Miniaturization and multiplexing of a lateral flow immunoassay for the detection of Rotavirus, Coronavirus, *Escherichia coli* F5 (K99) and *Cryptosporidium* in calf faeces

Lateral flow assays (LFAs) are designed as a reliable, fast, easy to handle and low-cost diagnostic platform for direct on-site testing (point-of-care, POC). Over-the-counter (OTC) pregnancy tests for home use are one of the most prominent LFAs. Besides those, a large number of diverse LFAs are available targeting e.g. biomarkers, toxins or pathogens in a wide range of samples like urine, blood, serum or faecal specimen. Based on traditional production technology, conventional LFAs rely on lines (control and test lines) arranged perpendicular to the flow direction. Since this design has a limitation in multiplex degree and the possibility of depletion and inadvertent interferences of assay components in multiparametric tests, there is a need for advanced array-based lateral flow assay layouts. The development of an array-based multiplexed calves scours lateral flow assay has been chosen exemplarily as a case study for this application note.

Diarrhoea is a major cause of mortality in young cattle under the age of one month. This multifactorial disease is referred to as bovine neonatal gastroenteritis. It can be caused by viruses (Coronavirus or Rotavirus), by bacteria (*Salmonella*, pathogenic strains of *E. coli*) or by protozoa such as *Cryptosporidium*. The diagnosis of the etiological agent of diarrhoea has traditionally only been performed in laboratories because the clinical signs do not suffice to distinguish between these pathogens.

LFA is gradually emerging as a reliable alternative in the diagnosis of gastroenteritis due to its simplicity, rapidity, sensitivity and specificity.

Bio-X Diagnostics S.A. and SCIENION AG have jointly demonstrated the feasibility of transferring four existing singleplex line-based LFAs into a highly specific, sensitive and multiparametric array-based test strip.

Materials and results

Arrays were printed on nitrocellulose membranes using SCIENION's proprietary non-contact sciDROP PICO. Replicate dot patterns were printed onto 30 cm x 2.5 cm membranes prior to laminating and cutting into individual strips. Conjugate and absorption pads used were made of glass fibre and cellulose respectively. The multiplex LFA is based on the classical sandwich ELISA microarray format, using monoclonal capture antibodies (cAb) and monoclonal detection antibodies (detAb) conjugated to black nanoparticles. Serial dilutions of positively tested calve faeces were used as analyte standards. All antibodies and calve faeces were provided by Bio-X Diagnostics S.A. Immobilization of cAb onto nitrocellulose and labelling of detAb with black nanoparticles were optimized guaranteeing highly specific and sensitive detection of each pathogen in the miniaturized multiplex LFA. In addition, buffers for loading of conjugate pads and for sample dilution were developed ensuring a reproducible conjugate release and high signal-to-background ratios, which is specifically required to detect low analyte concentrations. The colorimetric readout was performed after 10 min assay duration with a sciREADER CL2.
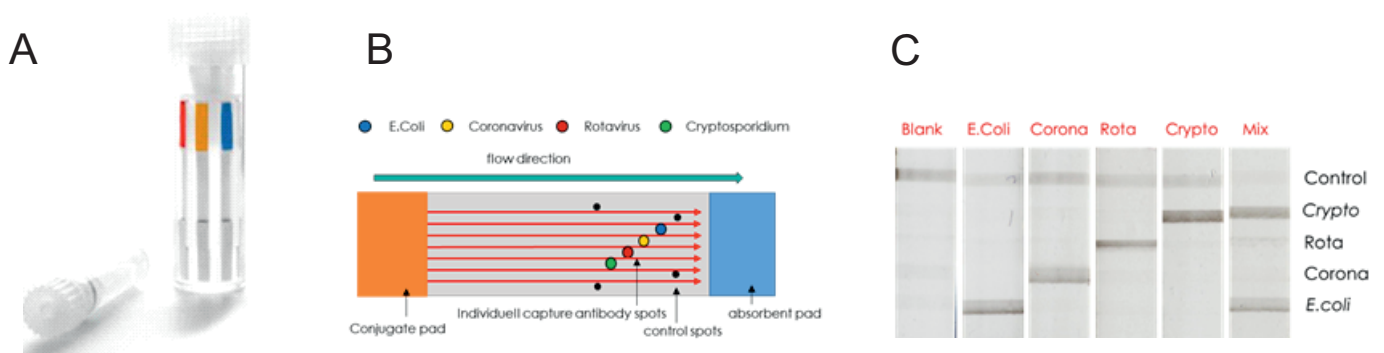

Fig. 1 (A) RAINBOW™ Calf Scours-BIO K 288 kit is a commercially available immunochromatographic device. Parallel processing of four strips in a single housing allows for multiparametric detection of *E. coli*, Coronavirus, Rotavirus and *Cryptosporidium* as causative agents in calves scours. (B) Schematic representation of the array-based pattern chosen for multiplexing of calf Scours-BIO K 288 panel. The staggered layout is arranged such that laterally flowing assay components are only passing a single capture zone avoiding depletion and interference unintendingly influencing assay results. (C) The multiparameter POC with 4 perpendicular arranged test lines and one control line shows the high risk of false negative signals. The Corona test line and the control are completely missing when all 4 pathogens are present in the sample.

Results

Subsequent to optimizing antibody labelling in singleplex assays and designing of the sandwich ELISA on the molecular level (data not shown), development of the multiplex LFA was initiated by establishing a compatible array layout, see figure 1B. The half-stick LFA setup shown in figure 2 was chosen to speed up assay development allowing efficient variation and optimization of critical assay components. The running buffer composition was optimized and detAb concentrations were titrated against analyte standards maximizing signal-to-noise ratios (data not shown). The evaluated parameters were finally combined in a full-stick LFA, RAINBOW™ Calf Scours-BIO K 288 panel in a single assay, see figure 3.
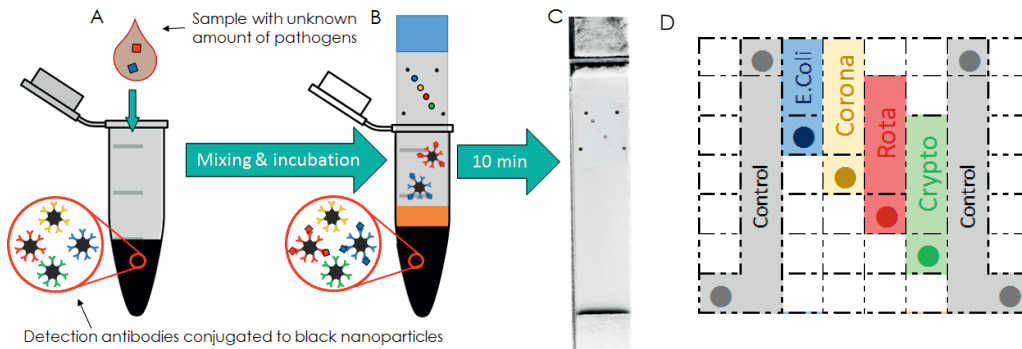

Fig. 2 Half-stick assay format chosen for assay development. (A) diluted scours samples containing target analytes are mixed with detAB-functionalized black nanoparticles in optimized running buffer. (B) a half-stick is incubated in 100 µl thereof. (C) readout after 10 minutes. (D) array layout for multiplexing the 4 pathogens with one stripe.
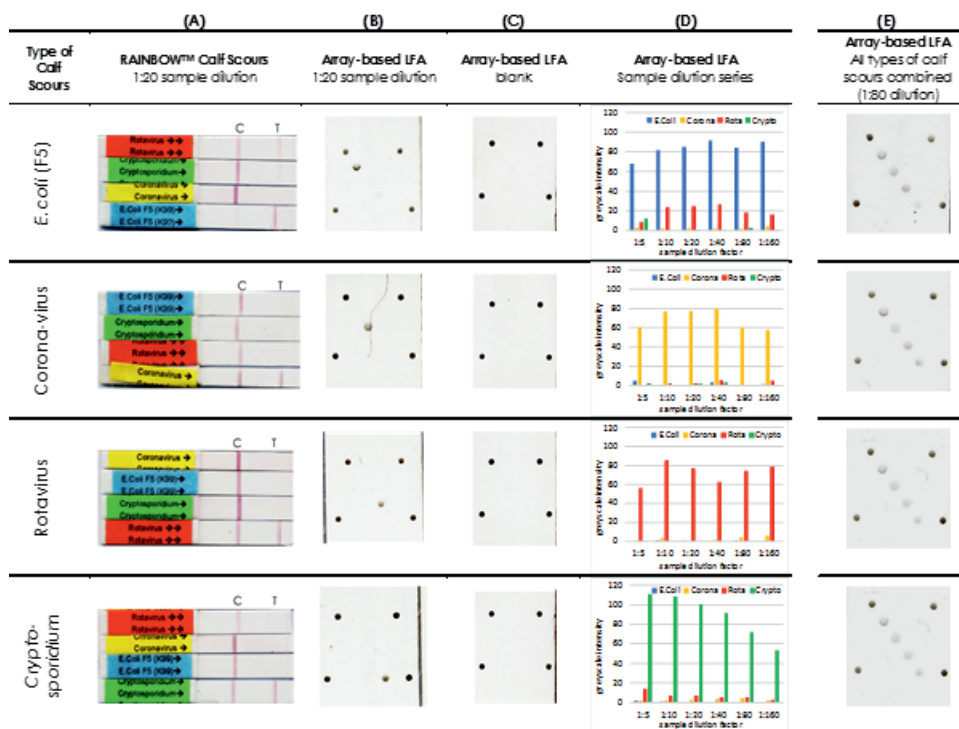

Fig. 3 Comparison of a 1:20 diluted sample between the (A) commercially available RAINBOW™ Calf Scours-BIO K 288 kit based on lines and the (B) newly developed array-based multiplex LFA. The *E.coli* calf scours shows a co-infection of rota virus and *cryptosporidium*. (C) highly specific array-based LFA with no observable false positive signals (D) with the array-based LFA the 4 pathogens in samples are detectable up to a 1:160 dilution. (E) Reliable and reproducible detection of all 4 pathogens simultaneously in one sample with a single stripe

Summary & Conclusion

1. Highly sensitive and specific multiplex sandwich ELISA-based LFA assay.
2. Spatial arrangement of captures as staggered dots rather than lines avoides depletion and interference of assay components (shown in figure 1).
3. Up to 65 % savings of stripe material, housing and antibody costs in a single 5.0 mm multiplex stripe instead of using four stripes with a width of 3.6 mm each.

Relying on the classical sandwich ELISA format, this miniaturized multiplexed POC test allows simultaneous detection of four different pathogens on a single 5 mm wide nitrocellulose strip using as little as 100 µl of sample. The staggered dot-based format leads to significant savings of capture, nitrocellulose and pad materials compared to classical line-based LFAs while avoiding unintended interference of assay components and depletion leading to false negatives.

Courtesy of Dr. Philippe Coppe from Bio-X Diagnostics S.A., Belgium - Email: p.coppe@biox.com
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