Video Article

ampliPHOX Colorimetric Detection on a DNA Microarray for Influenza

Kevin R. Moulton, Amber W. Taylor, Kathy L. Rowlen, Erica D. Dawson
InDevR, Inc.

Correspondence to: Erica D. Dawson at dawson@indevr.com

URL: http://www.jove.com/details.php?id=2682
DOI: 10.3791/2682
Citation: Moulton K.R., Taylor A.W., Rowlen K.L., Dawson E.D. (2011). ampliPHOX Colorimetric Detection on a DNA Microarray for Influenza. JoVE. 52. http://www.jove.com/details.php?id=2682, doi: 10.3791/2682

Abstract

DNA microarrays have emerged as a powerful tool for pathogen detection.[1-5] For instance, many examples of the ability to type and subtype influenza virus have been demonstrated.[6-11] The identification and subtyping of influenza on DNA microarrays has applications in both public health and the clinic for early detection, rapid intervention, and minimizing the impact of an influenza pandemic. Traditional fluorescence is currently the most commonly used microarray detection method. However, as microarray technology progresses towards clinical use,[1] replacing expensive instrumentation with low cost detection technology exhibiting similar performance characteristics to fluorescence will make microarray assays more attractive and cost-effective.

The ampliPHOX colorimetric detection technology is intended for research applications, and has a limit of detection within one order of magnitude of traditional fluorescence[11], with a main advantage being an approximate ten-fold lower instrument cost compared to the confocal microarray scanners required for fluorescence microarray detection. Another advantage is the compact size of the instrument which allows for portability and flexibility, unlike traditional fluorescence instruments. Because the polymerization technology is not as inherently linear as fluorescence detection, however, it is best suited for lower density microarray applications in which a yes/no answer for the presence of a certain sequence is desired, such as for pathogen detection arrays. Currently the maximum spot density compatible with ampliPHOX detection is ˜1800 spots/array. Because of the spot density limitations, higher density microarrays are not suitable for ampliPHOX detection.

Here, we present ampliPHOX colorimetric detection technology as a method of signal amplification on a low density microarray developed for the detection and characterization of influenza viruses (FluChip). Although this protocol uses the FluChip (a DNA microarray) as one specific application of ampliPHOX detection, any microarray incorporating biotinylated target can be labeled and detected in a similar manner. The microarray design and biotinylation of the target to be captured are the responsibility of the user. Once the biotinylated target has been captured on the array, ampliPHOX detection can be performed by first tagging the array with a streptavidin-label conjugate (ampliTAG). Upon light exposure using the ampliPHOX Reader instrument, polymerization of a monomer solution (ampliPHY) occurs only in regions containing ampliTAG-labeled targets. The polymer formed can be subsequently stained with a non-toxic solution to improve visual contrast, followed by imaging and analysis using a simple software package (ampliVIEW). The entire FluChip assay from un-extracted sample to result can be performed in about 6 hours, and the ampliPHOX detection steps described above can be completed in about 30 min.

Protocol

1. Sample amplification using RT-PCR

1. Extract viral RNA from clinical material or a viral isolate using the Qiagen MinElute Virus Spin Kit in conjunction with the QIAcube automated nucleic acid extraction platform. Extractions are performed on 200 µl specimen with a final elution volume of 60 µl. Store extracts at -70°C or lower for later use.
2. In a template free-area, prepare the RT-PCR master mix on ice according to the manufacturer's protocol. To incorporate biotin during RT-PCR, use a biotinylated dNTP mixture in place of the manufacturer supplied dNTP mix. The cost of using a biotinylated dNTP mixture is less than $1 USD/assay. Alternative methods of biotin incorporation such as the use of a biotinylated primer can also be used. Add primer mixes at final concentrations of 1.0 µM for Flu A, 1.0 µM for Flu B, and 0.14 µM for the internal control. The Flu A primer set amplifies the matrix gene segment (1032 nt product) and the Flu B primer set amplifies a non-structural gene segment (811 nt product). Each FluChip primer set contains one primer with a 5' phosphoryl group to facilitate the generation of single stranded DNA via enzymatic digestion with lambda exonuclease following PCR. The resulting single stranded product requires much shorter hybridization times than the double stranded equivalent and significantly shortens the overall assay time. A pre-prepared mixture of these primers as well as the internal control template RNA are available from InDevR to interested researchers on a limited basis.
3. Briefly vortex and distribute 18 µl of the master mix into thin-walled PCR tubes.
4. Transfer tubes on ice to a suitable work area for template addition, and add 2 µl template to each reaction tube.
5. Transfer PCR tubes to a thermal cycler, and run the following thermal profile: reverse transcription at 50°C for 30 min, enzyme inactivation/activation at 95°C for 15 min, 40 PCR cycles of 95°C for 30 s, 55°C for 30s, and 72°C for 1 min, and a final extension at 72°C for 10 min.

2. Hybridization of RT-PCR products to low density microarrays

1. In order to generate single stranded DNA for FluChip hybridization, prepare the enzymatic digestion mixture by combining 1.0 µl lambda exonuclease enzyme, 2.2 µl of the accompanying reaction buffer, and 0.8 µl of nuclease-free water. These amounts are for a single sample, but can be simply scaled for the total number of samples to be digested. Remove samples from the thermal cycler and add 4 µl of the prepared mixture to each reaction to digest the phosphorylated strand of the PCR product. Return samples to the thermal cycler and program the thermal cycler to 37 degrees Celsius for 15 minutes followed by 95 degrees Celsius for 10 minutes to complete enzymatic digestion and heat fragmentation steps.

2. The custom influenza microarrays used are printed on aldehyde functionalized glass slides by Applied Microarrays Inc. (Tempe, AZ). 5'-amino terminated capture sequences are combined with an optimized spotting buffer and printed at a final concentration of 20 µM (except for the positive control sequence which additionally has a 3'-biotin modification and is spotted at a final concentration of 500 nM). A non-contact spotting method is used, with an optimal spot diameter of 300 µm and center to center pitch of 700 µm.
3. Remove microarrays from the storage box and apply single-use hybridization wells around the microarrays by removing the protective sheet and pressing firmly around well perimeter.
4. Place slides in a wash bin containing 110 ml of purified water for a 5 min pre-hybridization wash using an orbital shaker at 60-90 rpm. Dry the array by gently touching a tissue wiper to the edge of the well and allowing water to be wicked away.
5. Combine 22 µl of 2x Hybridization Buffer with each of the fragmented ssDNA products, and pipet 40 µl of the hybridization solutions into the microarray wells.
6. Allow slides to hybridize in closed humidity chamber for 60 minutes.
7. Remove the slides from the humidity chamber, briefly rinse arrays with Wash Buffer D in a rinse bottle before placing into the slide rack. Typically the rinsing volume of Wash Buffer D is 2 ml per array. Place slide rack containing slides in wash bin containing 110 ml Wash Buffer A. Place bin on orbital shaker at 60-90 rpm for 1 minute.
8. Remove slide rack from Wash Buffer A, briefly rinse with Wash Buffer D, transfer slide rack to a bin containing Wash Buffer B, and shake at 60-90 rpm for 5 minutes.
9. Gently dry the array on each slide, and place dried slides in humidity chamber in preparation for ampliPHOX Colorimetric Detection steps.

3. ampliPHOX: labeling hybridized product and calibration chips with ampliTAG

1. Combine 10 µl of ampliTAG, 20 µl of 2x ampliTAG buffer, and 10 µl of purified water for each array to be processed. Reagent volumes can be simply scaled for the total number of samples. Make sure to prepare enough labeling mixture to account for all sample arrays and calibration arrays needed. The number of calibration chips needed depends on whether you are performing a full calibration (for a new instrument or new lot of reagents), or just an instrument check. For a full calibration, 3 calibration chips should be labeled, and for a reagent check, just a single calibration chip should be labeled. Please note that before the calibration arrays are labeled, they should go through the pre-hybridization washing step that was previously described for the influenza arrays.
2. Transfer 40 µl of label mixture to each array and allow labeling reaction to proceed in a closed humidity chamber for 5 minutes.
3. Immediately rinse arrays with Wash Buffer D in a rinse bottle before placing slides in a slide rack. Transfer rack into bin containing 110 ml of Wash Buffer C, and shake at 60-90 rpm for 5 minutes.
4. Using a second wash bin filled with purified water, perform three consecutive brief water dips to remove salt residue. Dry the arrays by gently touching a tissue wiper to the edge of the wells.
5. The microarrays are now properly labeled with ampliTAG, and the remainder of the ampliPHOX detection procedure can be performed. Photoactivation and imaging of labeled arrays should be completed within 24 hours, and additional arrays can be stored in a dark slide box until use.

4. ampliPHOX: calibration, signal amplification, and imaging

1. Turn ampliPHOX reader on and ensure ampliVIEW software is ready for photoactivation.
2. Determine the optimal photoactivation time using calibration chips. In addition to the brief introduction that follows, this procedure is also outlined in detail in the ampliPHOX Operation Manual. The calibration chips contain a series of dilutions of a biotinylated control sequence that are utilized to optimize the sensitivity of the assay, with the goal of maximizing the number of rows of the calibration chip that produce a positive signal as determined by the software.
3. Remove ampliPHY from 4°C, allow to warm to room temperature, and vortex briefly to mix. Pipet 3 µl of ampliPHY enhancer into the ampliPHY vial, and vortex thoroughly for 10 seconds.
4. Evenly transfer 40 µl of ampliPHY solution into the microarray well containing the ampliTAG-labeled array, ensuring that no bubbles are present. Close the ampliPHY vial between each application. Insert the microarray slide into the photoactivation bay of the ampliPHOX Reader.
5. For the first calibration array, use the default photoactivation time in the 'Time' box on the lefthand panel of the software, and click the green 'Start' button to start the photoactivation. Once completed, remove the array and rinse with purified water to remove excess ampliPHY. Clear polymer formation on some of the spots should now be visible.
6. Allow the polymer spots to dry for 2 minutes, then distribute two drops of ampliRED onto the array and allow staining to proceed for 2 minutes.
7. Next, quickly rinse microarray with purified water and dry with a tissue wipe.
8. Insert microarray into the imaging bay of the ampliPHOX Reader, and click the 'Capture New Image' button in the Imaging tab. Once imaged, adjust the crop and save the image.
9. In the Analysis tab, select the Calibration Chip mask and the 'Auto Placement' button to start the analysis. The software will automatically produce a 'Summary Record' showing the quantified results. Based on these results, the photoactivation time is adjusted by 10 seconds for the second calibration array, and the procedure is repeated.
10. Once the optimal photoactivation time has been determined, the sample arrays can be processed using the same signal amplification and imaging protocol used for the calibration arrays.
11. Once the image is captured for the sample arrays, a mask for your particular array layout such as the influenza array layout described here can be created.The ampliVIEW software is capable of performing automated image analysis and generating influenza subtyping results for each sample.

5. Representative Results:

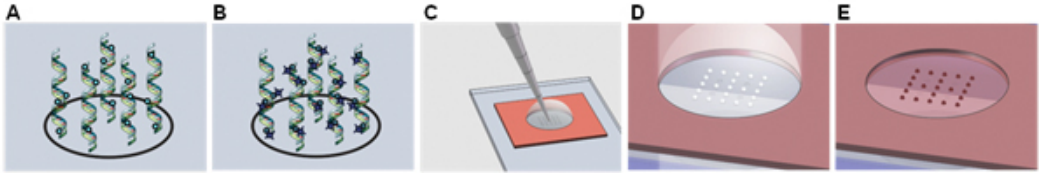

Figure 1. Schematic illustration of the ampliPHOX colorimetric detection method. (A) Biotinylated target DNA is hybridized to each spot in the array, and (B) labeled with ampliTAG. (C) ampliPHY solution is then added, and (D) exposed to light to form visible polymer spots. (E) The polymer spots formed are subsequently stained with a non-toxic dye to improve contrast.

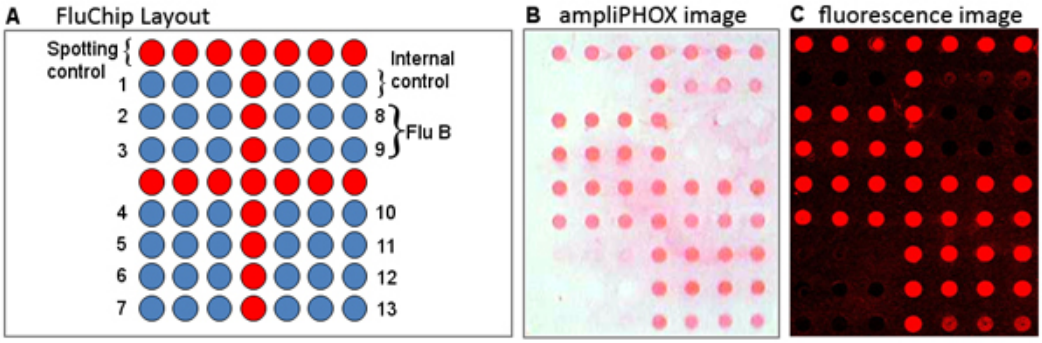

Figure 2. (A) Influenza low density microarray layout. Sequences 1-7 and 10-13 target influenza A, and sequences 8, 9 target influenza B. (B) ampliPHOX and (C) fluorescence images of a 2009 novel H1N1 ('swine flu') specimen showing the same detection pattern by both methods.

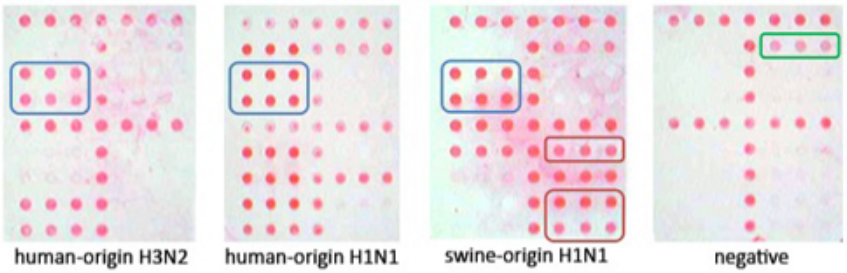

Figure 3. From left to right, representative ampliPHOX images for influenza A H3N2, human-origin H1N1, 2009 novel H1N1 (swine-origin), and a negative specimen. All 3 subtypes show visually distinct patterns on the array. Notice in the negative that only the MS2 internal control is seen, indicating the RT-PCR amplification was not inhibited.

Discussion

The ampliPHOX colorimetric detection technology presented here is a rapid, inexpensive alternative to single color fluorescence detection for lower density microarray applications. Shown schematically in **Figure 1**, the detection principle is based on the use of a photoinitiator label (1B). In the presence of a monomer-containing solution (1C), light exposure causes the photoinitiator (ampliTAG) to trigger a polymerization reaction only in labeled regions (1D). Although demonstrated here on a DNA array for influenza identification and subtyping, the technology can be extended to detect any biotinylated product captured on a microarray. As an example, our earlier work on photopolymerization-based detection using a different reagent chemistry was demonstrated on both nucleic acid and antibody-based arrays.[12] Others have also shown proof of concept for antibody and protein-based applications of a photopolymerization-based system. In these cases, different reagent chemistries requiring purging with an inert gas were utilized.[13,14]

A schematic of the influenza low-density microarray and representative images can be seen in **Figure 2**. Shown in Figure 2A, the array contains a spatial marker/spotting control (in red) and 14 unique capture sequences (in blue), each spotted in triplicate. The capture sequences are amino-terminated synthetic short (˜25 mer) oligonucleotides designed to capture influenza targets that are genetically representative of specific types or subtypes of influenza. The capture sequences are designed to produce distinctly different patterns on the chip for different influenza A subtypes. Figures 2B and 2C show a direct comparison of the detection of a 2009 novel H1N1 specimen by ampliPHOX and traditional fluorescence, respectively. The fluorescence result was generated using a confocal fluorescence microarray scanner (Genetix aQuire). The same overall detection pattern can be easily seen for both methods, however, the ampliPHOX result is visible to the naked eye.

Shown in **Figure 3** are ampliPHOX results from three representative influenza A positive specimens and one negative specimen as processed by the protocol described here. Figures 3A, 3B, and 3C show results for a human-origin H3N2, human-origin H1N1, and swine-origin H1N1 (2009 novel H1N1), respectively. A distinct difference in the overall detection pattern can easily be seen for these 3 images. For instance, it can be seen that sequences 2 and 3 produce signal for all of the influenza A subtypes shown (3A-3C), but that sequences 10, 12, and 13 only produce signal for the swine-origin H1N1 specimen (3C). This approach has been used previously to successfully type and subtype influenza viruses on a microarray.[6,8,10] Importantly, the negative specimen shown in Figure 3D shows signal on internal control sequence, indicating no inhibition or failure of the RT-PCR reaction.

The interpretation of these images is easily automated by the ampliVIEW software. The user first uses the software to define the microarray layout, and then to describe which targets should be present to generate a certain "answer" for this layout. For example, the influenza layout shown in Figure 2A could generate logical results such as "Flu A positive", "Flu B positive", "non-seasonal influenza A", "seasonal influenza A", and "negative", depending on the desired outcomes. Once these logical assignments are made and saved, the software can automatically interpret the images to provide a result with little user input.

ampliPHOX detection technology generates visual, colorimetric results for lower density microarrays with similar sensitivity to fluorescence in minutes. We believe the combination of easy-to-use, inexpensive reagents with a low-cost instrument provides an attractive alternative to traditional microarray detection methods, particularly as more targeted, lower density genomic/diagnostic microarrays become increasingly used in a variety of applications.

Disclosures

All of the authors are employees of InDevR, Inc., a for-profit entity. InDevR is commercializing the ampliPHOX technology described herein.

Acknowledgements

InDevR acknowledges NIH/NIAID U01AI070276 and R43AI077112 for funding this work.

References

1. Kumar, R.M. The Widely Used Diagnostics "DNA Microarray"-A Review. *Amer J Inf Dis* **5**, 207-218 (2009).
2. Miller, M.B., Tang, Y.W. Basic Concepts of Microarrays and Potential Applications in Clinical Microbiology. *Clin Microbiol Rev* **22**, 611-633 (2009).
3. Mikhailovich, V., Gryadunov, D., Kolchinsky, A., Makarov, A.A., Zasedatelev, A. DNA microarrays in the clinic: infectious diseases. *BioEssays* **30**, 673-682 (2008).
4. Call, D.R. Challenges and opportunities for pathogen detection using DNA microarrays. *Crit Rev Microbiol* **31**, 91-99 (2005).
5. Raoult, D., Fournier, P.E., Drancourt, M. What does the future hold for clinical microbiology? *Nat Rev Microbiol* **2**, 151-159 (2004).
6. Dawson, E.D., Rowlen, K.L. "MChip: A Single Gene Diagnostic for Influenza A", in *Influenza: Molecular Virology*, Wang, Q. and Tao, Y. J., eds. Norfolk, UK, Caister Academic Press (2010).
7. Gall, A., Hoffman, B., Harder, T., Grund, C., Ehricht, R., Beer, M. Rapid hemagglutinin subtyping and pathotyping of avian influenza viruses by a DNA microarray. *J Virol Meth* **160**, 200-205 (2009).
8. Townsend, M.B., Dawson, E.D., Mehlmann, M., Smagala, J.A., Dankbar, D.M., Moore, C.L., Smith, C.B., Cox, N.J., *et al*. FluChip: Experimental evaluation of a diagnostic influenza microarray. *J Clin Microbiol* **44**, 2863-2871 (2006).
9. Wang, Z., Daum, L.T., Vora, G.J., Metzgar, D., Walter, E.A., Canas, L.C., Malanosky, A.P., Lin, B., Stenger, D.A. Identifying influenza viruses with resequencing arrays. *Emerg Inf Dis* **12**, 638-646 (2006).
10. Kessler, N., Ferraris, O., Palmer, K., Marsh, W. and Steel, A. Use of the DNA Flow-Thru Chip, a three-dimensional biochip, for typing and subtyping of influenza viruses. *J Clin Microbiol* **42**, 2173-2185 (2004).
11. Kuck, L.R., Taylor, A.W. Photopolymerization as an innovative detection technique for low-density microarrays. *Biotechniques* **45**, 179-186 (2008).
12. Avens, H.J., Bowman, C.N. Development of fluorescent polymerization-based signal amplification for sensitive and non-enzymatic biodetection in antibody arrays. Acta Biomat 6, 83-89 (2010).
13. Sikes, H.D., Jenison, R., Bowman, C.N. Antigen detection using polymerization-based amplification. *Lab on a Chip* **9**, 653-656 (2008).

