments. The standard Kastle-Meyer's test for latent blood has a shelf-life of approximately 9 months [26], whereas Bluestar formulation luminol can last up to 3 years in dry storage at room temperature [16].

2.2. Immunological

Increasing the sensitivity and specificity of the application often requires the use of a more complicated test and the expertise required to use and interpret the result. Immunoassays are biomolecular toolkits for detecting or quantitatively measuring the levels of biomarkers in a fluid sample using an antibody-antigen interaction. Wet-lab immunoassays such as enzyme-linked immunosorbent assay (ELISA) are well-established and have very high specificity and sensitivity, and in recent years there has been an effort to apply this technology outside of the laboratory [27]. Several immunoassays are available for forensic use, such as rapid stain identification (RSID™) kits, which are lateral flow immunoassays. RSID kits have been developed for the rapid on-site detection of blood, saliva, and semen [28, 29, 30]. The sensitivity and specificity of all of these tests were found to be greater than their respective presumptive chemical tests. Immunoassays are promising alternatives to chemical testing for body fluids due to their ease of use, rapid action, reliable results and confirmatory nature. Cost analysis studies of these immunological tools have calculated their per-test cost at roughly £3-6 [31]. Moreover, they show extended shelf-lives between 12-24 months, giving them comparable stability to most available chemical tests. Quantitative immunological assays for non-human markers such as the precipitin test have been available for well over a century [32] and have been used by law enforcement officials to identify remains of particular animals protected by the law e.g. big game animals [33, 34].

However, due to poor reliability and resolving power between closely-related species [35] there has been difficulty in transferring precipitin tests to routine forensic use.

2.3. DNA-based testing

As on-site tests are designed to be performed outside of the laboratory, there is little opportunity for complex sample preparation [36]. This often limits their role to the diagnosis of crude biological samples to determine their identity (typically a body fluid such as blood, saliva, or semen or tissue). This is one reason that has prevented the development of field-based DNA applications as the PCR process is highly susceptible to inhibitors co-collected with the crime scene sample [37]. However, there are early examples of field-based forensic genetic analysis. MtDNA studies in the mid-1990's on whale products lacking morphological features (e.g. processed meat) sold at retail markets in Japan and South Korea found that some whale products sold as minke whale were actually obtained from endangered whale species such as humpbacks, the trade of which is heavily regulated and violates international whaling laws [38, 39]. This work was performed in a hotel room that had been loosely turned into a traditional laboratory with the existing tools and chemistry simply placed in this location. However, as DNA systems developed in the laboratory the assumption that they should automatically provide high quality robust results also grew. This creates another developmental hurdle to researchers and developers and again is prioritised collectively alongside other requirements.

3. Next-Generation Field-Based Systems

Research and development of field-based molecular approaches has been increasing significantly over the last 20 years. This is clear from a literature search of research papers and patents in the subject area show an increasing trend which peaks between 2010-2014 (Figure 1). Throughout the 1990's, improvements to chemical testing for body fluids were made [40, 41], whilst DNA profiling largely remained a laboratory-based technique [42]. By the 2000's, DNA-based detection methods had advanced significantly. Lab-on-a-chip technology was a much-publicised area of research, and as such by the end of the decade, several micro total analysis systems for forensic and clinical applications had been developed [43, 44, 45], accounting for the sharp rise in publications seen during this time span.

Similarly, non-DNA based methods also became more sophisticated during this time, benefiting from advancements in engineering and miniaturisation of existing technology to allow for on-site usage. Table 2 provides an overview of next generation forensic identification tests and their features.

3.1. Drivers for development

The key driver in the all this new development has been the end user group has changed between the 'Laboratory User' to 'Field User' and a set of 'end-user specifications' has been identified. This is largely due to the concerted effort of stakeholders in the criminal justice community directing research in this area. In 2006, the "Future of Forensic and Crime Scene Science Conference" identified some key system and technological drivers that came out of discussion with International Stakeholders in the forensic community [46], specifically:

- Miniaturisation to increase portability and ease of use.

- Faster analysis.

- Simple 'Black-Box' interpretation.

- Easy integration of case information.

- Low cost.

Being representatives of the end-user community the 'wish list' of the stakeholders formed an early market research exercise and set of specifications that developers could work towards. This conference identified a need by the criminal justice community that was not at the time being met and led to further research and development by industry, academic and government groups to develop a system that could meet these requirements and supercede the traditional laboratory process flow (Figure 2). The current laboratory process can delay obtaining results due to work backlogs [13]; transitioning to a primarily field-based approach would greatly reduce the current average time taken to produce full DNA reports for use as evidence in court. More formal end-user specification documents were later published by the National Policing Improvement Agency (NPIA) to represent the United Kingdom Criminal Justice Community [47] and also by the Federal Bureau of Investigation (FBI) to represent North American interests [48]. Once again, this feedback to developers has been instrumental for the successful

development and integration of such systems into operational use. Consequently, flexibility-of-use has been downgraded in order to simplify operation, while test robustness and sensitivity have decreased as a consequence of developing a single step process with data analysis primarily being software driven instead of requiring independent expertise. While these may be seen as an indication that systems need further optimisation before routine adoption, others believe it simply narrows their operational use [14]. Indeed as forensic analysis becomes more decentralised, it becomes more important to fulfil the needs and identify the requirements of the practitioner whilst also remaining informative in the wider context.

3.2. Non DNA-based

Techniques that have been pioneered elsewhere can have applications in forensic science. Raman spectroscopy is a technique that obtains information on the vibrational mode of molecules in a system excited by inelastic (Raman) scattering of monochromatic light [49]. Its primary use has been in analytical chemistry, as different chemical bonds have characteristic vibrational outputs, allowing the resulting spectra to be analysed to determine which chemical bonds are present in a sample. In the past few years, forensic analysts and researchers have sought to co-opt Raman spectroscopy as a tool for forensic science [50]. This is based on the observation that body fluids analysed by Raman spectroscopy display their own unique peak signatures, making the results relatively simple to analyse by comparing them to the known spectral peaks of various biological substances. Raman spectroscopy is also a confirmatory test, as the position of the spectral peaks are tied to particular molecules and thus is extremely unlikely to bring up false positives [13]. Another advantage of performing Raman spectroscopy in a forensic setting is that it is both non-destructive to the sample (as no contact with the sample is required) and does not require time-consuming sample pre-treatment steps [13]. Several on-site Raman spectroscopy devices currently exist [51, 52, 53, 54] which have been demonstrated to work effectively in the on-site detection of chemical and biological samples. These devices are simple to use and deliver results rapidly. Portable Raman spectroscopy possesses many of the "ideal" on-site test but is more expensive than chemical-based detection and may require some training prior to use in order to ensure that results are correctly interpreted. As the only requirement for Raman spectroscopy to take place is that there is an optically visible stain present, it dispenses with the need for any reagents that

would normally be used in the treatment of unknown stains for analysis. This means there is no associated storage requirements or expiry date of the Raman instrumentation, making it ideal for use in de-centralised locations.

3.3. DNA-based - Human

Perhaps the most important technique in modern forensic crime scene investigation is the detection of human DNA. This analysis has traditionally been carried out by trained forensic experts as biological samples require complex pre-treatment to extract DNA, and obtaining a full STR profile can take upwards of 8 hours [55]. This estimate does not include the time taken to process samples from a crime scene to obtain a full DNA report, which has been found to take an average of 66 days in the UK for serious crimes [56]. This highlights a need for a shorter and more simplified workflow. In doing so, this would also facilitate the use of STR profiling by individuals with no/limited forensic background that routinely require STR profiling work, such as law enforcement officials. In the past few years, there has been considerable progress towards user-friendly DNA detection and identification. This has been achieved by advancements in microfluidics [43], as well as miniaturisation of thermal cycling [57] and optimisations to the PCR process such as implementation of rapid inhibitor-tolerant polymerases (e.g. Phusion™ Flash) [58]. Another major enabling technology is the automation of steps in STR profiling, including DNA extraction, PCR amplifaction, separation, and detection by a single instrument allowing for a rapid "sample in, result out" workflow without any additional input from the user. Several automated laboratory-based DNA detection instruments exist, such as RapidHIT® and DNAScan™ [59, 60, 61]. Inputs for these assays include "neat" biological samples such as buccal swabs and blood, but can also analyse indirect samples such as swabs from drinking glasses or cigarette butts that may hold forensic evidence, making them suitable for forensic case work where there may not be large amounts of biological material for direct sampling. Validation studies of these systems have found that the RapidHIT ID system has a high sensitivity, able to generate full STR profiles from ~500 pg of DNA applied to a cotton swab. [62]. The DNAScan system was designed to produce full STR profiles from 1.0 µg of template DNA present on buccal swabs but can also produce partial profiles with a lower input [63]. Accuracy for these systems is also very high, demonstrating a 100% genotype concordance with

known reference profiles [62, 63, 64]. Both systems take ~90 minutes to build a full STR profile, and have low (<15 minutes) handling times for the user [62, 65]. Further issues of these systems is the high cost associated with them, both in terms of the machinery itself and the reagents required for its use. Both DNAScan and RapidHIT instruments utilise single-use cartridges as their inputs, with the operator only having to load a reference swab onto them and insert into the machine, which automatically handles the sample processing and outputs an STR profile. While this system is convenient for the end-user, processing high volumes of samples may become prohibitively costly. The sample cartridges and reagents have a shelf-life of up to 6 months at room temperature [63, 66].

These laboratory-based detection methods have many of the features recommended for an ideal on-site assay such as robustness and ease of use, but are not considered field portable. The ParaDNA® Field Instrument from LGC [67] can perform two separate tests in ~75 minutes each; a screening test for determining the presence of human DNA in a sample and an intelligence test for building an STR profile across 5 loci (D3S1358, D8S119, D16S539, D18S1358 and TH01) [68]. Both of these tests also profile for amelogenin to simultaneously determine gender. The ParaDNA screening system is a presumptive test as it measures the presence/absence of DNA through PCR of two STR loci (D16S539 & TH01) with fluorescent Hybeacon® human-specific probes, and outputs a percentage chance of the sample containing DNA suitable for laboratory analysis as a relative assessment score [69, 70]. This circumvents a major issue in submission policy for forensic case work as the screening data is an objective measure and so eliminates the need for speculation by the end user, helping to reduce waste and improve laboratory processing. A cost analysis of the ParaDNA screening system against standard in-house STR profiling found that although the per-test cost of ParaDNA is relatively high (~$50), it is potentially able to save thousands of dollars per annum by eliminating the screening costs of negative samples that may be processed under other screening methods [71]. Validation studies of the screening test show high accuracy for blood, saliva, and semen [70] but some conflicting results with touch DNA samples. The screening test is able to obtain a gender result in >80% of samples with a sufficient presence of DNA (>62.5 pg), though is more sensitive to male samples than female due to software reporting a male result whenever a Y target is amplified [69]. There has also been some assessment of whether the assay interferes with existing forensic processes with research demonstrating that pre-

treatment of samples with Phadebas and luminol/Bluestar® did not impact the ability of ParaDNA to reliably screen these samples for DNA [72]. However, despite the ease of use and portability, the ParaDNA screening test is intended to augment the existing processes of sample submission and case management, and not designed to replace existing tools [73] meaning that costs savings may be more difficult to identify. Independent validation studies of the ParaDNA intelligence test found that full DNA profiles of 12 alleles could be produced from an input of 500pg DNA and that 99.8% of allele calls by ParaDNA was concordant with those of other STR typing kits [68, 73]. However, some cross-reactivity of the system is observed with some primate DNA samples [70]. As the output of the ParaDNA intelligence test is an STR profile, this does require some training in order to interpret, but is minimal compared to the training needed to perform STR analysis in-house. Both tests are noted for their ease of use by non-expert handlers [70] and have demonstrated reliability and high potential for cost-saving. Recent work by LGC has looked at developing an assay for field-based mRNA analysis for the identification of body fluid samples [74].

The RapidHIT ID system is a compact version of the laboratory-based RapidHIT system that utilises the same GlobalFiler® Express chemistry and has been optimised for use in decentralised environments, such as police stations or border control posts [66]. Validation studies have shown that the RapidHIT ID system is also capable of obtaining full concordance from assumed single-source DNA samples, with complete STR profiles obtained from as low as 12,500 cells per sample swab [75]. Multiple RapidHIT ID systems across geographic locations can be networked together with RapidLINK™ software for remote access to results and control of the RapidHIT ID instruments themselves. [76]. This allows for easy access and processing of data from remote locations by a centralised laboratory prior to uploading resulting DNA profiles to a database.

3.4. DNA-Based - Non-Human

While detection and identification systems of human DNA have received the majority of the research attention there have also been some big steps towards the application of portable molecular tools for non-human and food standards applications. The ability to perform on-site species identification is particularly important in the food standards and conservation fields. On-site detection means that

samples do not have to be transported to centralised laboratories. From a food standards viewpoint, food samples of unknown origin do not have to be seized from retail markets or restaurants and can be identified on-site, improving workflow while also reducing the possibility of a genuine sample being needlessly analysed by an equipped laboratory. For conservation work, suspected trade of endangered animal products in violation of CITES seized at international borders can have the species identified on-site without having to transport samples to a wildlife forensic laboratory, which may be situated in another country. On-site potential for species detection has been greatly improved in the past decade due to advancements in isothermal amplification and miniaturisation of sequencing instruments. Recombinase polymerase amplification (RPA) is a highly sensitive and specific isothermal method of DNA amplification that can take place in a single tube, dispensing with the need for a thermal cycler [77, 78]. This technology has been commercialised by TwistDX, who supply the portable battery-powered Twirla® mixing incubator and provide several kits for food safety and species identification. One such kit is specific to DNA from the Red Snapper [79] and is designed to prevent mislabelling fraud. The assay requires a one-step biochemical reaction prior to incubation which can be performed by non-experts and the result of the assay is processed automatically and delivered by the instrument in <20 minutes as either positive or negative for Red Snapper. A similar on-site assay for non-expert usage is the real-time nucleic acid sequence-based amplification (RT-NASBA) assay devised by Ulrich et al. [80] which has demonstrated similar efficiency to laboratory-based benchtop RNA purification with mildly lower sensitivity. However, this assay takes longer to perform (~80 minutes). NASBA is a well-established molecular biology tool for the isothermal amplification of RNA sequences [81]. RT-NASBA works by combining this method with fluorescent molecular beacons, allowing for the real-time detection of target sequences using a handheld fluorometer, which also doubles as a heater to keep samples at an optimal temperature for the reaction to progress.

A novel on-site RNA/DNA sequencing instrument is the MinION™ real-time sequencing device from Oxford Nanopore Technologies [82]. The device is smaller than most mobile phones and contains a flow cell for spotting DNA/RNA for sequencing. MinION can be powered by a laptop after hooking up to one with a standard USB 3.0 port, making it extremely useful for field work in even highly-remote

locations. The device costs approximately $1000 USD, though this also includes everything needed to begin sequencing right away, with a very low per-test cost. Early-access studies also found the system to be useful for taxonomic analyses [83, 84], which would be of great benefit to on-site species identification. Although the potential of this technology for true on-site DNA detection is very high, performance studies have shown error rates as high as 38% [83, 85, 86]. Although this rate can be reduced to as low as ~5% with improved data analysis [87], it also suggests that extensive optimisation and specialist knowledge of both the system and the DNA sequence of interest is required.

In summary, it is important to clarify that field-based testing does not necessarily dispense with the requirement for expert training that is often needed to operate large, bench-mounted apparatus. Indeed, portability of a system and the expertise required to successfully operate it exists on a spectrum. When considering the ease of use vs the portability it is possible to see that the systems presented do not always achieve all of the identified end user requirements (Figure 3). For many end users, there is a desire for tests that are both highly portable and easy to use as these are the most practical in the field and have the greatest potential user-base. However, the development of such tools is compounded by factors such as manufacturing costs (both of the device itself and the reagents required on a per-test basis), availability of appropriate miniaturisation technology, and the flexibility of use.

4. Future Innovations in Field-Based Analysis

Advancements in forensic science are often the result of co-option of existing technologies routinely used in other fields, particularly medical science. A selection of techniques that have recently been applied to on-site forensic analysis of various biological samples show some great potential and may form the basis of the next phase of development in field-based molecular identification.

4.1. Loop-mediated isothermal amplification

Development of reliable and rapid isothermal PCR would be of great benefit to on-site forensic analysis as it would transfer one of the most important laboratory-based forensic techniques into the field, greatly increasing the potential of on-site investigation. As discussed previously, there has already been some application of isothermal PCR to forensics with RPA and the TwistDX system, however this is not the only method of low-temperature PCR. Loop-mediated isothermal amplification (LAMP) is a PCR method that can be performed at relatively low temperatures (60-65°C) [88, 89], making it suitable for on-site work when paired with a portable battery-powered heater. LAMP differs from conventional PCR in that it uses multiple sets of primers, which form loops in the synthesised DNA strand that facilitate further rounds of amplification without the need for a bench-mounted thermal cycler. LAMP has previously been used extensively in clinical science for the detection of harmful bacteria in complex biological samples such as blood and sputum [90, 91] and would be applicable to detection of biomarkers of forensic importance. Very recently, several rapid on-site LAMP assays were developed for drug detection and species identification, all of which have shown very promising results with comparable efficiency to laboratory-based methodology whilst heavily reducing the cost that would normally be incurred from using any specialised equipment [92, 93, 94]. This highlights the potential of LAMP for forensic use and warrants further development to produce a commercialised LAMP assay. There are several benefits to using LAMP over other PCR methods. Firstly, the amplification product is much simpler to visualise with LAMP than standard PCR, using photometry to measure the turbidity of the sample post-amplification. Although LAMP is used for the detection of DNA, it is possible to combine the technique with a reverse transcription step (RT-LAMP) to enable the detection of RNAs [88, 95]. Complex biological samples such as blood contain inhibitors (e.g. Immunoglobin G [96, 97] that affect the PCR reaction, and is a common cause of amplification failure [37]. LAMP is more resistant to these inhibitors than standard PCR, which would make LAMP a more ideal method for detecting DNA in body fluid samples and would require less prior sample preparation such as DNA/RNA extraction [98]. However, the use of LAMP is limited by the difficult design of primers, requiring the use of software kits [99], as well as being more restricted in its range of designs compared to conventional PCR markers. This is particularly a concern for forensic applications as some biomarkers may have transcriptional variants, where the design of primers would have to be centred

around a common sequence. Another issue with LAMP is that due to the increased number of primer sets required compared to standard PCR, it increases the likelihood of primer-primer dimer interactions occurring in a multiplex reaction. As such, LAMP is typically reserved for single-target detection. It is possible to utilise LAMP in a multiplex detection assay [100], but this would require complex processing that would be difficult to transfer to on-site practice.

4.2. Synthetic Biology

The field of synthetic biology has made considerable strides in the past decade, with the aim of characterising genetic "parts" to create programmable biological devices with complex functions. Despite the numerous hurdles faced by the field [101], it remains a promising avenue of research for a number of fields, including forensics. Researchers from the University of Dundee have attempted to use synthetic biology in conjunction with microsphere technology to provide an all-in-one body fluid identification assay ("FluID") that would eliminate the need for multiple body fluid tests [102]. This assay would consist of a liquid formulation ("BioSpray") containing fluorescent microspheres that have binding ligands specific to biomarkers present in various body fluids immobilised onto their surfaces. When the ligands come into contact with their respective molecules, a binding interaction takes place which leads to fluorescence of the microspheres. Biochemically functionalised microspheres are an ideal delivery method for the binding proteins as they have been shown to not impact the effect of biochemical molecules immobilised onto their surfaces [103], and also display high sensitivity [104]. Similar to luminol, BioSpray can be applied to surfaces and then examined for fluorescence in darkness. The primary advantage this offers over established body fluid identification tests is that the BioSpray formulation would contain binding proteins for biomarkers from a number of body fluids, including blood, saliva, semen, and urine, allowing for only one test to be used when normally multiple tests would be required. As a cell-free approach is used, this means that there is no contamination of crime scenes with foreign DNA. It is also hoped that fluorescence from BioSpray would last longer than other fluorescent methods and there would be no interaction with chemicals that interfere with forensic investigations at crime scenes e.g. bleach. Using synthetic cell-free systems, researchers were

successfully able to overexpress and purify binding proteins against haemoglobin and spermidine for the detection of blood and semen, respectively. However, work is still required to purify binding proteins agains biomarkers in saliva and urine, and for the formulation to have its performance tested against crude samples. An issue with transferring biology to field-based applications lies in the stability of biological components at ambient temperatures. DNA sequences, PCR products, and many other reagents require storage at temperatures $\leq$-20°C and can lose their functions or shear DNA through repeated freeze-thaw cycles. Room-temperature storage of components would allow for more applications to be performed outside of the laboratory. Pardee et al. [105] have demonstrated a method of storing cell-free protein expression systems and synthetic gene network DNA for long periods of time (at least 1 year) at room temperature by freeze-drying these components onto ordinary filter paper discs ~3mm in diameter. The synthetic gene network sequence is complementary to a desired mRNA sequence, and will activate expression of a reporter gene (e.g. GFP/LacZ) to visualise detection when the embedded paper is rehydrated with this sequence (Figure 4). Research in this area has recently advanced with the development of the SHERLOCK (Specific High sensitivity Enzymatic Reporter unLOCKing) platform developed by MIT and Harvard University. Taking a slightly different approach, the research combines isothermal amplification with CRISPR Technology to develop a 4-channel single reaction multiplexing method, capable of detecting unique DNA and RNA frangments [105.5]. Using a lateral flow readout to detect presence or absence of amplification target, this device enables rapid detection of multiple fragments for field-based diagnosis with early evidence suggesting allele specific amplification from saliva is possible. The use of paper as a substrate and the negligible amount of materials required makes this process extremely cheap, with a per-test cost of 4-65¢ USD [105]. These features allow for cheap, easily portable, and stable diagnostics of desired nucleotide sequences (such as those from infectious diseases) in remote locations. The novel design of the synthetic gene networks used in these experiments also gives them the potential to detect virtually any desired sequence [106], greatly increasing their utility across a range of fields.

4.3. Smartphone Forensics

Smartphones are a near-ubiquitous aspect of the modern world. They are lightweight, small, versatile in their range of applications, and in recent years have become reasonably cheap. This makes them ideal candidates for use as on-site forensic detection instruments. Many modern smartphone models are equipped with high-resolution cameras and applications are available to measure the RGB output from images, allowing for colorimetric analysis. Researchers have devised a novel method ("Smart Forensic Phone") of estimating the age of bloodstains using a colorimetric analysis of bloodstain images taken with a smartphone over a fixed time period [107]. However, this analysis can only be performed on bloodstains <42 hours old which severely limits its potential use and requires more optimisation to be used on older bloodstains.

Another study examining the range of on-site applications of smartphone cameras modified Raspberry Pi cameras available for smartphone market to repurpose them as ultra-violet (UV) imaging equipment [108]. This is particularly beneficial due to the wide applications of UV detection, and the very low cost of manufacturing smartphone cameras. Smartphones are also capable for use as analytical and data processing devices. Researchers have developed an electrochemical chip that utilises a rapid (<20 minutes) quantitative enzyme analysis for gender identification of body fluids deposited at a crime scene. This chip can then interface with smartphones utilising a special user-friendly application that guides the user through the detection process [109]. The chip is compatible with several different models of smartphone and as many smartphones possess wi-fi capabilities, any data recorded using the application can be uploaded to a central "cloud" network for storage or downloaded to other devices. Performance studies of the electrochemical chip found high specificity and sensitivity (88.9% and 88.3%, respectively) on analysis of real samples, but also noted slight interference from substances such as ascorbate. An upcoming device from Oxford Nanopore Technologies LTD is the SmidgION nanopore sequencer, currently in beta testing and expected to release in 2017 [110]. This device works similarly to the MinION sequencer (also from Oxford Nanopore) described previously, but can instead be connected to a smartphone, or any similar mobile device. This would further reduce the cost of equipment needed to sequence DNA/RNA in the field, whilst also enabling its use with a wider user-base and in more remote locations.

Smartphone technology has also been combined with handheld PCR. Biomeme have developed a mobile handheld RT-PCR device equipped with a heater and fluorometer for tracking reaction progress. This device can be docked to a smartphone, which runs an application that controls the device and utilises the smartphone's camera to track changes in fluorescence [111]. This highlights a particular advantage of smartphone forensics in that the user interface of smartphones are designed to be as ergonomic and user-friendly to operate as possible. Combining this technology with standard forensic techniques has the potential to reduce the expertise barrier of entry and allow for simple use of the device by non-laboratory users.

5. Further Considerations

While the development and use of field-based molecular tools for non-laboratory trained individuals offers great potential, there are a number of issues that need further consideration by both developers and end-users, specifically the necessity for independent validation of novel technology, prior to use. In the traditional cycle of forensic product development and release there has been a build-up of scientific support from practitioner scientists and academics over time that have highlighted and reported on procedural oddities, errors, reproducibility and overall effectiveness of the system. Either because these individuals represented the target forensic user or the systems under evaluation also supported academic research, the publication and dissemination of this research makes it easy for the forensic community to assess and critically appraise new issues as they arise. The advent of field-based, non-expert user systems may change the nature of this. The identified end user for many of these systems include, police, customs (border) officials, military personnel, and crime scene examiners, many of whom have little or no scientific background but may be expected to a) have an opinion on whether they believe the device supports their work, b) identify erroneous results, and c) report on any new issues observed to the wider community. It is worth considering each of these in turn to assess the overall risk that under-developed and poorly characterised systems may have and how performance is recorded.

5.1 Development and Release

With respect to the development of new technology it is common to describe it in term of a 'Technology Readiness Level' (see figure 5) [112]. There is routinely a well characterised and documented path from first principles to product release which reduces the likelihood of products being early. In order to warrant a TRL measure of 9 it is common for industry groups to perform a 'beta-release', whereby new assays, kits and technology are distributed among a small number of practitioners for limited testing and feedback in an operational setting. Beta testing is typically only performed once the product format and protocols have been optimised and represents the finished product. Concurrent to this user evaluation, industry scientists also perform developmental validation studies which seek to characterise the approach and identify its '*efficacy and reliability for forensic casework*' [113]. While there is an obvious conflict of interest, the publication of data in support of a commercial application by industry scientists is not new, is not unethical but (like all scientific studies) should not be considered singularly. Publishing practices exist that require conflicts of interest to be declared and the peer review process means that the data and findings are independently critiqued. Indeed, given that quality and accuracy forms a large part of brand identity for biomolecular products it is counterproductive for industry to release technology early. Further independent assessment is also performed as many of the end users are represented by wider working groups or have ties to third party expertise who perform this function. For example the UK police forces work closely with the Centre for Applied Science and Technology (CAST) who have a remit to assess and publish their findings on novel developments that support policing [114, 115]. In turn the US police forces work closely with both the Federal Bureau of Investigation (FBI) and the National Institute of Standard and Technology (NIST), members of which have a long history of independent assessment and publication of novel forensic genetic methods [61, 116, 117]. There are also specific groups representing military interests such as the Defence Science and Technology Laboratory (DSTL) and the U.S. Army Research Office and the Defence Forensic Science Center (DFSC) that closely assess novel developments. As such it seems unlikely that novel technology will reach the end user without some form of independent assessment, although the well-publicised sale of ineffective bomb detectors to the UK military would suggest that on occasion the assessment process may not be as robust as needed [118]. While the oversight of a third party reduces the chance of underdeveloped systems entering operational use, it is also worth noting that the findings

of some groups are difficult to find, represent an internal discussion, is commercially sensitive, or are simply referred to (but not explored) in government records [e.g. 119, 120].

5.2. Identifying Errors

Once the instrumentation has been cleared for operational use it is still required to function well and perform as expected. If the instrumentation begins to drift and results change over time it is important that this is captured. Employing pre-existing Quality Control (QC) measurements and Good Laboratory Practices (GLP) may aid in the detection of such events, but the emphasis is again on the end-user to modify or develop their existing procedures, which again raises issues. One strategy is to have the machine self-calibrate. Such features are common in many non-expert user systems [65, 121, 122] and takes the emphasis off the end-user but also removes any independence. Further preventative measures commonly adopted include full annual servicing of instrumentation and the development and adoption of positive control samples and are likely to allow detection performance issues. However, the lack of support for the publication and dissemination of such data may mean that performance issues go undetected more widely by the forensic community despite initial validation.

Other potential operational impacts that require some control include the possible crime scene contamination through PCR. The traditional approach of DNA profiling uses the process of PCR to amplify small numbers of target DNA molecules into billions of copies that are then detected by the instrumentation. In a centralised laboratory there is a strict separation between pre- and post-PCR activities with a uni-directional workflow, provisions to prevent post-PCR work more than once a day, and positive pressure rooms that vent low contamination into high contamination areas rather than the other way round. Developing a robust anti-contamination strategy for crime scene analysis or custody suite analysis can simply mean following established procedures but may also mean the development of bespoke anti-contamination procedures and routine environmental sweeps of the instrumentation. Indeed there are perhaps fewer hurdles to the custody suite approach than the crime scene approach given that the custody suite remains remote from the scene of the crime. In the absence of an effective and proven anti-contamination strategy the default option is to reduce the evidential weight of the data obtained and seek further quality controlled data from a centralised laboratory. This may not be as

counterproductive as it sounds given that many of the popular applications of field-based testing are presumptive and may require further laboratory testing anyway. Being aware of the limitations of new technology and developing practices that seek to minimise the impact of errors is something that is core to the criminal justice community and there are already robust strategies in place for both minimising and reporting analysis and contamination issues so it is considered unlikely, but not impossible, that the adoption of genetic technology by non-laboratory trained users will result in mass errors given proper training.

5.3. Fitting End User Requirements

One of the driving forces behind the development of the next generation field-based technology has been cost. As identified in 2006 [14], cheaper forensic science was identified as a key end user requirement. However, assessing cost effectiveness is not a simple calculation for both developer and user. From the commercial development perspective there is a need to secure a profit margin that ensures the longevity of the product. The cost can neither be unaffordable or too cheap which may lead to unrealised commercial profit. Cost is determined by the size of the market, potential uptake and the business model under use [123]. From the end-user perspective there is the calculation of how much currently is spent on the existing processes, how much will get spent on the new process, and whether there is any time lag to seeing any savings. Both are business decisions and often not made by the scientific staff who are either developing or using the techniques. There is currently little *independent* evidence that the use of any of these devices leads to a greater monetary saving and increased sample success rate and more research is needed in this area. It is therefore important the forensic and law enforcement communities are aware of the potential for an 'Emperor's new clothes' outcome whereby no one can admit a novel application does not do what they want, and also to avoid the 'Concorde fallacy' whereby an application is deemed 'too big to fail' as too much investment (time, effort, raw cost) has already been put in. Ultimately there is no single group who takes responsibility for this assessment and long term assessment of an instruments utility requires the continued cooperation between government advisory groups, end users and industry partners

It is also important to recognise that the 'end user' in question is in fact the criminal justice community, not just the users of the instrument, and it's important to consider the wider impact of adopting new technology. With regards to Rapid DNA devices, it is likely that soon some traditional processes will no longer be performed in the laboratory. At what point in time this occurs is debatable but there are fewer hurdles now than there were 10 years ago. The next question is whether this shift in user is going to create a period of instability in the centralised laboratory as Law Enforcement Agencies submit less evidence to a laboratory. The answer to this question relies on both the quality of the data obtained and what other analysis options the laboratory can offer. Currently the forensic genetic community is assessing the ability to offer information on a genomic level. The adoption of Massively Parallel Sequencing by forensic laboratories offers an application that cannot currently be met through the use of a single field-based instrument. However the implementation of this technology is also currently under assessment with more work required to determine how well the system compares to existing approaches in terms of cost and performance but also from an ethical stance regarding whether it is appropriate to answer questions regarding race and ethnicity when the reported probabilities remain relatively low. Indeed while this offers an exciting potential it is likely to take five to ten years before forensic laboratories make the full transition to this platform [124]. Until this transition the laboratory continues to offer greater quality, greater sensitivity and greater evidential weight attached to the data it provides.

6. Summary

Molecular techniques for the forensic detection/identification of body fluids, individuals, and species have rapidly advanced in the last 30 years. During this time, the technology has transferred from trained forensic specialists working from an equipped, centralised laboratory to field users such as law-enforcement officials working at crime scenes. Most of this new generation of field-based forensic tests are characterised by their ease of use, rapid action, robustness, and comparable efficiency to similar laboratory-based assays. In their current state, on-site forensic assays are demonstrably effective methods of identifying body fluids, assessing the presence of DNA, or performing amplification of high quantity of genetic material for DNA profiling. However, the widespread adoption of on-site forensic

toolkits has been somewhat hampered, as it is still necessary for many on-site tests to be used in conjunction with laboratory analysis - due to either their presumptive nature or inferior activity. As such, future development work should seek to improve upon their performance and provide confirmatory results to achieve a true on-site forensic workflow. Some field-based techniques still suffer from issues surrounding component storage which will need to be addressed if they are to become standardised techniques. There are also procedural questions remaining about how to effectively utilise and these systems as they migrate from the lab to the crime scene or police station.

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Table 1 – An overview of the features, cost, and current end-user groups of established forensic techniques used in the detection of body fluids, DNA, and the identification of non-human species.

Primary Detection	Method	Input	Assay Name	Molecular Target	Field-based	Current End User Group	Result	Sensitivity/Detection Limit	Specificity	Cost	References
Body Fluid Detection and Identification	Chemical Test	Raw Sample	Kastle-Meyers Test	Haemoglobin	Yes	Police/CSI	Pink colour change	Positive results down to 10^{-7} dilution of neat blood	High rate of false positives in presence of hypochlorite or ferrous sulphate	Very Low	[20]
		Raw Sample	TMB	Haemoglobin	Yes	Police/CSI	Blue-green colour change	Low rate of false negatives	False positives in presence of plant peroxidases	Very Low	[21]
		Raw Sample	Alpha-naphthyl phosphate and Brentamine Fast Blue	SAP, GDA, CAP	Yes	Police/CSI	Colour change	65.60%	96.40%	Low	[13]
		Raw Sample	Luminol	Iron	Yes	Police/CSI	White/blue chemiluminescence	Positive results down to 10^{-6} dilution of blood (dependent on substrate)	False positives in presence of fibre foods, paints/varnishes, and mettalic ions	Very Low	[16, 17, 18, 19]
		Raw Sample	Phadebas test	α-amylase	Yes	Police/CSI	Blue colour change + absorbance measurement	0.5µl pure saliva	Positive results may not yield enough DNA to obtain a profile	Very Low	[22]
	Alternative Light Source	Raw Sample	Polilight	Whole Cells	Yes	Police/CSI	Fluorescence of stains	Any optically-visible stain	50%	Medium/High	[13]
	Immunological	Raw Sample	RSID	GPA/α-amylase/Semenogelin	Yes	Police/CSI	Positive Band	250nl/ul (blood) 0.5ng/ul (saliva), 2.5nl/ul (semen)	Cross-reactions with sweat and urien (saliva test)	Low	[28, 29, 30]
		Raw Sample	ELISA Membrane Assay	Haemoglobin/PSA, SVSA/α-amylase	Yes	Police/CSI	Colour change	~1mM of molecular target present	No cross-reactivity known	Low	[27]
Species Identification	Size Separation of DNA Fragments	Purified DNA	PCR amplification	Whole Genome	Office-based	Researchers	Bands on agarose gel	Dependent on gel resolution	Dependent on gel resolution	Medium	[3, 36, 42, 57]
	Reaction of sample with a species-specific antigen	Species-specific biological sample	Precipitin Test	Various	Yes	Police/CSI	Formation of antibody:antigen complexes	Detection up to 1:16,000 dilution of matching antisera	Cross-reactivity between closely-related species	Very Low/Low	[32, 33, 34, 35]

Cost is given as total cost considering both per-test and instrumentation expenses. Very low = <£100, Low = £100-£999, Medium = £1000-9999, High = <£100,00-100,000, Very high = >£100,000

Table 2 – An overview of the features, cost, and current end-user groups of next-generation forensic techniques used in the detection of body fluids, DNA, and the identification of non-human species.

Primary Detection	Method	Input	Assay Name	Molecular Target	Field-Based	Current End-User Group	Result	Sensitivity/Detection Limit	Specificity	Cost	References
Body Fluid Detection and Identification	Raman Spectroscopy	Raw Sample	Portable Raman Spectroscopy Device	Whole Fluids	Yes	Police/CSI	Unique Spectroscopic Peaks	Any contactable stain	No false positives	Medium	[49, 50, 51, 52, 53, 54]
	Endpoint analysis of mRNA	Raw Sample	ParaDNA Body Fluid ID Test	DNA	Yes	Police/CSI/Clinic	Presence / absence of marker	86%	93%	High	[74]
DNA Detection, Individual Identification, and Sample Matching	Endpoint analysis of STRs	Raw Sample	ParaDNA Intelligence Test	TH01, Amelogenin, D16	Yes	Police/CSI	DNA profile across 5 STRs	62.5pg of DNA	No cross-reactivity	High	[68, 70, 73]
	Endpoint analysis of DNA	Raw Sample	ParaDNA Screening Test	Short Tandem Repeats	Yes	Police/CSI	Relative quantitative assessment score (%)	500pg of DNA	99.8%, some cross-reactivity with primate DNA	High	[68, 69, 70]
	RAPID-DNA	Raw Sample	RAPIDHit, DNAScan	Short Tandem Repeats	No	Police/CSI	STR profile	500pg-1µg DNA	100%	Very High	[59, 60, 61, 62, 63, 64]
Species Identification	Recombinase Polymerase Amplification	Purified DNA	TwistDX	Various	Yes	Researcher	Presence of positive result band	Single copies of DNA/tens of RNA	Specific to target species	Medium	[77, 78, 79]
	Real-Time Nucleic Acid Sequencing	Species-Specific Biological Samples	RT-NASBA	Various	Yes	Researcher	Raw fluorescence data	80.30%	No known cross-reactivity	Medium/High	[80, 81]
	Real-Time DNA/RNA sequencing	Purified DNA	MinION, SmidgION	Whole Genome	Yes	Researcher	DNA/RNA sequence data	200ng of high molecular weight DNA	~0.01%	Low/Medium	[82, 83, 84, 85, 86, 87, 110]

Cost is given as total cost considering both per-test and instrumentation expenses. Very low = <£100, Low = £100-£999, Medium = £1000-9999, High = <£100,00-100,000, Very high = >£100,000
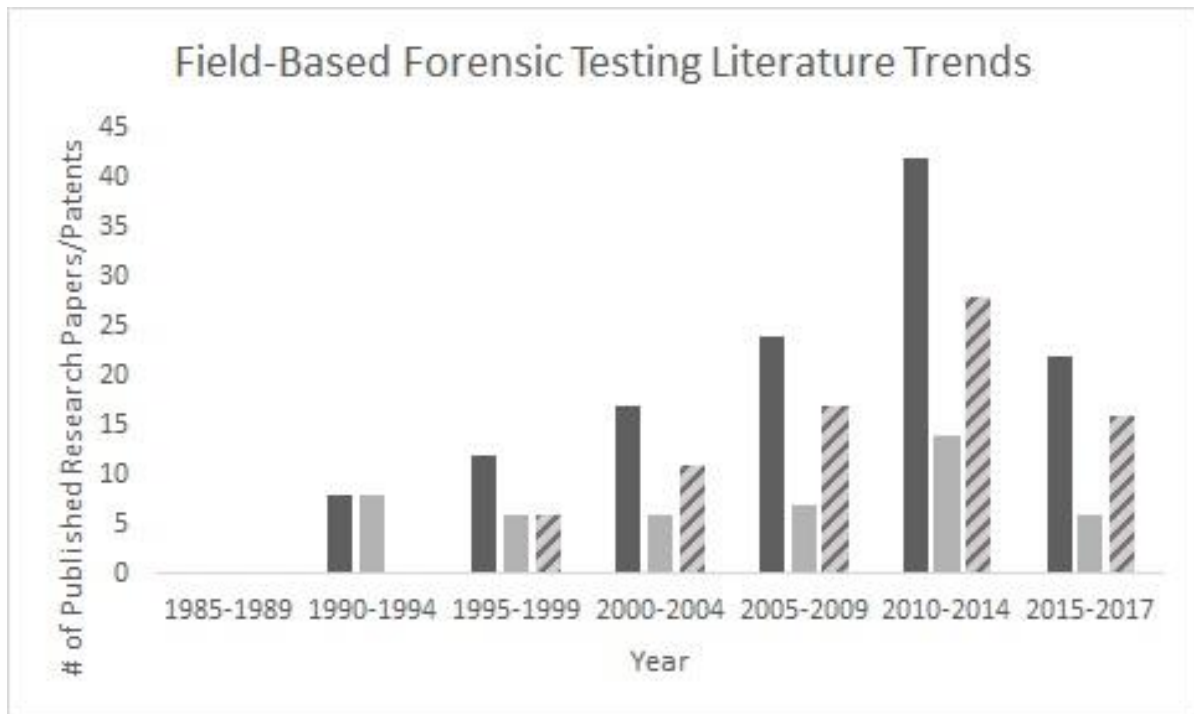
Fig 1. The number of research papers and patents published in the study of on-site forensic analysis, excluding drug and environmental methods, combined from Google Scholar and Scopus search engines. Black bars = Total number of publications, grey bars = proportion of publications concerning non-DNA-based (e.g. chemical reaction) methods for forensic detection, striped bars = number of publications concerning DNA-based methods for forensic detection. Search keywords: All of the words "onsite, forensic", exact phrase "forensic", at least one of the words "detection, identification, DNA, body fluid, rapid", without the words "drug, environmental".
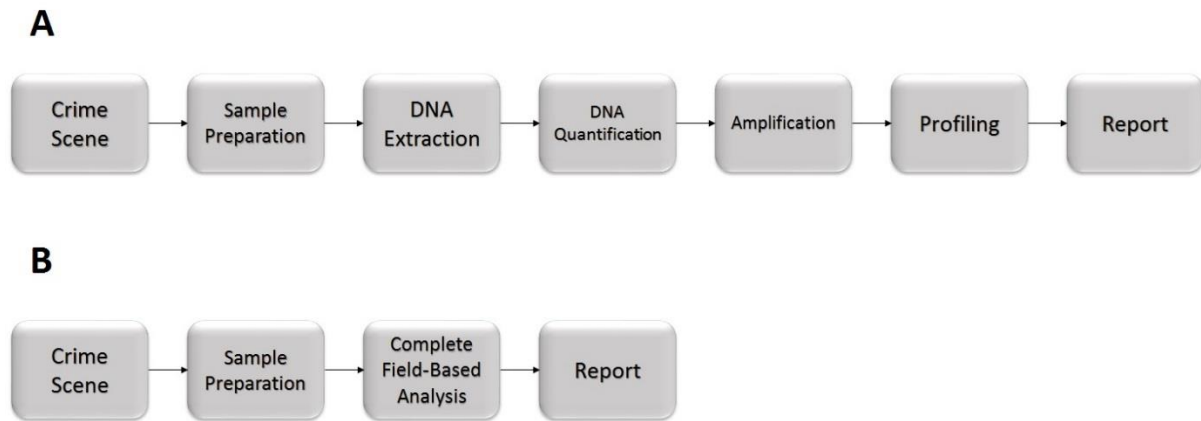
A

B

Fig. 2. A simplified workflow for obtaining a complete DNA report from a sample collected at a crime scene. A = The current process, which includes transfer of evidence to a centralised laboratory to extract and amplify DNA to obtain an STR profile. B = A potential future process, whereby all DNA profiling steps are carried out in the field via automated systems, largely cutting down the time and number of steps required at present.
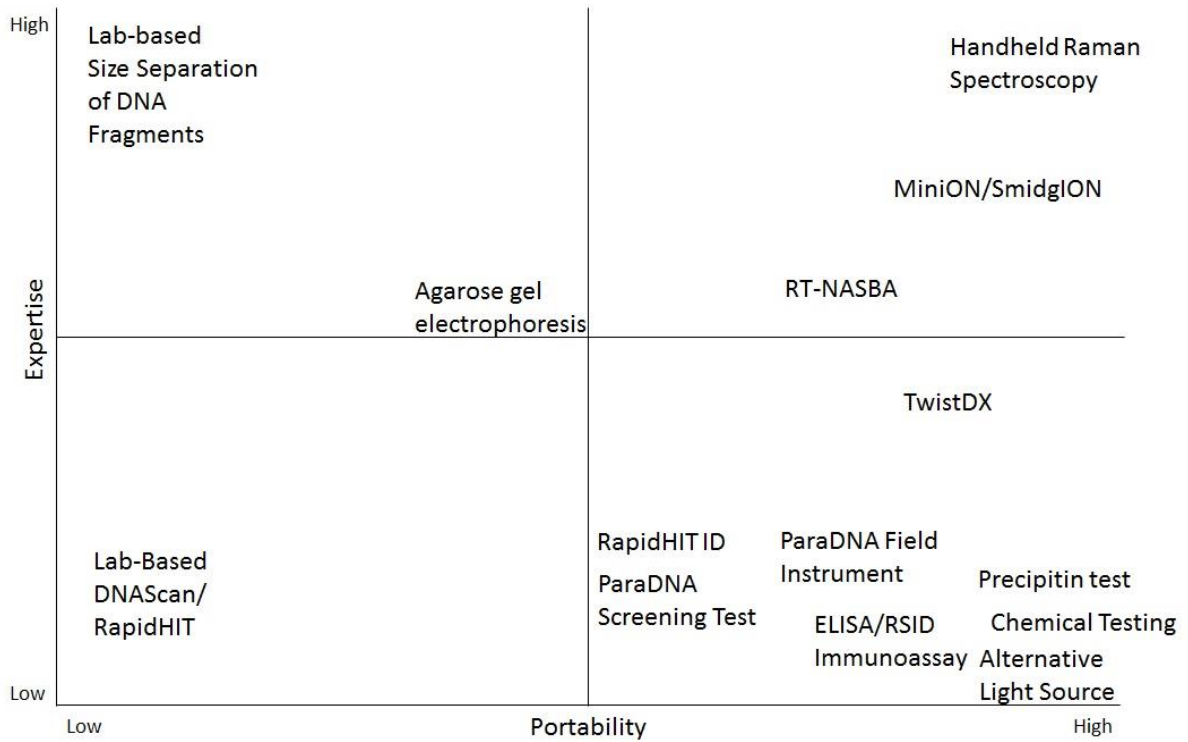
*Fig. 3: A sample of established and next-generation forensic techniques arranged by their portability and the expertise required to operate or interpret results from them. **NB**: Placement on the chart is defined by the authors' experience with the techniques and interpretation of the surrounding literature.*
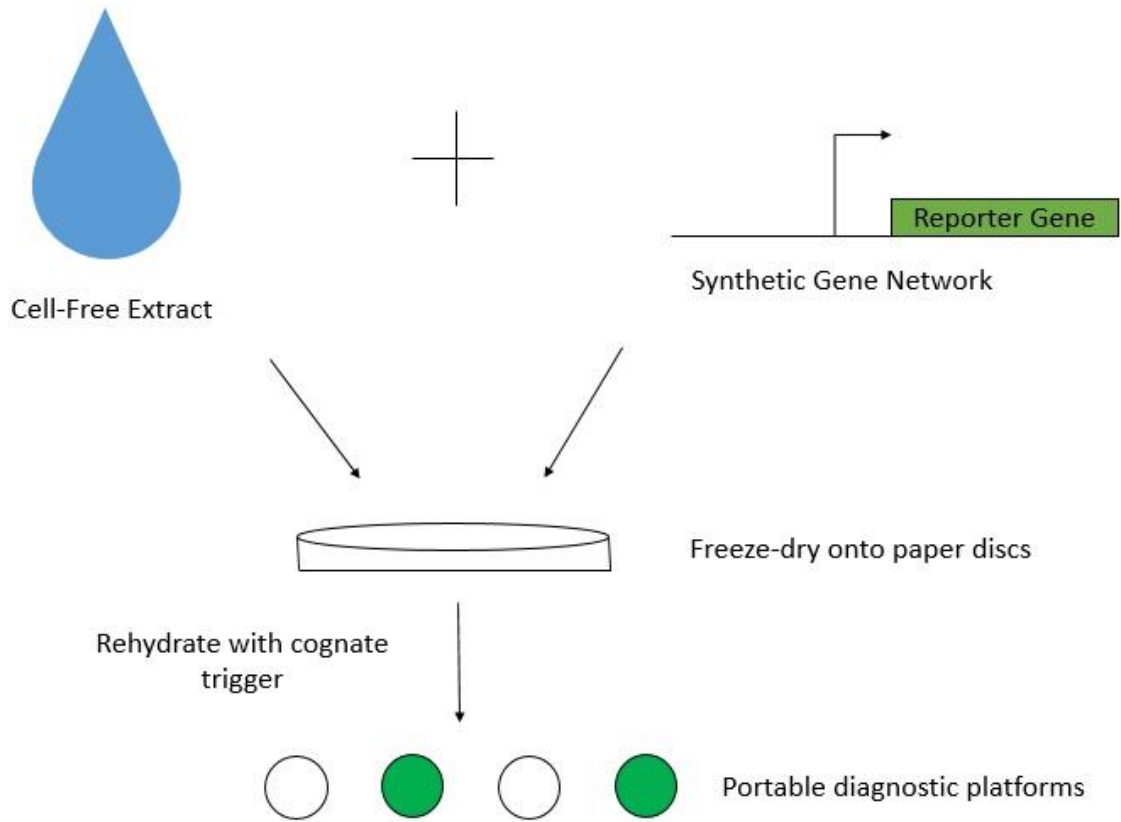
Fig. 4: Process of embedding cell-free machinery and synthetic gene networks onto paper discs for portable diagnostic capability. Adapted with permission from Pardee et al. (2014) [104].
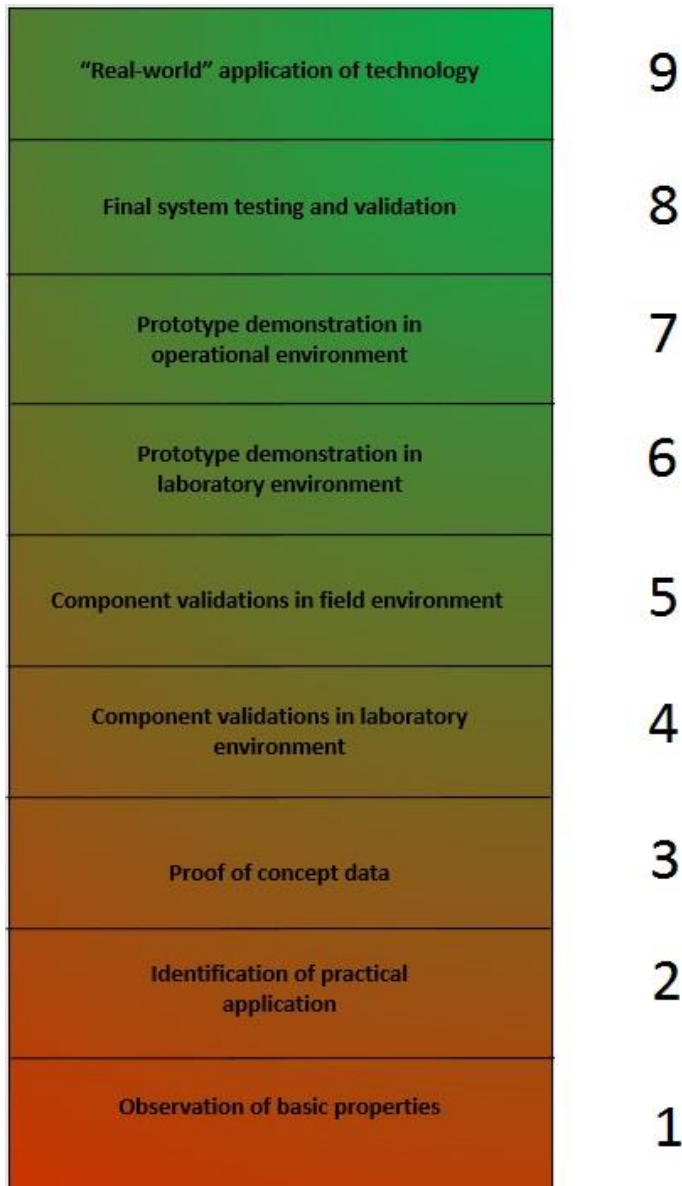
Fig. 5: A technology readiness level chart used to estimate the maturity of a novel technology during development. A higher position on the chart indicates a greater maturity and progress towards the finished product working under intended operational conditions.