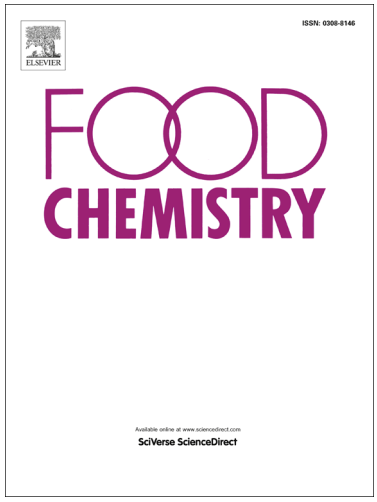

Lateral flow test for meat authentication with visual detection

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Lateral flow test for meat authentication with visual detection

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ABSTRACT

A new lateral flow assay has been developed for meat authentication. The assay is based on gold nanoparticles and enables visual detection, by the naked eye, of meat species-specific DNA sequences. The procedure includes DNA isolation from fresh meat samples, amplification of specific DNA sequences and detection by the lateral flow assay. The proposed assay is rapid and has been applied for the identification of four animal species: horse, pork, beef and sheep. The detection is completed within 25-30 min after amplification. The assay offers high detectability and good selectivity and reproducibility. As low as 0.01% of horse and 0.02% of pork DNA were detectable in binary mixtures by the reported lateral flow device.

1. Introduction

The adulteration of meat and food products in general, is a global challenge that is constantly increasing. Meat is one of the most vulnerable food products to fraud. Meat fraud usually involves adulteration or substitution of a type of meat with meat of lower nutritional value and/or quality, and therefore of lower price, aiming for economical profit. Besides the consumer financial protection issues, another important matter is the health risks posed by the occurrence of allergic reactions. For these reasons, regular controls of meat products are in great demand, in order to protect consumers and producers from fraud, and ensure food safety and public health. Sensitive, fast, simple, inexpensive and effective analytical methods are required to confirm the animal origin of meat-based products and detect meat adulteration (Abbas et al., 2018; Xu et al., 2018).

Several analytical methods have been developed during the last decades for meat authentication based either on animal-specific proteins or species-specific DNA sequences. However, DNA is the preferred analyte for food authentication tests, due to its enhanced thermal stability, especially during food processing. DNA is also present in the cells and tissues of all animal species and retains sequence-specific information (Alikord, Momtaz, Keramat, Kadivar, & Rad, 2018; Ali, Razzak, & Hamid, 2014; Rodríguez-Ramírez, González-Córdova, & Vallejo-Cordoba, 2011). Molecular techniques offer accurate, specific and reliable testing for food authentication and species identification regardless of age and condition of the samples (Lo, &

Shaw, 2018; Rahmati, Julkapli, Yehye, & Basirun, 2016). Various DNA-based methods have been introduced for meat authentication, including end-point PCR, isothermal amplification, real-time PCR, droplet digital PCR, sequencing-based techniques and DNA microarrays. The limit of detection (LODs) of these methods is reportedly in the range of 0.01 - 0.1% of adulteration (Alikord, Momtaz, Keramat, Kadivar, & Rad, 2018; Lo, & Shaw, 2018; Hong et al., 2017; Abbas et al., 2018).

Most recently, biosensors were developed for fast, sensitive and inexpensive detection of meat species. The main advantage of biosensors is that they do not require highly qualified personnel (Xu et al., 2018; Singh et al., 2016). Azam et al. (2018) developed a new and rapid luminol-based electrochemiluminescent sensor for the detection of porcine DNA. The method was able to detect as low as 0.1 pg/μl of amplified DNA and 0.001% of porcine DNA in binary mixtures with chicken DNA. The method, however, lacks good specificity (Azam et al., 2018). A new method based on loop-mediated isothermal amplification combined with a lateral flow device was developed for mammalian DNA, offering a detection limit of 10 pg of isolated DNA and 0.01% of mammalian DNA in mixtures of processed meat (Xu et al., 2017). An amperometric biosensor based on magnetic beads was constructed for the detection of 0.5% horse meat in beef meat (Ruiz-Valdepeñas Montiel et al., 2017). A real-time loop-mediated isothermal DNA amplification method was also performed in compact disc micro-reactors detecting 10 μg/g of bovine DNA (Santiago-Felipe et al., 2016). Another approach involves the rapid visual detection of eight meat species using optical thin-film biosensor chips with a detectability of as low as 0.001% of deer and beef meat powder in pork powder (Wang, Zhu, Chen, Xu & Zhou, 2015).

In the present work, we have developed a fast and simple lateral flow assay based on gold nanoparticles as reporters for meat authentication by the naked eye. The lateral flow assay was applied to the detection and identification of four meat species: horse, pork, beef and sheep. Furthermore, binary DNA mixtures were prepared and analyzed. We were able to detect as low as 0.01% of horse DNA and 0.02% of porcine DNA in beef and sheep DNA, respectively.

2. Experimental section

2.1. Materials and apparatus

The Immunopore FP membrane for the construction of the strips was from Whatman (Florham Park, NJ). The sample pad, absorbent pad, and glass-fibered conjugation pad were from Schleicher & Schuell (Dassel, Germany). Gold nanoparticles, 40 nm in diameter, were from BBI Solutions (Cardiff, UK). Triton X-100, Sephadex G-25 and a 2× Fast Starter Master Mix, containing a mixture of the reaction buffer, *Taq* DNA Polymerase, $MgCl_2$ and dNTPs were from Sigma-Aldrich (St Louis, MO). Bovine serum albumin (BSA) was purchased from Serva (Heidelberg, Germany), streptavidin (SA) was from Roche (Basel, Switzerland), sulfosuccinimidyl-6-(biotin-amido) hexanoate (EZ-Link sulfo-NHS-LC-Biotin) from Pierce (Thermo Fisher Scientific, Waltham, MA) and sucrose from AppliChem (Maryland Heights, MO). The terminal transferase reaction kit, containing the enzyme, the terminal transferase buffer and 2.5 mM $CoCl_2$ solution, was from New England BioLabs (Ipswich, MA), while deoxythymidine triphosphate (dTTP) and deoxyadenosine triphosphate (dATP) were obtained from Invitrogen (Carlsbad, CA). The NucleoSpin Tissue kit was from Macherey-Nagel (Düren, Germany). Agarose and DNA molecular weight markers (ΦX174 DNA, *Hae*III digest) were obtained from HT Biotechnology (Cambridge, UK) and mono- and dihydrate sodium phosphate were purchased from Lachner (Neratovice, Czech Republic). Oligonucleotides and primers were from Eurofins Scientific (Brussels, Belgium). The sequences of all the probes and primers used throughout this work are presented in Table 1 (Köppel, Ruf & Rentsch, 2011). Meat products or blood samples were obtained by local markets or farmers. Phosphate-buffered saline (PBS) solution consisted of 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4 with pH adjusted to 7.4. The 6× Saline-Sodium Citrate (SSC) buffer consisted of 900 mM sodium chloride and 90 mM sodium citrate, adjusted to pH 7.0. The 0.1 M Phosphate Buffer (PB) solution (pH 6.8) consisted of 46.3 mM Na_2HPO_4 and 53.7 mM NaH_2PO_4. The hybridization buffer contained 0.22 M NaCl, 0.1M Tris (pH 8.0) and 0.88 ml/l Triton X-100. The developing solution contained 2% glycerol, 10 mg/l BSA and 2 ml/l Tween-20 in PBS pH 7.4.

A TP 600 Gradient thermal cycler from TaKaRa Bio (Japan) was used for PCR amplification. The TLC applicator CAMAG Linomat 5 (Muttenz, Switzerland) and a Dedalos – 4c oven obtained from KEL (Patras, Greece) were used for the construction of the test and the control zones onto the diagnostic membrane of the strips. Finally, a common smartphone camera was used for lateral flow assay documentation.

2.2. Samples and DNA isolation

DNA was isolated from fresh meat samples of different animal origin (pork, beef and sheep) and from horse blood samples using the NucleoSpin Tissue kit from Macherey-Nagel according to the manufacturer's instructions.

2.3. Tailing reaction

The oligonucleotide dT(30) was elongated with dTTP by terminal deoxynucleotidyl transferase (TdT), while a poly(dA) tail was added to all the specific probes through the following tailing reaction. Tailing reactions were performed in a volume of 20 µl containing 50 mM potassium acetate, 20 mM Tris-acetate and 10 mM magnesium acetate at pH 7.9, 250 µM $CoCl_2$, 20 units of TdT, 700 pmol of dT(30) or sequence-specific probe and 3.5 mM dTTP or dATP, respectively. The mixture was incubated at 37 °C for 1 h and the reaction was stopped by adding 2 µl of 0.5 M EDTA (pH 8.0).

2.4. Biotinylation of bovine serum albumin (BSA)

The biotinylation was carried out in 250 µl containing 200 µg of BSA, 1 mg of sulfosuccinimidyl-6-(biotin-amido) hexanoate (EZ-Link Sulfo-NHS-LC-Biotin) and 50 µl of 0.5 M $NaHCO_3$, pH 9.1. The mixture was incubated at room temperature for 1 h with occasional stirring. Finally, the biotinylated BSA (b-BSA) was purified twice by size-exclusion chromatography using a pre-packed Sephadex G-25 spin-column and a 2-min centrifugation at 800 g.

2.5. Construction of the lateral flow strip

The lateral flow assembly (strip), had a size of 4 mm×70 mm and was constructed by a plastic adhesive backing pad where an immersion pad, a glass fiber conjugate pad, a nitrocellulose membrane and an absorbent pad were placed on with overlapping ends, ensuring

the flow of the developing solution, the sample and the reagents. Biotinylated BSA (b-BSA) and poly(dT) sequences were immobilized onto the membrane forming the control and the test zone of the lateral flow device, respectively. More specifically, a solution containing 300 ng/μl BSA in 5% (v/v) methanol, 2% (w/v) sucrose in PBS buffer was deposited at a density of 150 ng/strip onto the control zone of the membrane using the TLC applicator. Then, a solution having 10 pmol/μl poly(dT) oligonuleotide in 2% (v/v) methanol and 2% (w/v) sucrose in 6×SSC buffer was also loaded at the test zone of the membrane with a density of 30 pmol/strip. The immobilization was carried out at 80 °C for 2 h. The strips were then properly assembled.

2.6. Preparation of streptavidin-functionalized gold nanoparticles (SA-AuNPs)

The pH of a 200-μl aliquot of gold nanoparticles, 40 nm in diameter, was adjusted to 6.0 with approximately 10 μl of 20 mM Phosphate Buffer (pH 6.8). Then, 3 μg of streptavidin (SA) were added and the mixture was incubated at room temperature for 2 h with occasional hand-stirring. Subsequently, BSA at a final concentration of 10 mg/l was added to block the unreacted parts of the nanoparticles. The mixture was incubated for 30 min at room temperature. The streptavidin - gold nanoparticles conjugates (SA-AuNPs) were collected by centrifugation at 3300 g for 20 min and resuspended in 20 μl of 10 mM phosphate buffer pH 6.8. The conjugates were finally stored at 4 °C. A 3-μl aliquot of SA-AuNPs was directly applied onto the conjugation pad of the lateral flow assay for visual detection of meat-specific DNA sequences.

2.7. Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in a total volume of 25 μl containing 1× Fast Start Master Mix (Sigma), 0.6 μM of each of the primers and 5 μl of isolated DNA. The amplification reaction was carried out as follows: initial denaturation at 95 °C for 5 min and 45 cycles of 95 °C for 20 s, 50 °C for 30 s and 72 °C for 30 s, followed by a final incubation at 72 °C for 10 min. The concentration of the PCR products was determined by 2% agarose gel electrophoresis with ethidium bromide staining followed by densitometric analysis.

2.8. Hybridization of the PCR products to the complementary dA-tailed probes

Each PCR product was hybridized to a complementary oligonucleotide probe specific to each meat species. An aliquot of the PCR product, diluted properly in 0.1 g/l BSA, was denatured at 98 °C for 3 min in the thermal cycler and rapidly placed on ice for 2 min. A 5-μl volume of the denatured PCR product was then added to a pre-heated, at 37 °C, solution containing 0.5 pmol of the dA-tailed specific probe and a proper volume of hybridization buffer to reach a final volume of 10 μl. The mixture was finally incubated for 15 min at 37 °C.

2.9. Lateral flow assay

A 5-μl aliquot of the pre-hybridized double-stranded PCR product along with 3 μl of streptavidin-functionalized gold nanoparticles (SA-AuNPs), were applied onto the conjugate pad of the lateral flow strip. The strip was then immersed into 300 μl of the developing solution. As the developing solution moves upwards and after 10-15 min, visual red lines were formed on the nitrocellulose membrane of the strip due to the accumulation of functionalized gold nanoparticles. Finally, the strip was removed from the developing solution, while a common smartphone camera was used for image capturing. The images were further processed using the open source image processing program ImageJ. In more detail, the colour density of the test zones (negatives and positives) on grayscale mode was measured by the ImageJ software.

3. Results and discussion

In this study, a lateral flow assay was developed for meat authentication. More specifically, a new lateral flow device (strip) was constructed for the visual detection, by the naked eye, of DNA sequences specific to four meat species: horse, pork, beef and sheep. Horse and pork were selected as models for the development of the proposed method, as they are widely used in meat adulteration of both beef- and sheep-derived meat products. The lateral flow assay was finally applied to the detection of meat-specific DNA sequences in binary mixtures of DNA isolated from fresh meat or blood samples, as a test for meat adulteration detection. In more detail, DNA was isolated from fresh meat products and blood samples. Each DNA sample from the four different animal species was subjected to an amplification reaction using a biotinylated primer.

The biotinylated product was then heat-denatured and hybridized to the complementary oligonucleotide probe that carries a poly(dA) sequence at one end. The hybridized product is applied onto the conjugation pad of the strip next to streptavidin-functionalized gold nanoparticles and the strip is dipped into the developing solution. As the developing solution migrates upwards, a red line is formed at the test zone of the strip, in the presence of DNA target, because (a) the hybrids are captured by poly(dT) sequences immobilized at the test zone of the strip due to dA-dT hybridization and (b) the functionalized gold nanoparticles bind to the biotinylated pre-hybridized PCR product due to streptavidin-biotin interaction. A second red line is formed at the control zone of the strip as the excess of gold nanoparticles conjugates are captured by the immobilized b-BSA at this zone to confirm that the lateral flow assay works properly (Figure 1). The colour density of the test zones is also quantified by the ImageJ software to avoid false-positives by naked-eye detection.

The sizes of the amplification products specific to horse, pork, beef and sheep were 107, 154, 83 and 88 bp, respectively. The effect of the amount of the PCR product on the test zone intensity was initially studied. For this purpose, various amounts of the PCR products of the four meat species ranging from 0.8 – 100 fmol were applied to the proposed lateral flow sensor. We observed that the intensity of the colour of the test zone increases, as the amount of the PCR product increases. Finally, as low as 1.6 fmol of the PCR product specific to horse and 6.2 fmol of pork and sheep-specific PCR products, were detectable by this assay (Figure 2).

We observed that the beef-specific PCR product gave a low positive signal at the test zone of the strip and was finally analyzed as follows: a mixture containing 5-μl of the specific to beef PCR product and 0.5 pmol of its corresponding probe with a poly(dA) tail, was denatured by the addition of 5 μl of 0.4 M NaOH solution for 10 min at room temperature. Then, 5 μl of 0.4 M HCl and 5 μl of 0.4 M Tris buffer (pH 7.5) were rapidly added and the mixture was incubated for 15 min at 37 °C. A volume of 10 μl was finally applied to the lateral flow assay, resulting in a significant increase in the density of the red colour of the test zone. The other amplification products for horse, pork and sheep, were also tested by the lateral conditions (heat denaturation and alkaline denaturation), but no changes were observed. We observed that as low as 0.4 fmol of the PCR product specific to beef could be detected by the proposed assay (Figure 2).

Subsequently, binary mixtures of horse-beef and pork-sheep DNA were prepared by mixing appropriate amounts of isolated DNA from meat and blood samples derived from the four

different animal species. The mixtures contained different amounts ranging from 0.01 to 5% of horse or pork DNA in beef or sheep DNA, respectively. The mixtures were amplified using the primers specific to horse or pork DNA sequence, accordingly. Finally, the amplified products were analyzed by the lateral flow assay. The results are presented in Figure 3. As low as 0.01% of horse DNA and 0.02% of pork DNA in beef and sheep DNA, respectively, were detectable by the proposed assay.

To assess the accuracy of the method, a cross-reactivity study was performed. An amount of 50 fmol of beef PCR product was analyzed by the lateral flow assay using the horse-specific probe, while the same amount of the sheep PCR product was analyzed with the probe specific to pork DNA. The intensity of the colour of the test zones of the strips was determined by densitometric analysis using the free software ImageJ. We found that the cross-reaction for both probes specific to horse and pork was lower than 2% (Figure 4).

Finally, the reproducibility of the lateral flow assay was assessed. For this purpose, amplified products corresponding to the 0.2% content of horse-beef and pork-sheep binary mixtures were analyzed in triplicates by the lateral flow assay. The results are presented in Figure 5. The density of the coloured test zones was measured using the ImageJ software and the coefficient of variations (CVs) were calculated. The % CVs were equal to 5.5 and 1.6 % for horse and pork DNA sequence, respectively, revealing the excellent reproducibility of the assay.

4. Conclusions

A new lateral flow assay based on gold nanoparticles for visual detection of meat adulteration was constructed. The assay was applied to the detection of four different DNA sequences of four different meat species derived from horse, pork, beef and sheep. The development of the method has been based on real fresh meat samples only and no interreferences were observed. Moreover, binary mixtures of the isolated DNA were also prepared to assess the detectability of the method. As low as 0.01% of horse DNA in beef DNA and 0.02% of pork DNA in sheep DNA were detectable. The proposed lateral flow assay is simple, rapid, sensitive and cost-effective. Compared to other DNA-based lateral flow assays for meat authentication, the proposed assay offers simultaneous detection and identification of the meat-derived DNA sequences, is universal and can be applied for the detection of any animal-

specific DNA sequence. The whole procedure including DNA isolation from fresh meat or blood samples, PCR amplification and detection by the lateral flow assay is completed within 3-3.5 h, whereas the detection is carried out within 25-30 min after PCR amplification. The detection is accomplished by the naked eye without the need of expensive instruments or well-qualified personnel. Finally, as DNA is the most stable marker, compared to proteins, especially during thermal food processing, the method could be applied for the detection of meat adulteration in processed meat samples.

Declarations of interest

None.

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Figure Captions

Fig.1. Schematic principle of lateral flow assay. The biotinylated PCR products specific to four meats (horse, pork, beef and sheep) are hybridized to their complementary probes that carry a poly(dA) sequence at one end and applied to the strip. A red line is formed in the presence of DNA target at the test zone of the strip, as the hybrids bind to immobilized poly(dT) sequences and detected by streptavidin-gold nanoparticles. A second red line is formed as the excess of streptavidin-gold nanoparticles are captured by immobilized biotinylated BSA to confirm the proper function of the strip. CZ: control zone, TZ, test zone, B: biotin, BSA: Bovine Serum Albumin, SA: streptavidin, Au: gold nanoparticles.

Fig. 2. Effect of the amount of amplification product on the intensity of the test zone of the strip for four meat-specific PCR products. The lateral flow assay was performed for four meat-specific PCR products. Different amounts of each PCR product (0.8-100 fmol) are analyzed separately by the lateral flow assay. CZ: control zone, TZ, test zone.

Fig. 3. Analysis of binary DNA mixtures of horse in beef and pork in sheep DNA ranging from 0.01-5% by the lateral flow assay. As low as 0.01% of horse DNA and 0.02% of pork DNA were detectable by this assay. CZ: control zone, TZ, test zone.

Fig. 4. Cross reactivity study.

Fig. 5. Reproducibility of the assay. Samples containing 0.2% of horse and pork DNA in binary mixtures were analyzed in triplicate by the lateral flow assay. The CVs were 5.5 and 1.6 % for horse and pork DNA sequence, respectively. CZ: control zone, TZ, test zone.

Table 1: Sequences of all the primers and probes used in this study (Köppel, Ruf & Rentsch, 2011)

Oligonucleotide	Name	Sequence (5´ → 3´)	Size (nt)
PCR primers	Beef R	b-GGCCAGACTGGGCACATG	18
	Beef F	GTAGGTGCACAGTACGTTCTGAAG	24

	Pork R	b-CTGGGGACATGCAGAGAGTG	20
	Pork F	GGAGTGTGTATCCCGTAGGTG	21
	Sheep R	GGAACTGTAGCCTTCTGACTCG	22
	Sheep F	b- CCAACATGCCTTTAAACCCTCAA	23
	Horse R	b- GTTAAAGCTTGGCTCGACACG	21
	Horse F	CCAACTTCATCATGGACAACGC	22
Probes	Beef probe	CGGCACACTCGGCTGTGTTCCTTGC	25
	Pork probe	TCTGACGTGACTCCCCGACCTGG	23
	Sheep probe	TGCCTTTCCTTCCCCGCCAGTCTC	24
	Horse probe	AAGTGCATCCCCGTGGCCCCTCA	23

*b: biotin

HIGHLIGHTS

- Meat authentication by a gold nanoparticle-based lateral flow strip test
- Detection of four meat-specific DNA sequences of horse, pork beef and sheep
- Detection of 0.01% of meat adulteration in binary mixtures
- A rapid, cost-effective, sensitive and universal lateral flow assay was developed

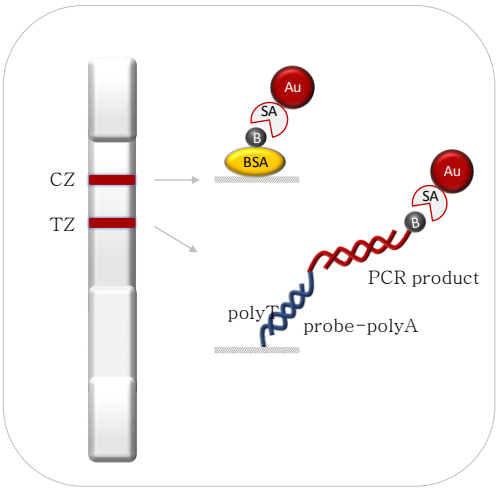

Figure 1

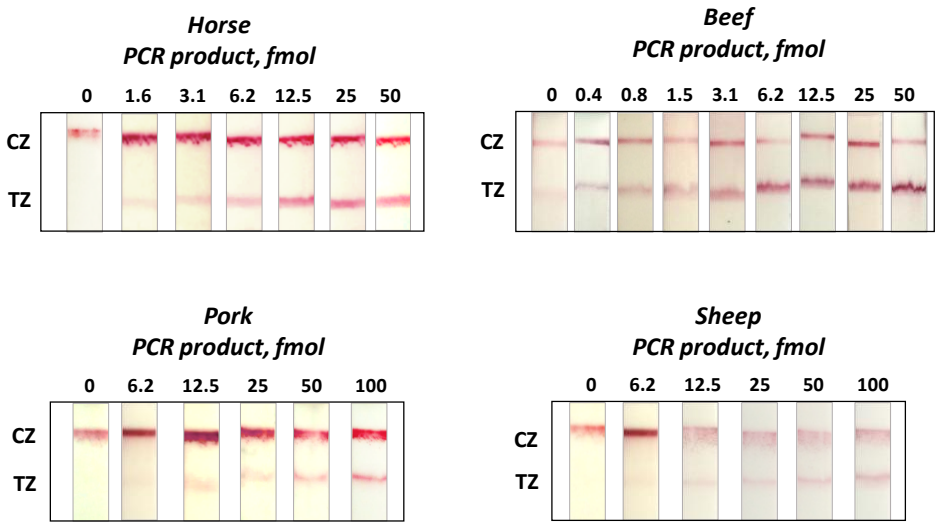

Figure 2

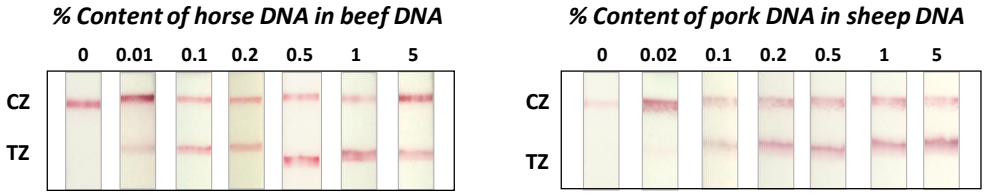

Figure 3

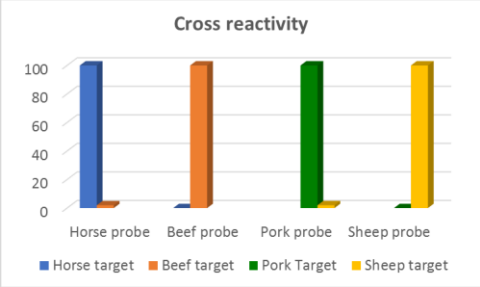

Figure 4

Reproducibility

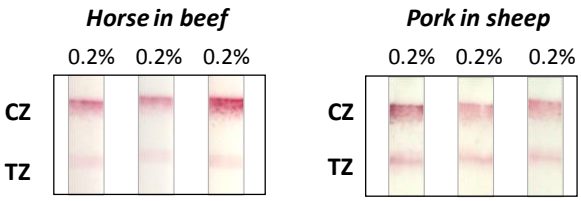

Figure 5