The authors would like the thanks the editor and the reviewers for their careful consideration and evaluation of their manuscript. After having addressed the reviewers' comments as below, the authors believe that their manuscript's quality has significantly improved and they are thankful for reviewers' time and expert comments.

The authors reproduce below the Reviewer's questions in bold followed by the authors' responses in roman font. The revisions in the manuscript appear highlighted in red.

Editorial comments:

The manuscript has been modified and the updated manuscript, 60489_R0.docx, is attached and located in your Editorial Manager account. Please use the updated version to make your revisions.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Proofreading has been performed on the final revised version of the manuscript prior to submission

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Elsevier copyright agreement has been added to the submission

3. Please use h, min, s for time units.

Time units were formatted as recommended throughout the manuscript.

4. Please avoid long steps (more than 4 lines).

P.2: Step 1.2 of the protocol was edited to reduce its length.

5. Please specify the antibody in the manuscript.

P.3: Step 1.2.3 The antibodies are listed as part of the protocol for surface modification. Also, a reference is made to Table 1, which contains a list of the detection antibodies and the pathogens.

6. Step 3.5.1-3.4.6: Please write each step in complete sentences and in the imperative tense.

P.6, The procedure steps, 3.5.1 to 3.5.6 were rewritten using an imperative tone.

7. Please sort the items in alphabetical order according to the name of material/equipment.

The list of materials was sorted in alphabetical order (A-Z) as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript describes a digital microfluidic (DMF) platform operated based on electrowetting-on-dielectric (EWOD) principle for a programmable and automated enzyme-linked immunosorbent assay (ELISA), utilizing magnetic beads as the protein carrier. The platform seems to be highly robust and authors describe the experimental protocol in detail with sufficient data to well support the reliability and efficiency of the method. However, as the demonstrated method/platform performs single ELISA per assay, the authors should provide discussions on how this work compares with conventional ELISA kits, which process assays in parallel in 96-well plates, in terms of overall cost and throughput. Given that the following comments will be addressed, this work should meet the criteria and standard expected by the readers of the JoVE.

Major Concerns:

1. As mentioned in the summary, the authors should provide discussions on how this platform/method compares with conventional ELISA kits in terms of cost and throughput. Based on the working voltage utilized to demonstrate the representative assays, it seems the reusability of the actuation plate would be low (~2-3 assays per plate), and considering the standard cost for printing chrome masks, this may not be a viable method for places where it is really needed. Additional discussions over the scalability of the platform should help to address the potential issue.

P. 11, L. 522: The discussion section was extended towards reduction for the cost per actuation plate.

"The presented platform utilised chrome printed EWOD chip, which could be reused reliably for consecutive measurements up to four times at operating voltage of 120 V and intermediate plate cleaning after each experiment. Plates were recycled, to reduce the cost per experiment, by decontaminating (brushing the surface with undiluted Neutracon before thourouglhy rincing) the biofouled Cytop® coating and spin-coating a fresh one on top of the plate. However, actuation plate recycling requires manual handling, costly reagents (Cytop®) and specialized equipment (spin-coater). Alternative EWOD chips are investigated successfully with cost-efficient substrates such as paper, acetate films or printed circuit boards (PCBs). Such disposable consumables can facilitate reliable and affordable use of the DMF platform and provide means to sidestep the biofouling issue."

P.12, L.549: A paragraph was added to discuss the difference in costs and implementations between standard ELISA and EWOD-based ELISA method.

"The automated DMF platform is designed to perform a single sandwich ELISA test per run while using microliter volumes for both reagents and analyte. When it is required, conventional sandwich

ELISA kits exist based on pre-coated 96-well or 384-well plates that in combination with auxiliary laboratory equipment result in higher throughput per run; based on reagents price only, the approximate cost per assay/well is 6.04USD (580USD/96) and 0.33USD (2×580USD/384) respectively. This renders the conventional ELISA methods ideal for a large number of samples processed typically by trained technical personnel at centralized laboratory facilities. However, in remote locations, the detailed cost analysis of ELISA for environmental monitoring showed that when capital costs (*i.e.* laboratory operating costs, recurrent costs, sample transportation, supplies and personnel) were included the actual price per ELISA was 60USD of which 34USD were for supplies per sample. In contrast, the proposed DMF platform is portable, requires minimum training to operate and with pre-coated beads can provide sample-to-answer analysis in minutes. Hence, the presented technology can be deployed to point-of-need locations and complement analyses otherwise available in centralized laboratories."

2. The presented DMF platform is oil-free. the authors should provide discussions on any potential issue with drying of the solution during operation, or provide description if there were any components in the platform that helped prevent drying.

The duration of the ELISA assay is less than 10min long, no droplet drying has been observed. Despite being oil-free, the EWOD actuation plate is covered by a hydrophobically coated wafer that confines the droplets and prevents evaporation.

3. The authors emphasize the comparable performance of their platform with Wheeler's system. Authors should provide similarities/differences between the systems for the readers to be able to better understand the potential applicability of the methods described in this work.

More details are provided in the introduction (page 2 paragraph 2) when comparing our system to Wheeler's as follow:

Both Wheeler's and our system, being transportable, self-contained, fully automated with included on-chip, real-time chemiluminescent measurements are arguably among the most advanced DMF biodetection systems available. The two systems have been design with very different applications in mind. Wheeler's system targets biomarker to allow biomedical dianostic on patient whereas our biodetection system was built around defence requirement for direct detection of pathogen previously sampled from air. The similarity between the two stresses the broad range of life impacting sectors that EWOD based technology can impact. It is believed that DMF-based detection platform and associated EWOD system can have key implication in health (biomedical diagnostic); military and civilian protection (threat detection); Agri-tech (crop monitoring) and work safety (controlled environment monitoring)

Minor Concerns:

1. The authors should make sure that all abbreviations used in the manuscript are spelled out at least once they are first introduced.

All abbreviations are written in full when they are first introduced.

2. Page 2, line 119: In what solution are the microbeads stored? Are there any contamination issue when stored for a month? Is there any sterile environment required in preparing the reagents and microbeads? Please provide explanation to clarify.

P. 2, L. 120: To explain the handling of the beads, this step was added: "1.2.1 Aliquot 0.2 ml beads (2 mg) into a plastic tube and remove the supernatant."

P.2, L. 109: All preliminary steps were completed in a sterile environment to avoid possible contamination of the buffer solutions and microbeads.

To the best of the authors' knowledge, there have not been any contamination issues; the issue with the long-term storage is two-fold, the beads tend to clump together and there may be antibody degradation after month storage at 4^{o}C.

3. Page 3, line 144: What concentration of PBS is used? Please provide.

P.3, L.140: The required information was added, 100 mM PBS.

4. Page 3, line 150: What concentration of Luminol is used? Please provide.

For the detection step, SuperSignal™ ELISA Femto Substrate was used, which consisted of two reagents, Luminol/Enhancer Solution and Peroxide Solution. The supplier, ThermoScientific, does not share information about the exact content of the two reagents. However, studies on the effect of luminol concentration reported that the maximum strength of the signal was achieved at concentration 0.3 mM Luminol [Chen H, Gao F, He R, Cui D, Chemiluminescence of luminol catalysed by silver nanoparticles. *J Colloid Interface Sci.* 315(1), 158-63 (2007)].

5. Page 4, line 206: The authors should cite the previous work where introduces the DMF platform

P. 5, L. 228: The citation was added to step 3.1, referencing the detailed description of the DMF.

6. Page 4, line 215: The authors should provide explanation on how the electrical contacts are made over the parylene C and Cytop films.

P. 4, L. 200: The following two steps were added to explain the protection of the electrical contacts, "2.4 Stick masking tape over the electrical contacts in order to isolate them during the two coating steps." Further down the text, another step was introduced, "2.7 Remove the masking tape from the electrical contacts."

7. It is recommended to providing labels in Figure 1 and Figure 5 to help better understand the described protocol.

The labels are included in the new Figure 2 with a detailed description in the caption for that figure

and references from the text are made. It was found that Figure 5 quality wasn't satisfactory as it hindered reader's understanding of the process. Figure 5 was remade with better quality images.

Reviewer #2:

Manuscript Summary:

The entire manuscript concerns on the application of EWOD for the combination of functionalised magnetic beads that enables a Lab-on-a-chip platform for sample preparation and identification of pathogens using ELISA.
The methodology followed is clear. However, the paper must be significantly improved before publication. The comments are given below.

1. Need to add reference(s) mentioned regarding the oil-based EWOD chip. It is mentioned as, the material collection in oil based systems impossible. But there are papers which explain the material collection (elution buffer) in oil based environment. Need to explain more on this portion.

The authors recognise that the statement concerning collection of material on a surface can be easily confused with the beads collection step presented in the manuscript. However, the authors refers to a different application. Minor modification of their original sentence and adequate references were added to remove possible ambiguity.

2. In Table 1, it seems to be a separate headline called stock. But there is nothing related to it below. Check Table 1 & the details provided.

The reviewer is absolutely correct. As the column "stock" was not relevant to the current article, it was removed from the table.

3. You should explain the size of the used magnetic beads. If size of M.B. matters, how do you inject the beads in between the gap, before or after placing the top plate on bottom plate.

Pierce™ NHS-Activated Magnetic Beads were used throughout this study. Beads' surface is activated with N-hydroxysuccinimide (NHS) functional groups on a block magnetic bead surface. The mean bead diameter is 1 µm, and the bead density is 2 g cm^{-3}. The bead concentration is 10 mg ml^{-1} in DMAC solution. According to the supplier (Thermo Fischer Scientific), bead binding capacity is equal or more 26 µg of rabbit IgG mg^{-1} of beads.

The beads are pipetted onto the EWOD chip with all the remaining reagents and the analyte before placing the top plate. Given that the bead diameter is significantly smaller than the shim/gap height (500 µm), the bead diameter would not influence the DMF assembly.

4. The volume of the generated droplet depends on the gap between the top plate and bottom plate, other than the size of electrodes. So if you say, the electrode can generate 1.5 µL to 3 µL volume of droplet, it is mandatory to explain the gap also.

The reviewer is correct, 1.5 µl is the minimum droplet size that can be actuated. It correspond roughly to the droplet (projected) countour being circumscribed to the square pad. However, there

isn't any theoretical limit in size other than caused by the resistance (friction) of the body of fluid. The recommended volume limit for reliable actuation using a 500 µm gap, as constated by the author, is 3 µl. Of course, as the reviewer rightfully pointed out, this actuatable range is modified by the gap thickness.

This clarification is included to the NOTE following 2.1 in page 4

5. It is hard to remove the bio-residues from chip fully after each experiment by using DI water only. So need more explanation on how to clean the chip?

Indeed it is very hard to remove the residues from the surface, which is discussed in the paragraph on biofouling (P.12, L 534). Currently, the DMF platform is utilised with chrome printed glass EWOD chip and intermediate cleaning with tissues and DI water. The chip reusability is limited and the surface may accumulate residues from previous runs. However, disposable consumables are investigated that can substitute the printed glass EWOD chip thus providing a pristine surface for each experiment (P.12, L.529-532).

6. In figure 2, the mixing zone is not shown. What are the steps taken for reducing the contamination of regents due to the overlapping of mixing paths?

In figure 2, current figure 1 in the manuscript, the mixing zone is outlined by a red line. Also, the caption of figure 1 has a list of the pads that comprise the mixing zone, to improve the readability.

Several steps are embedded in the chip design and the experimental protocol to reduce the contamination of reagents in a single run. In terms of design, (*i*) sperate loading electrodes are defined for each reagent/sample, (*ii*) path lengths to the mixing zone are minimised for droplets with critical reagents in them. The protocol is optimised such that there are no overlapping paths for two reactive droplets. For instance, Luminol/peroxide mixture is loaded onto pad No.35 (D), adjacent to the mixing zone; the enzyme carrying droplet is placed on pad No. 5 opposite to the mixing zone. And the two droplets come in contact with the beads on pad No.33, central for the mixing zone.

Independent experiments are followed by a thorough cleaning with DI water and lint-free tissue. And after 3 to 4 runs, depending on the working voltages, the EWOD chip is replaced.

7. Give more clarity on Figure 5. The separation of magnetic beads from reagents is not clear in the figure.

It was found that Figure 5 quality wasn't satisfactory as it hindered reader's understanding of the process. Figure 5 was remade with better quality images. Labels were added to the panels in figure 5 point out the separated beads.

8. Most of Figures have low resolution, need to offer higher resolution for publication.

Better quality images were provided and remade where necessary.

Reviewer #3:

The manuscript presents a protocol for a DMF based automated ELISA assay for the detection of different analytes. While this work sounds valuable, the manuscript lacks presenting enough information regarding the device and the operations. The manuscript could be acceptable following significant modifications. Bellow are some comments that I have for improving the manuscript and clarifying the missing information.

1- The full form of every abbreviation should be presented first they appear in the text. Samples are HRP in the Abstract and RT-PCR in the Introduction sections.

This comment was addressed. All abbreviations are written in full when they first appear in the text.

2- Figures are not addressed in the right order in the text. If figure 2 is addressed before figure 1 (also the rest of the figures) then their names must be switched.

Rearrangements were made to reference the figure in the correct order. Thank you for pointing out the issue.

3- In step 1.2 include further details about the temperature at which the conjugation is done, is the process static or involves shaking/rotation?

Clarification of the immobilisation conditions is available in Step 1.2.3.

P.3, L.134-137: Step 1.2.3 Bind the selected antibody (Anti-Human Serum Albumin [15C7] , Rb anti-BG polyclonal, Rb anti-*E.coli* MRE 162 polyclonal or Goat anti-MS2 polyclonal), 40 µg ml^{-1} in 67 mM Borate Buffer, covalently to the beads for 1 h with shaking at 37^{o}C. Table 1 contains the list of all antibodies used with the current protocol and the antigen pathogens [Coudron *et al.*, 2019].

4- For the case of storing the beads up to a month, is it necessary to add any material to the solution to avoid agglomeration or keeping the antibodies functional?

To avoid contaminations all solutions are handled in a sterile environment.

P.2, L.119-121: All preliminary steps must be conducted in a sterile environment to avoid contaminations. Sodium Azide should not be used for storage as it would inhibit the activity of the horseradish peroxidase (HRP) enzyme.

The beads are stored at 4C in Lysis/Wash buffer (P.3, L. 145: Step 1.2.7) without any modification of the solution. Should agglomeration occur, mild tapping of the vial should break the precipitate and resuspend the beads in solution. After one month the antibodies may be degraded, therefore freshly modified batch should be used.

5- In 2.1 the first NOTE, the electrodes are 1.7 by 1.7 mm. To achieve a 1.5 uL droplet on these electrodes, the height has to be around 500 um, and for 3 uL droplets it would be over 1 mm. It is well known that there is a height threshold (usually height/electrode size ratio) for successfully splitting droplets, which is typically an order of magnitude smaller than the electrode size. The authors must provide more details of the DMF chip design and measurements. They should also present the characterization of droplet handling, specifically splitting, using their system. Since the

device performs an entire detection assay, I suppose it involves multiple fluidic operation including splitting/dispensing.

The reviewer is correct, 1.5 µl is the minimum droplet size that can be actuated. It correspond roughly to the droplet (projected) countour being circumscribed to the square pad. However, there isn't any theoretical limit in size other than caused by the resistance (friction) of the body of fluid. The recommended volume limit for reliable actuation using a 500 µm gap, as constated by the author, is 3 µl. Of course, as the reviewer rightfully pointed out, this actuatable range is modified by the gap thickness. This clarification is included in the NOTE following 2.1 in page 4.

Concerning splitting, the author chose to work with a relatively large gap so that the droplet actuation isn't impacted by potential variability across in the gap thickness across the device. It is true that with the gap value used by the author, splitting is compromised, however, the current application doesn't require any splitting other than the extraction of the magnetic pellet (which isn't the kind of splitting the reviewer is referring to in his comment).

6- Since a grid layout is used in the electrode design for transparency, the image of the fabricated electrode must be presented. This could be an independent figure or as a panel in figure 2.

A new panel showing a micrograph of the microgrid design of the electrode has been added to the former figure 2, now figure 1

7- One big challenge in all DMF based assays is surface biofouling. I did not see any information regarding avoiding surface biofouling, specially because the solutions used in this work include proteins and peptides which are well known for their biofouling effects on hydrophobic surfaces. This must be clearly explained in the manuscript and the proof of the durability of the device should be presented.

Biomolecule adsorption is a serious hinderance. And the article discusses biofouling and acknowledges the shortcomings it implies on the DMF functionality as well as strategies to reduce it (P. 12, L.534-547). The durability of the chip and its limitations are shown in the first paragraph of the result section (P.8, L.388-395) and in Figure 3. Further in the discussion reusability of the EWOD chip is reported and best practices are recommended improving on DMF durability (P. 11, L.522-532).

8- Figure 1 is not informative. Figures are usually provided to show certain information visually. At least different components could be labeled to better explain the architecture of the device.

The comment of the reviewer was taken on board for all the figures in the article. Specifically, Figure 2, former Figure 1, now contains extra images with labelled components plus a detailed caption.

9- Since this manuscript presents a protocol, it is necessary to show the images of the actual samples being operated on the device. I recommend the authors add one figure and include a few representative sequences of the operation on the device, labelling the droplets of the samples and their movement direction through the detection process.

A new figure (7) presenting the overall on-chip protocole is proposed.

– End of reviewers' report –

To conclude, the authors would like to thank the reviewer once again for the careful evaluation of their work. We would like to acknowledge the Reviewer's evident expertise of the subject as we felt that a lot of the comments were very sound and sensible and, after we hope having addressed them all, we recognise that our manuscript is significantly improved.