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Rapid PCR-free meat species mitochondrial DNA identification using Electric Field Induced Release and Measurement (EFIRM®)

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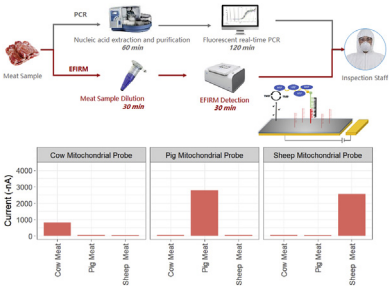
HIGHLIGHTS

- A novel electrochemical DNA biosensor method for detection of mitochondrial DNA was developed for identifying animal species.
- This analysis method is simple, PCR-free, and only requires sample immersion in a 2% SDS solution.
- Assay detection was rapid, requiring less than 2 hrs and as little as 40 mg of sample.

GRAPHICAL ABSTRACT



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ABSTRACT

This work details the usage of EFIRM® (Electric Field Induced Release and Measurement) for PCR-free rapid electrochemical detection of mitochondrial DNA. EFIRM® was able to perform highly sensitive detection of animal species for meat contamination testing without multistep sample lysis, DNA extraction, or PCR amplification steps, demonstrating the capability to detect the presence of foreign meat species that only constituted 0.1% of the total mass of a food sample (achieving sensitivity equivalent to that of PCR). The EFIRM® strategy utilizes surface immobilized nucleic acid probes that complement to mitochondrial sequence of Ovis Aries, Sus Scrofa, and Bos Taurus and are immobilized in a polypyrrole matrix on a 96-electrode array. Quantification was performed through amperometric measurement of oxidation-reduction reactions on a streptavidin-peroxidase enzyme chain that completes the nucleic acid complex. All electrochemical procedures were performed using a high-throughput potentiostat system that allows parallelized electrochemical measurement and interfacing to the 96-electrode array.

1. Introduction

In the modern food production economy, the ever-increasing competitiveness of the global market has led some to pursue the practice of adulterating food. Deceptive practices such as mislabeling products, filler additives, and ambiguous marketing practices have led consumers and inspection agencies to recognize the

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need for more stringent food quality testing. A particular area of concern is meat adulteration testing: In certain regional markets, for example, merchants have been found to produce counterfeit lamb meat, substituting pork meat.

In the recent years, a variety of analytical techniques have been pursued to determine whether foodstuffs have been adulterated. A survey of the literature indicates that food testing and meat adulteration test methods include a variety of different analytical methods such as polymerase chain reaction (PCR) [1–3], mass spectroscopy (MS) [4], and immunoassays [5] in order to identify animal species present in meat samples. Meat identification methods have also been reduced down to commercial test kits, with kits such as the PCR based method from ThermoFisher [6] or an ELISA based assay from Elisa Technologies Inc [7]. Each technology differs in the method of sample processing, detection principle, detection speeds, and sensitivities.

The commercially available PCR-based kit, 10–20 g of meat specimens samples must first be processed by being digested with a series of proteinase K and lysis buffer digests for 1–16 h and then extraction of DNA through the usage of ethanol and spin-column purification [8]. Following this processing and DNA extraction, the extracted DNA sample is then incubated with appropriately designed TaqMan® probes and subject to 36 cycles of amplification, equating to a real-time PCR assay that totals 55 min. According to the available public datasheet the assay is capable of detecting a sample that is 0.5% (w/w) of the total foodstuffs.

The immunoassay kit method for meat identification does not require extensive digests and lysis buffers, but instead involves the immersion of 1 g of diced meat sample into 9 mL of 0.9% w/v sodium chloride solution, agitating for 10 s, and then letting the sample settle for 10 min. The supernatant is then diluted into an assay buffer solution and placed into a well precoated with a species specific antibody and incubated for 20 min at room temperature. Following that, a reporting antibody and enzyme is added to complete the assay and perform measurement, with the assay in total being approximately 50 min. The product insert states that the assay is able to detect a meat species when it is 1% (w/w) of the total foodstuffs.

When the methods are evaluated, relative disadvantages of the method can be noted. The PCR-based detection method requires numerous steps for purification of relevant DNA and long multisteps procedures of extraction and amplification that make a total sample testing time of at minimum 110 min. The ELISA based identification method, while reducing the complexity of sample extraction and assay time still reduces the sensitivity of the assay and the usage of an antibody increases the cost of a test. Consequently, it is worth considering if there are alternative methods that may still achieve levels of sensitivity similar to the PCR-based approach while reducing assay complexity.

This work details a highly sensitive and rapid method that simplifies the identification of meat species, eliminating the need for PCR and multi-step lysis and DNA extraction steps for sample processing. This method achieves sensitivity levels similar to conventional PCR-based techniques while greatly reducing the amount of operations necessary for assaying, making it more suitable for practical application in food testing environments. The method used in this work, titled EFIRM® (Electric Field Induced Release and Measurement) has been used previously for examining circulating tumor DNA [9,10], RNA [11], exosomes [12], and protein targets [13]. The basis of the EFIRM® method involves the usage of a polypyrrole conducting polymer with doped DNA probes [13,14] that efficiently hybridize to molecular targets present in a biological specimen. Following hybridization, a detector probe and a reporter-enzyme chain is used to transduce electrochemical signal (see Figure). The measured result of the EFIRM® is electrochemical current which increases in magnitude if more of the target species is captured.

Instead of using traditional PCR-amplification and multistep DNA-purification techniques, the EFIRM® technique used in this work only requires a small cutting of sample be immersed in a surfactant solution (see Fig. 1A). A small volume of the surfactant extraction solution (10 μL) is then pipetted to the surface of the biosensor surface for analysis. Our strategy pursues identification of meat species by having surface immobilized capture probes hybridize to mitochondrial DNA sequences of commonly consumed animal species (*Ovis Aries*, *Sus Scrofa*, and *Bos Taurus*). Once the target sequence has been hybridized to a surface bound probe, a biotinylated detector probe is hybridized to the target sequence downstream of the area hybridized by the capture probe. A streptavidin-peroxidase reporting enzyme is then to generate a signal that is measured with the reader device.

This strategy of targeting mitochondrial DNA is advantageous because of the preponderance of mitochondrial organelles present in samples, which translate to a large copy number of DNA sequences that can be captured. Furthermore, the naturally occurring state of mitochondrial DNA is significantly shorter compared to genomic DNA (17 kb vs 50,000 kb), equating to smaller fragments that may be more easily detectable by the surface based probes that are used in EFIRM®, as longer fragments of nucleic typically are more difficult to detect with surface based DNA biosensor techniques [15].

For this project, data has been generated demonstrating the viability of the EFIRM® based approach for identification of animal species. Initial tests are performed on synthetic oligonucleotide-based targets to verify the probes can detect the sequence of interest. Following these tests, experimental work is performed to show the ability to detect mitochondrial sequences from cuttings of meat samples, demonstrating that the probes do not cross-react with other animal species. Finally, tests are performed that suggest that the method is able to perform detection when a meat species is only0.1% (w/w) of total sample weight. These results demonstrate the viability of the EFIRM® to create a simple and highly sensitive test.

2. Materials and methods

2.1. Electrochemical and experimental equipment

All experiments utilized in experimental work using the EZLife Bio EFIRM® electrochemical platform (Los Angeles, CA). This platform is a multichannel potentiostat that can be used to perform chronoamperometry on 96-channels simultaneously. The instrument has a pogo-pin structure integrated that allows an EZLife Bio 96-electrode plate (Los Angeles, CA) to be placed on top and clamped in place for performing electrochemical techniques. This gold electrode was prepared by laser ablation of bonded gold-PET sheets and printed conductive silver that allows interface with the electrode using the pogo pins. The 96-electrode array possesses the form-factor of a traditional 96-well microtiter plate commonly used in biological applications, and consequently is integrated with traditional plate washers, such as the Tecan Hydroflex 8-Strip plate washer (Mannedorf, Switzerland) used in this work.

2.2. Reagents and chemicals

Pyrrole, potassium chloride solution, formamide, and Tween-20® was purchased from Sigma-Aldrich (St Louis, MO). 1% Blocker Casein, 20% SDS Solution, and 1-Step Ultra TMB® was purchased from ThermoFisher (Waltham, MA). Streptavidin Poly-80 Conjugate was purchased from Fitzgerald Industries (Acton, MA).

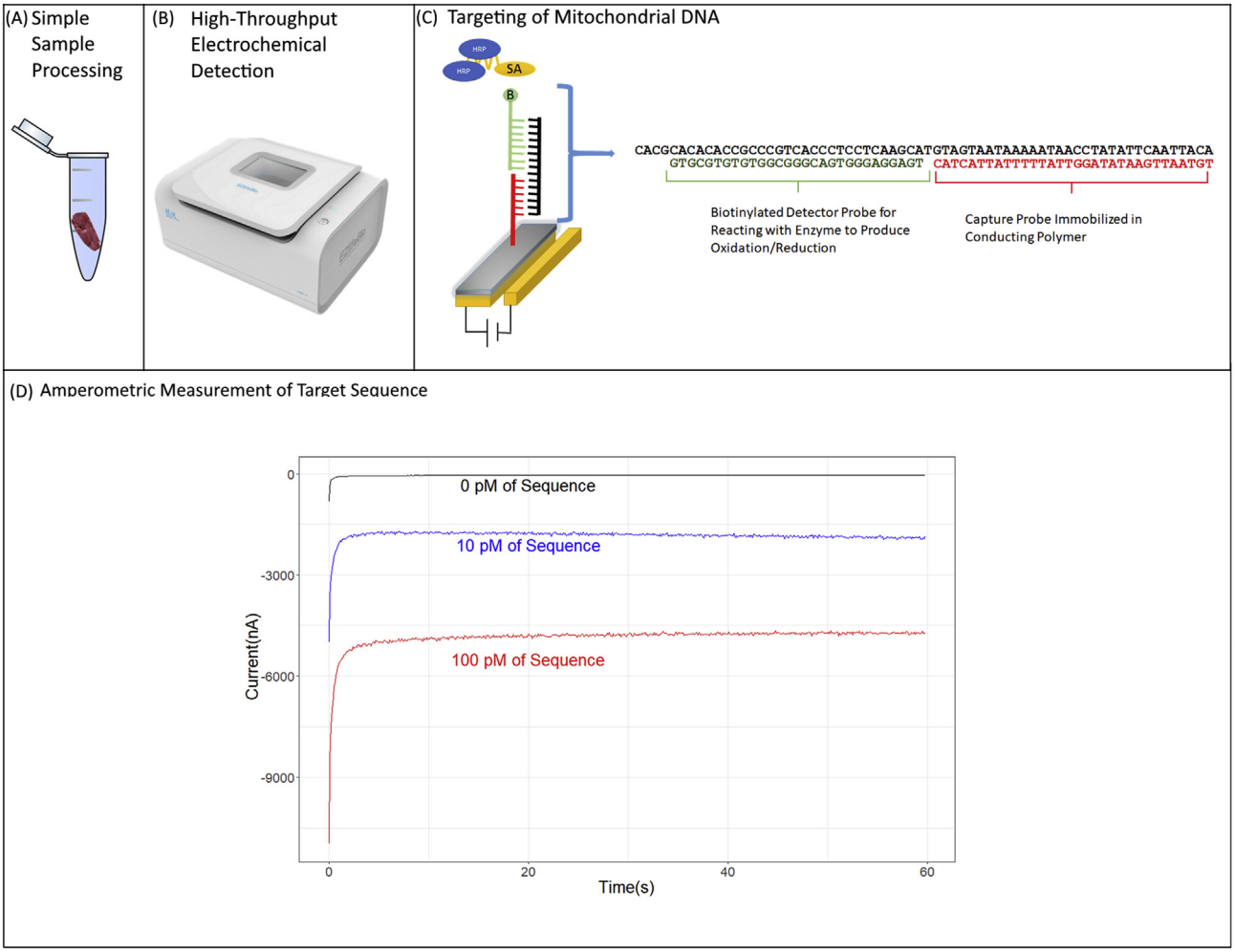

Fig. 1. Overview of key features of the assay (Panel A) Sample processing in this study on requires immersion of meat foodstuffs into a microcentrifuge tube with an SDS solution (Panel B) Supernatant from sample tube is pipetted into an electrode and measured electrochemically on a 96-channel electrochemical hybridization device (Panel C) Assay configuration on the electrode involves a capture probe (shown in red) that captures a target mitochondrial DNA sequence (shown in black) and a biotinylated detector probe (shown in green) and enzyme complex that will be used for electrochemical measurement (Panel D) Example amperometric readout that is made using the EFIRM® method. The higher the concentration of the target species, the larger the current magnitude that will be measured due to the enzyme-reporter mechanism. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3. Capture and detector probe sequences

These mitochondrial sequences were readily searchable and identifiable through the BLAST database and the probes designed were made to complimentary bind to the sequences using a previously described method of probe design [9]. Capture probes that were complimentary to the mitochondrial DNA sequences within *sus scrofa* (pig), *ovis aries* (chicken), and *bos taurus* (cow) were designed and synthesized by Integrated DNA Technologies (Coralville, IA), these capture probes being intended to be doped into a polypyrrole matrix on the surface of the electrode and used to hybridize specifically with a species specific sequence in the mitochondrial DNA. The capture probes also have regions consisting of DNA sequences that are designed to not hybridize to the target mitochondrial DNA but for elongating the probe and reducing steric hindrance during the sample capture portion of the experimental work. Detector probe sequence corresponded to a sequence that was universally found on mitochondrial DNA of all 3 species, and detector probes also had a biotin group modification inserted on the 3' end for enzymatic electrochemical measurement. Additionally, synthetic oligonucleotide sequences that corresponded to the animals were synthesized and tested on the probes as positive controls. The paired probes are listed in Table 1.

2.4. Conducting polymer capture probe immobilization

We have previously described our work in Wei et al. [13], but to describe in brief: An electropolymerization solution consisting of 0.3 M KCl, 10 mM pyrrole monomer, and 2.5 μM mitochondrial DNA capture probe was prepared by combining in a microcentrifuge tube and vortexing for 3–4 s. Following preparation of the solution, 30 μL was pipetted to each of the individual electrode wells and the EFIRM® high-throughput potentiostat used to apply a pulsed square waveform of +350 mV for 1 s and +1100 mV for 1 s at 4 cycles (for a total of 8 s) to initiate the polymerization of the DNA probes on the electrode.

After this polymerization is applied, the electrode was washed with a Phosphate Buffer Saline with 0.05% Tween-20 (PBST) solution using the automated plate washer. Every wash cycle in this particular application consisted of 2 cycles of wash where 400 μL is directly dispensed to the bottom of the electrode, a secondary wash of 400 μL at the top of the plate well, and then an aspiration of the remaining liquid.

Table 1
Oligonucleotide sequences used for probes and targets. The capture probe sequence is what is immobilized on the biosensor surface, while the detector probe is in a hybridization buffer solution.

Name	Sequence
Ovis Aries Capture Probe	AATAGGTTTAAGTATATCATATTTAAATAGGTTTAAGTATATCATATTTAAATAGGTTTAAGTATATCATATTTA
Sus Scrofa Capture Probe	AAAAAAAAAAAAAAAAAAAAAAAAAGTGTAATTGAATATAGGTTATTTTTATTACTAC
Bos Taurus Capture Probe 1	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACAATAAGGTTAGATGCATTGAATC
Bos Taurus Capture Probe 2	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACAATAGGGTTAGATGCACTGAATC
Pan-Species Capture Probe	CCGGTCTGAACTCAGATCACGTAGGACTAAACAAACAA
Ovis Aries Target Sequence	CACGCACACACCGCCCGTCACCCTCCTCAAGTAAATATGATATACTTAAACCTATTT
Sus Scrofa Target Sequence	CACGCACACACCGCCCGTCACCCTCCTCAAGCATGTAGTAATAAAAATAACCTATATTCAATTACACAACCATGCAAGAAGAGACAAGTC
Bos Taurus Target Sequence	AAGCACGCACACACCGCCCGTCACCCTCCTCAAATAGATTCAATGCATCTAACCTTATTTAAACGCACTAGCTATATGAGAGGAGACAAG
Pan-Species Target Sequence	GGTTCGTTTGTTCAACGATTAACAGTCCTACGTGATCTGAGTTCAGACCGGATAATCCAGGTCGGTTTCTATCTAT
Pan-Species Detector Probe	TGAGGAGGGTGACGGGCGGTGTGTGCGTGTCCTGC/3Bio/
Bos Taurus, Sus Scrofa, Ovis Aries Detector Probe	TGAGGAGGGTGACGGGCGGTGTGTGCGTGTCCTGC/3Bio/

2.5. Sample preparation and incubation

Sample was prepared by taking 0.05–0.10 g of meat foodstuff and adding into a 2% SDS lysis buffer solution. The meat specimen is then incubated for 30 min at 25 °C, and then 10 μL of supernatant was pipetted into the electrode plate. Next a 20 μL of a solution consisting of 37.5% v/v formamide, 5x SSC, and 1.5 μM concentration of detector probe is pipetted to the sensor surface and incubated for 30 min. Following this incubation, 2 cycles of washing using a PBST wash buffer solution is performed.

2.6. Enzyme reporter solution

An enzyme reporter solution was prepared by diluting Streptavidin Poly-HRP 1000-fold in Casein/PBS. 30 μL of this reporter solution is added to each individual well and incubated at 25 °C for 30 min. Following the incubation, the excess reporter solution is washed off using 2 cycles of the PBST wash buffer solution.

In order to perform electrochemical measurement of the captured DNA species, 60 μL of TMB and hydrogen peroxide reporter solution (1-Step Ultra TMB from Thermofisher) is added into each individual well. The EFIRM® potentiostat reader was then set to perform a chronoamperometric readout, where a fixed potential at −200 mV is applied and electrochemical current measurement is made for 60 s. Signal for each well is measured in nanoamperes (nA), with the value used for evaluating each well based on the average of the last 10 s of the current measurement phase.

3. Results and discussion

3.1. Development and verification of oligonucleotide probes

In order to perform initial verification of the method for detection of species-specific DNA sequences, target sequences that corresponded to the regions were targeted by the capture and detector DNA probes were synthesized and ordered. When oligonucleotide sequences were diluted in hybridization buffer and tested on the coated electrode sensor method, results showed that

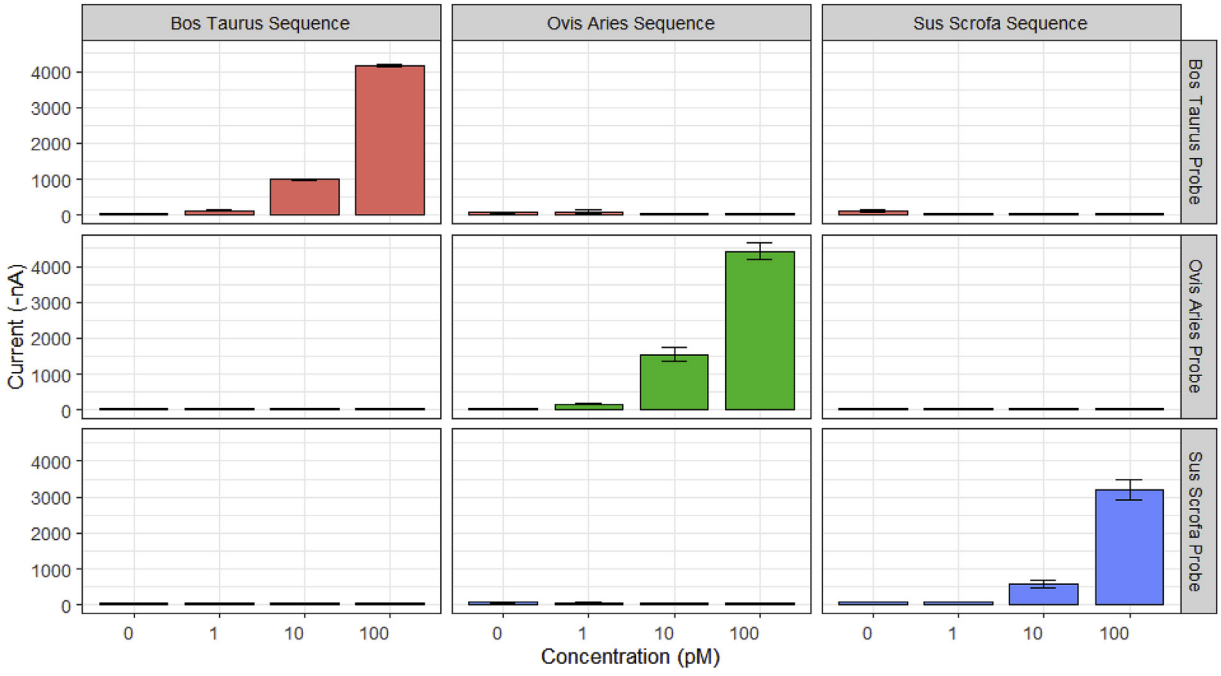

Fig. 2. Combinatorial testing of the different capture probes and target sequences. Each column represents a different capture probe that targets the mitochondrial DNA sequences, and each row represents target sequences that were tested with the probes. This graph demonstrates different concentrations of synthesized oligonucleotide target sequences that were tested with the probe.

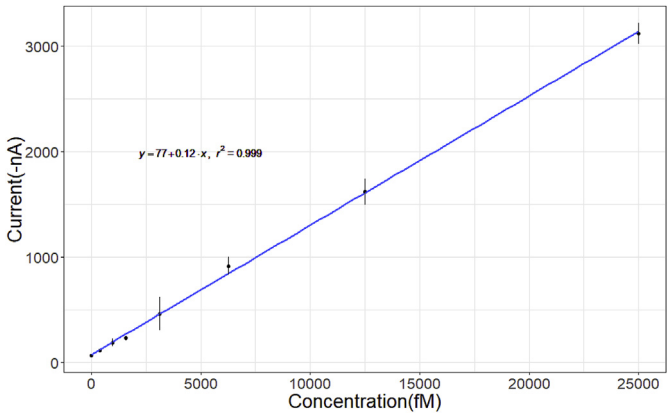

Fig. 3. Calibration curve establishing assay linearity for Bos Taurus oligonucleotide samples tested using the meat identification assay.

the probes were able to achieve picomolar sensitivity (all probes easily achieved 10 pM sensitivity) and high specificity detection, with over 500 nA difference between a 10 pM sample of the correct matched species and sequences of other species. Fig. 2 shows the capture probe sequences being paired with target sequences of the other animal species, demonstrating the specificity between sequences.

In order to evaluate the assay linearity and relationship between oligonucleotide concentration and measured electrochemical current, oligonucleotide reference standard sequences were also serially diluted from the 25 pM to the 390 fM concentration in order to evaluate the linearity of the assay. This series of dilution showed a highly linear (R^2 = 0.99) relationship between the measured electrochemical current and the concentration of the oligonucleotide reference (see Fig. 3).

3.2. Testing of sensor ability to differentiate meat samples

Meat samples from a local grocery store were cut and weighed to a range of 300–350 mg, and immersed in 200 μL of sample treatment solution and assayed according for their species, as per the methods section. Results determined that the sensor performed statistically significant differentiation between different cutting of samples, with an over 1000 nA difference between the appropriately matched samples and the species that incompatible with the DNA probes (see Fig. 4). A probe that was designed to detect sequences that were homologous between multiple animal species was also tested and evaluated, with each sample having a signal level being at least 150 nA normal sensor background levels.

Alongside testing the probe sequence for specificity and ability to detect animal species from a cutting of meat, the solution that the foodstuffs was immersed in was also evaluated for its effect on the assay performance. As Fig. 4 indicates, immersing the cuttings of sample into a distilled water solution was able to yield a measurable signal, but the assay ability to differentiate between samples was significantly reduced compared to samples were immersed in a 2% SDS solution, a possible mechanism being that the 2% SDS aids in the lysis of cells and the release of mitochondrial DNA content from the meat samples.

3.3. Testing optimal concentration of meat in extraction solution

In order to evaluate the total amount of meat necessary in solution for detection, cuttings from a sample of *sus scrofa* were taken and weighed and immersed in solution in proportion to weight (i.e. a 200 mg cutting of meat would be diluted in 200 μL of solution for a 1 mg/mL sample). The sample was then incubated at room temperature for 30 min, and dilutions of the sample analyzed using the EFIRM® technique in duplicate on the electrode plate. Results demonstrated that as the concentration of meat mass in solution decreases, there is a proportional decrease in measurable electrochemical current (see Fig. 5). Analysis shows that the EFIRM® assay was able to have a statistically significant difference between 0 mg/mL and 0.1 mg/mL sample of meat to extraction buffer with no nonspecific signal effects (as demonstrated by the fact that no signal was detected when the same samples were tested using the *Ovis Aries* assay).

3.4. Sensitivity of assay (differentiation between species)

Cuttings of beef and pork meat were weighed and mixed together in the 2% SDS treatment solution (adding 2% SDS extraction solution until the concentration of meat in solution was 1.75 mg/mL) and then subjecting the sample to analysis using the *Sus Scrofa* EFIRM® assay. Different amounts of pork mass in

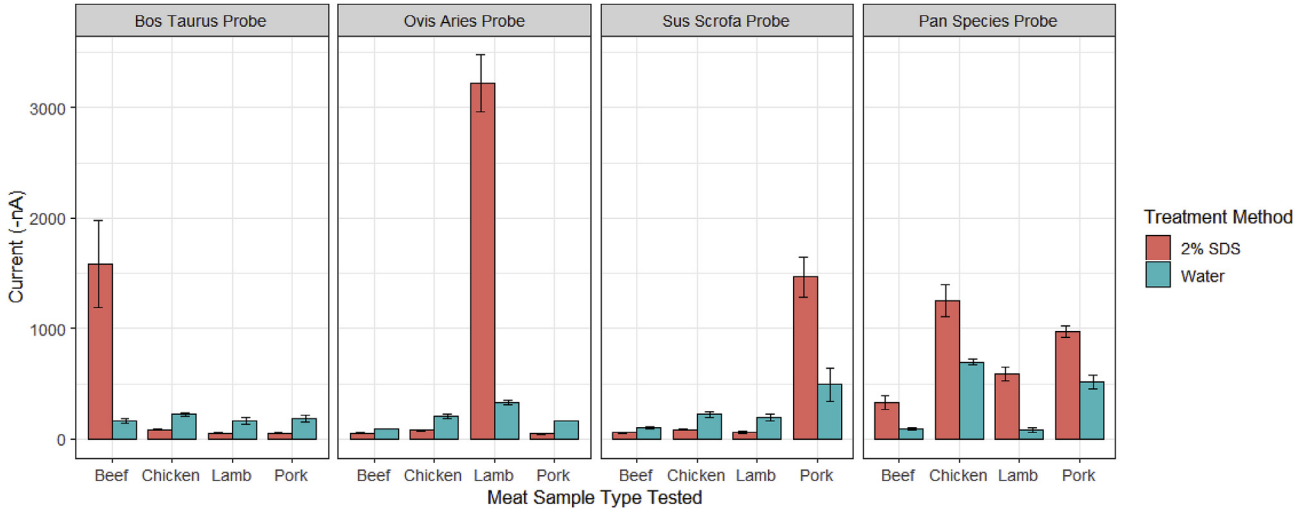

Fig. 4. Each panel represents the species that was assayed for using EFIRM®, while the x-axis markings correspond to the meat product that was tested with the probe. Samples were tested with different extraction buffers of ultrapure water or a 2% SDS solution.

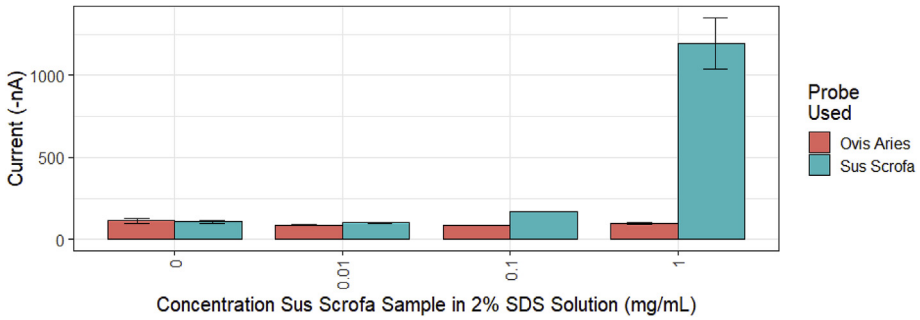

Fig. 5. Tests on assay sensitivity based on total concentration of meat in sample using dilutions of Sus Scrofa sample. Sample from Sus Scrofa was tested at different concentrations to determine the minimal detectable amount of sus scrofa sample necessary for analysis using EFIRM®.

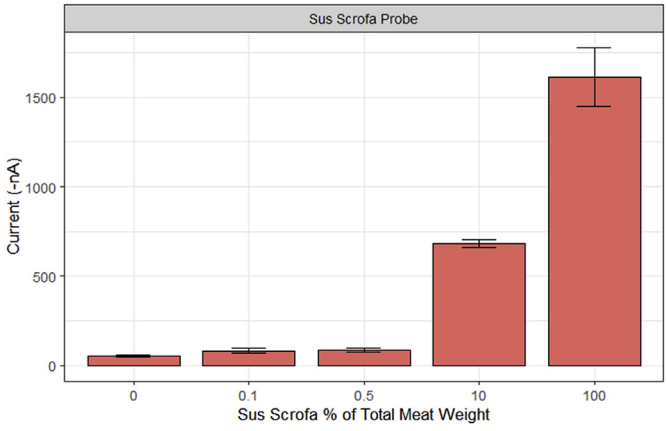

Fig. 6. Tests on assay ability to identify Sus Scora from a mixture of pork and beef cuttings that were mixed in different proportions.

proportion in proportion to a total mixture of beef and pork were tested (e.g. a 10% sample would have 1 g of pork in 10 total grams of meat), with 0.1%, 0.5%, 10%, and 100% values of pork in proportion to beef meat being tested. EFIRM® results shows that for the *Sus Scrofa* assay the measured current was directly proportional to the total percentage of pork that was present in the meat mixture (see Fig. 6).

4. Conclusion

This experimental work began by demonstrating through oligonucleotide samples that EFIRM® was able to detect specific mitochondrial DNA sequence with no major cross-reactions. Following this initial demonstration, actual meat samples were tested, with results show that the EFIRM® technique is able to successfully identify the species of a meat samples. This identification does not require multistep column-based extraction, but requires only testing the supernatant of meat that has been immersed in a 2% SDS solution. Initial characterization of the performance characteristics of the assay were conducted, showing that the assay was able to be achieve sensitivity levels comparable to existing tests.

Consequently, this work presents a notable enhancement over existing methods of PCR based meat identification tests in that it can perform high sensitivity DNA based tests without the complexities of sample extraction or amplification. This method of performing identification is also an improvement over the immunoassay-based method because it is a rapid and simple nucleic acid based test, eliminating the need to develop and manufacture antibodies for new animal species.

Multiple courses of investigation can be used to further increase the utility of the assay method described in the work, such as the development of assays for other animal species that are of interest to foodstuffs analysis and further exploration of methods to isolate and efficiently detect species specific nucleic acid content. Further exploratory work could also be done in developing standardized method in order to determine whether the assay can be a quantitative analytical tool for meat foodstuffs. Nonetheless, the work performed in this work has substantially demonstrated immense capability of using the EFIRM® technique for greatly simplifying testing of foodstuffs.

Author contributions

Michael Tu: Conceptualization, methodology, software, validation, supervision, investigation, resources, writing – original draft, writing – review and editing, visualization. **Man Yee Wong:** Methodology, Investigation, **Xiaoxia Sun:** Conceptualization, methodology, investigation, **Minxian Dai:** Investigation, validation. **Renping Huang:** Investigation, validation, **Ying Chen:** investigation, validation, supervision, **Xiaoyan Lin:** Conceptualization, methodology, investigation, supervision, **Qiuyue Zheng:** Conceptualization, Supervision, **Aifu Yang:** Conceptualization, Supervision, **Wei Liao:** Conceptualization, supervision, Writing – Original draft, funding acquisition, project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Many authors (MT, MW, XS, MD, RH, YC, XL, WL) of this work were employed by EZLife Bio during the scope of this research project. Wei Liao is founder/CEO of EZLife Bio Inc and owns equity in EZLife Bio Inc. Michael Tu owns equity in EZLife Bio Inc. Work performed in this study was funded by EZLife Bio Inc.

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