Video Article

Multiplex Detection of Bacteria in Complex Clinical and Environmental Samples using Oligonucleotide-coupled Fluorescent Microspheres

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Abstract

Bacterial vaginosis (BV) is a recurring polymicrobial syndrome that is characterized by a change in the "normal" microbiota from *Lactobacillus*-dominated to a microbiota dominated by a number of bacterial species, including *Gardnerella vaginalis*, *Atopobium vaginae*, and others[1-3]. This condition is associated with a range of negative health outcomes, including HIV acquisition[4], and it can be difficult to manage clinically[5]. Furthermore, diagnosis of BV has relied on the use of Gram stains of vaginal swab smears that are scored on various numerical criteria[6,7]. While this diagnostic is simple, inexpensive, and well suited to resource-limited settings, it can suffer from problems related to subjective interpretations and it does not give a detailed profile of the composition of the vaginal microbiota[8]. Recent deep sequencing efforts have revealed a rich, diverse vaginal microbiota with clear differences between samples taken from individuals that are diagnosed with BV compared to those individuals that are considered normal[9,10], which has resulted in the identification of a number of potential targets for molecular diagnosis of BV[11,12]. These studies have provided a wealth of useful information, but deep sequencing is not yet practical as a diagnostic method in a clinical setting. We have recently described a method for rapidly profiling the vaginal microbiota in a multiplex format using oligonucleotide-coupled fluorescent beads with detection on a Luminex platform[13]. This method, like current Gram stain-based methods, is rapid and simple but adds the additional advantage of exploiting molecular knowledge arising from sequencing studies in probe design. This method therefore provides a way to profile the major microorganisms that are present in a vaginal swab that can be used to diagnose BV with high specificity and sensitivity compared to Gram stain while providing additional information on species presence and abundance in a semi-quantitative and rapid manner. This multiplex method is expandable well beyond the range of current quantitative PCR assays for particular organisms, which is currently limited to 5 or 6 different assays in a single sample[14]. Importantly, the method is not limited to the detection of bacteria in vaginal swabs and can be easily adapted to rapidly profile nearly any microbial community of interest. For example, we have recently begun to apply this methodology to the development of diagnostic tools for use in wastewater treatment plants.

Video Link

The video component of this article can be found at https://www.jove.com/video/3344/

Protocol

This method was used in the research reported in Dumonceaux *et al. J. Clin. Microbiol.* 47, 4067-4077, doi:10.1128/jcm.00112-09 (2009).

A schematic diagram depicting the overall procedure is presented in Figure 1.

1. Bead coupling

This describes the methods to be used for coupling oligonucleotide probes to polystyrene Luminex beads (see Table 2). Volumes are adapted slightly for evaluation of new capture probes on a trial basis; these volumes are indicated in parentheses.

1. Remove 1-Ethyl-3-(3-dimethylamiopropyl) carbodiimide HCl (EDC) powder from -20°C dessicator and warm it to room temperature.
2. Resuspend the microspheres by sonciation in a waterbath sonicator for 20 seconds, then vortexing approximately 20 seconds.
3. Transfer 400 µl (100 µl) of microspheres to an Eppendorf tube (this corresponds to $5x10^6$ or $1.25x10^6$ microspheres). Luminex suggests the use of low protein binding microcentrifuge tubes (e.g. Eppendorf Protein LoBind tubes, catalog number 0030 108.094) to prevent the uncoupled beads from sticking to the tubes and interfering with bead recovery. We have not found this to be a problem using standard polypropylene microcentrifuge tubes, which we typically use (e.g. VWR catalog number 87003-298).
4. Pellet at 14000 x g for 1 minute. Remove and discard the supernatant.
5. Resuspend the microspheres in 50 µl (12.5 µl) of room-temperature 0.1 M 2-(N-Morpholino)ethanesulfonic acid (MES) pH 4.5.
6. Prepare a fresh solution of EDC at 10 mg/ml in water.

1. Prepare less than 1 ml of this solution by weighing 5-10 mg of EDC on an analytical scale, then adding water to 10 mg/ml.

7. Add 1 nmol of 5'-amino C12-modified capture oligonucleotide (Table 2) to microspheres and mix by vortexing. 1 nmol is 5 µl of 200 µM oligonucleotide.
8. Add 2.5 µl of fresh EDC solution to the microspheres and mix by vortexing for 5 seconds.
9. Incubate at room temperature for 30 minutes in the dark.
10. Discard the EDC solution (step 1.6) and prepare a fresh sample of 10 mg/ml EDC in water as above (step 1.6).
11. Add another 2.5 µl of fresh EDC solution to the microspheres and vortex for 5 seconds.
12. Incubate at room temperature for 30 minutes in the dark.
13. Wash the beads by adding 1 ml of 0.02% Tween 20. Vortex (optionally sonicate for 20 seconds as well) to resuspend the beads.
14. Centrifuge 14000 x g 1 minute. Remove and discard the supernatant.
15. Wash the beads again by adding 1 ml of 0.1% sodium dodecyl sulfate (SDS). Vortex (optionally sonicate for 20 seconds as well) to resuspend the beads.
16. Centrifuge 14000 x g 1 minute. Remove and discard the supernatant.
17. Resuspend the beads in 100 µl (25 µl) of Tris-EDTA (TE) buffer [10 mM Tris-Cl pH 8.0, 1 mM EDTA, pH 8.0].
18. Enumerate the beads in a haemocytometer or Coulter counter to determine the stock concentration.
19. Prepare a Microsphere Master Mix by diluting each bead to a final concentration of 100 beads/µl in TE buffer. Pool coupled beads corresponding to the desired plex of the assay (e.g. for a 10-plex assay, mix 10 different coupled beads at a final concentration of 100/µl of each bead).
20. Store the Microsphere Master Mix at 4°C in the dark. The mixture can be stored for months if kept under these conditions.

2. Chaperonin 60 universal target (cpn60 UT) amplicon production and generation of single strands.

1. Generate polymerase chain reaction (PCR) product for each sample. Include the phosphorothioate- and biotin-modified 5' primer set (Table 1). See Table 3 for volumes to mix and concentrations.
2. Immediately after the PCR is complete, add 2 µl (20 units) of T7 exonuclease to each PCR tube (the PCR buffer will suffice for the T7 reaction). Incubate the reaction at room temperature (~22-25°C) for 40 minutes.
3. At the end of this incubation, add 12.5 µl of 0.5 M ethylene diamine tetraacetic acid (EDTA) pH 8.0 and mix. This gives a total of ~64.5µl of single-stranded PCR product.

3. Hybridization of single-stranded PCR product to oligonucleotide-coupled polystyrene beads.

1. Prewarm the instrument to 60°C to maintain hybridization temperature during analysis. Turn on the platform heater using the instrument's software and be sure to use the brass heating block that fits the PCR-style plate that contains the hybridized bead mixture.
2. Resuspend Microsphere Master Mix (step 1.20) with a pipette, dispense an appropriate amount into an Eppendorf tube, cap the tube, and sonicate in a waterbath sonicator for 2 minutes. Alternatively, to ensure absolute consistency in bead resuspension, the microsphere master mix can be resuspended by vortexing and pipetting, then sonication as above, then dispensing the desired amount into a polypropylene Eppendorf tube.
3. Dispense 17 µl of single-stranded PCR product (step 2.2) into the appropriate wells of a low-profile 96-well Thermowell PCR plate (Table 2). Add 33 µl of resuspended, sonciated bead mixture to each well. Cover with silicone cover (Table 2) and tap gently.
4. Put the plate into the thermocycler with a program of: 95°C for 5 min, 60°C for 10 min, 60°C hold, 60°C for 5 min, end. Start the program.
5. Make fresh streptavidin-phycoerythrin (SA-PE) solution; you will need 25 µl per well (make several wells extra). Make SA-PE solution by diluting stock SA-PE (1 mg/ml) 1:50 to 20 µg/ml with 1x tetramethylammonium chloride (TMAC) buffer (3 M TMAC; 0.1% Sarkosyl; 50 mM Tris-HCl, pH 8.0; 4 mM EDTA, pH 8.0).
6. When the thermocycler gets to the 60°C hold step, open the lid, remove the silicone cover and add SA-PE solution directly to each well (DO NOT take the plate out of the thermocycler). Replace the silicone cover, close the thermocycler lid and resume the program.
7. When the program is complete, take the plate out and quickly transfer it to the BioPlex machine to read. Plate must be read within 10 minutes. Read at 60°C; ensure that the BioPlex has been prewarmed to this temperature. Be certain that the probe height has been adjusted to accommodate the plate utilized, as described in the user's manual for the instrument used.
8. For accurate bead and signal detection, the gate settings on the BioPlex must be set according to the type of microspheres being used. BioRad polystyrene beads require a gate setting of 4,335-10,000 while magnetic microspheres require a setting of 5,000-25,000. There is also the option of running the plate using the High PMT setting which increases the reporter gain value. This can increase the intensity of lower signals however it is important include an appropriate negative control as background signal will also be increased.

4. Representative results:

One of our goals in developing and implementing this assay was to make it as simple and streamlined as possible. We therefore optimized the critical post-amplification steps, including T7 exonuclease treatment time and hybridization time in order to develop an assay that can be completed in a reasonable time frame. As shown in Figure 2A, T7 treatment of the amplicon is essential for signal generation, as most probes had little or no signal with short or no T7 treatment. The signal increased linearly until approximately 40 minutes, at which time the signal increase slowed. No degradation of the signal was observed even at 2 hours of T7 treatment time, indicating that the phosphorothioate modification of the primers is highly effective in preventing target strand degradation as described[15]. We chose 40 minutes for T7 treatment time to minimize the overall protocol time, but it is clear from Figure 2A that T7 treatment can go on for much longer. We also determined the effect of hybridization time on signal generation (Figure 2B) and found that 10 minutes was sufficient for maximum signal, as no further increase in signal was observed even after 4 hours of hybridization. Therefore, a 10 minute hybridization step was chosen, again to minimize the overall

assay time. With these results in mind, and with rapid DNA extraction techniques such as InstaGene (Bio-Rad), the overall assay including DNA extraction, PCR, and Luminex analysis can be completed in 4-5 hours.

On a cautionary note, the level of aggregation of the polystyrene beads during a Luminex or BioPlex run can have a major impact on the efficiency of the assay. The BioPlex software will display the level of bead aggregation, which occurs when more than one bead is detected in the laser path and results in the exclusion of the hybridization signal from the aggregate. Apparent bead aggregation can also be caused by any particulate matter that is not the proper size for a single bead, and can even be caused by air bubbles. In any of these events, the signal from the bead aggregate, air bubble, or particle is discarded. In most cases, we find that bead aggregation is minimal (Figure 3A) and the targeted level of 100 counting events for each bead is easily achievable. Occasionally, however, the beads show a moderate (Figure 3B) or severe (Figure 3C) level of aggregation. In these cases, since most of the data is discarded, the instrument may have trouble reaching 100 events per bead type and the results may be questionable. The sonication step (step 3.2) is meant to minimize bead aggregation. In addition, storing the beads as a dilute microsphere master mix (step 1.20) may help. We have noticed that excluding TMAC from the hybridization buffer – adding it to the SA-PE diluent instead (step 3.5) – helps to minimize bead aggregation. Moreover, while we have not tested this, the newer magnetic beads available from Luminex or BioRad are thought to display less of a tendency to aggregate.

The results of the application of a 5-plex Luminex array targeting *G. vaginalis*, *A. vaginae*, *L. iners*, *L. crispatus*, and *L. gasseri* along with corresponding Gram stained vaginal swab smears are shown in Figure 4. These samples were taken from a single individual at multiple time points. At time 0, the individual was diagnosed with BV based on Gram stain (Figure 4A) and the Luminex results from this same sample (Figure 4B) show that of the organisms represented in the array, *G. vaginalis* was most prevalent, while *A. vaginae* and *L. iners* were also positive. Nine days later, the individual was still BV positive and the signal for *G. vaginalis* had increased substantially while *A. vaginae* and *L. iners* were still positive. Importantly, after this time the individual began to transition to a normal microbiota as Gram positive bacilli became detectable in the smears (Figure 4A) while the signal for *G. vaginalis* waned and the signal for *L. iners* increased (Figure 4B). By our original definition of BV with this method (samples positive for *G. vaginalis* and/or *A. vaginae* were considered BV positive)[13] , this individual was BV positive at all time points, although the Gram stain failed to detect *G. vaginalis* in the latter two time points. With the Luminex method described here, trends can be easily compared to sequencing-based methods, and the results of the Luminex assay typically corroborate Gram stains[13] while providing additional information on organism identity and abundance.

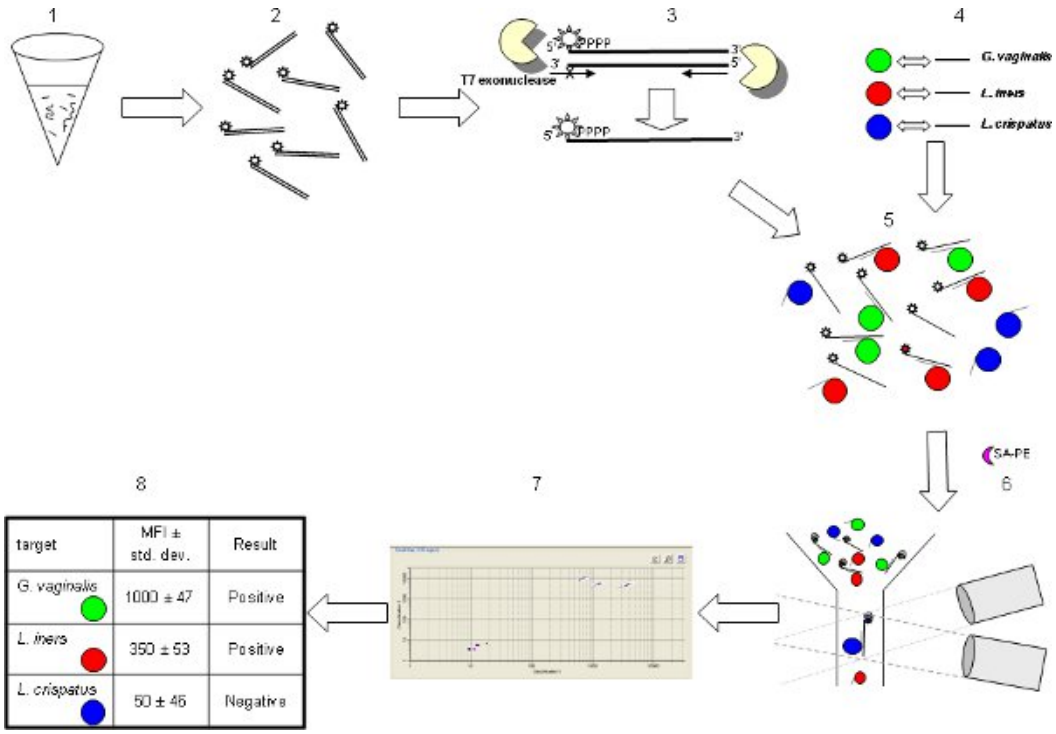

Figure 1. Schematic diagram of the Luminex protocol for determining the microbiota profile of a complex clinical or environmental sample. The protocol starts with template DNA that has been extracted from the sample of interest. (1) Generate PCR product from template DNA using strand-specific biotinylated, phosphorothioate-modified *cpn60* UT PCR primers coupled with unmodified *cpn60* UT PCR primers (Table 1); (2) This generates a PCR product pool representing the microbiota with the biotin-phosphorothioate modification on one strand; (3) Digest the double-stranded PCR product with T7 exonuclease, which cannot degrade the phosphorothioate-modified strand and therefore generates single-stranded DNA that is modified at the 5' end with biotin; (4) Couple polystyrene beads to species-specific *cpn60* UT probes – each bead has a unique spectral address (indicated by bead color) that is discernable by the Luminex or Bio-Plex instrument; (5) Hybridize the single-stranded PCR product generated from the sample of interest to the suite of species-specific oligonucleotide-coupled beads; (6) Add streptavidin-phycoerythrin conjugate that binds to the biotinylated single-stranded PCR product and acts as an indicator of hybridization; (7) Determine the spectral address (bead identity) and hybridization signal intensity using a Luminex or Bio-Plex instrument. At least 100 beads are counted for each bead identity and the median fluorescence intensity (MFI) of the phycoerythrin signal is reported as the output. Up to 100 different bead types can be evaluated simultaneously but an assay showing the discrimination of three bead types is illustrated. (8) When the MFI of PCR replicates generated from the same sample is significantly greater than the negative control for a given bead (one-tailed student's t-test, $p<0.05$), the sample is considered to be positive for that organism.

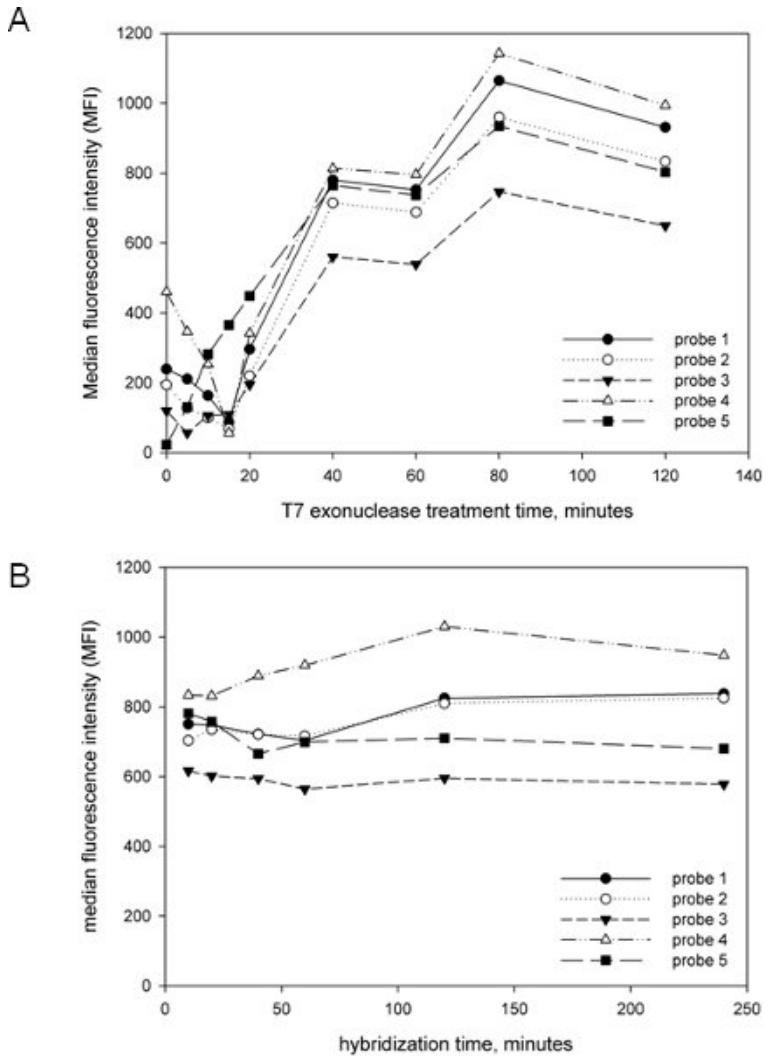

Figure 2. Optimization of Luminex assay parameters. Five different probes targeted to *Peptostreptococcus anaerobius* were used with amplicons generated from a mixed template comprising plasmids containing the cloned *cpn60* UT of 20 bacteria known to be associated with the vagina, including *P. anaerobius*. (A). Effect of T7 exonuclease treatment time. The protocol described above was followed but the time of treatment with T7 exonuclease was varied prior to hybridization and the median fluorescence intensity (MFI) was determined for all probes. (B). Effect of hybridization time. The protocol described above was followed with a variety of hybridization times. The same sets of probes were used with an amplicon generated from the same template as in A and the median fluorescence intensity (MFI) was determined for all probes.

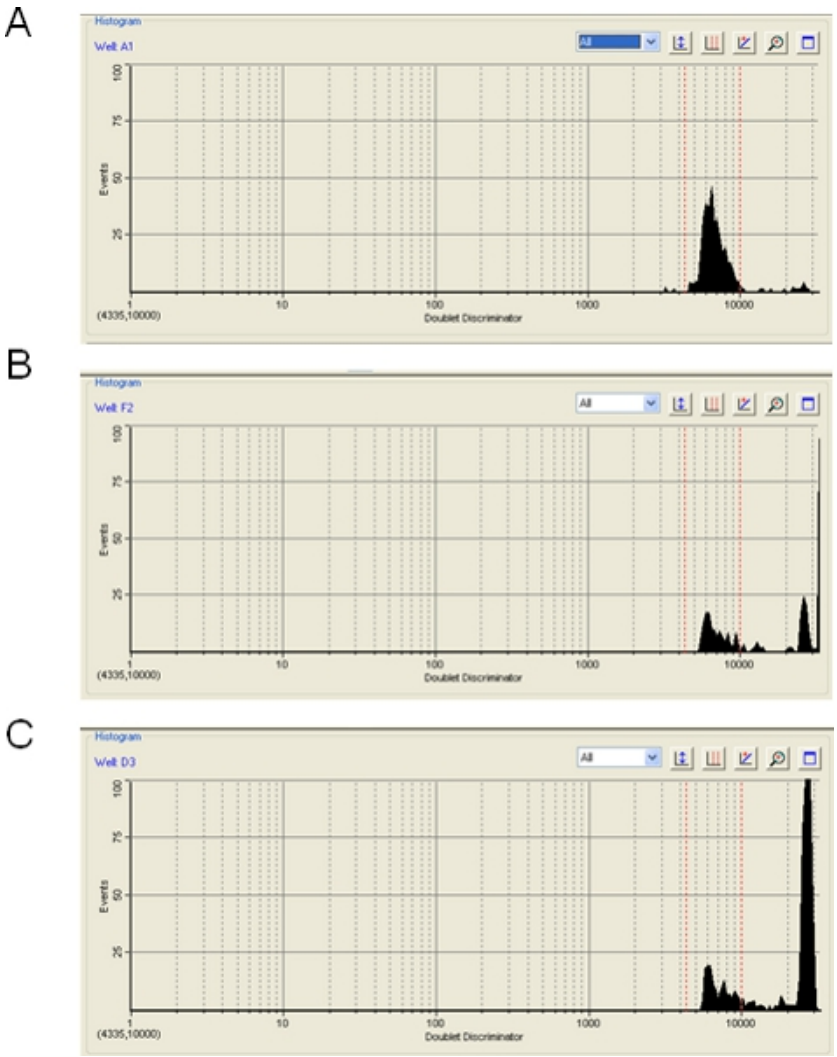

Figure 3. Determination of bead aggregation using BioPlex software. Three examples are given of BioPlex runs in which the level of bead aggregation is (A) acceptable (5%); (B) borderline (50%); and (C) unacceptable (80%).

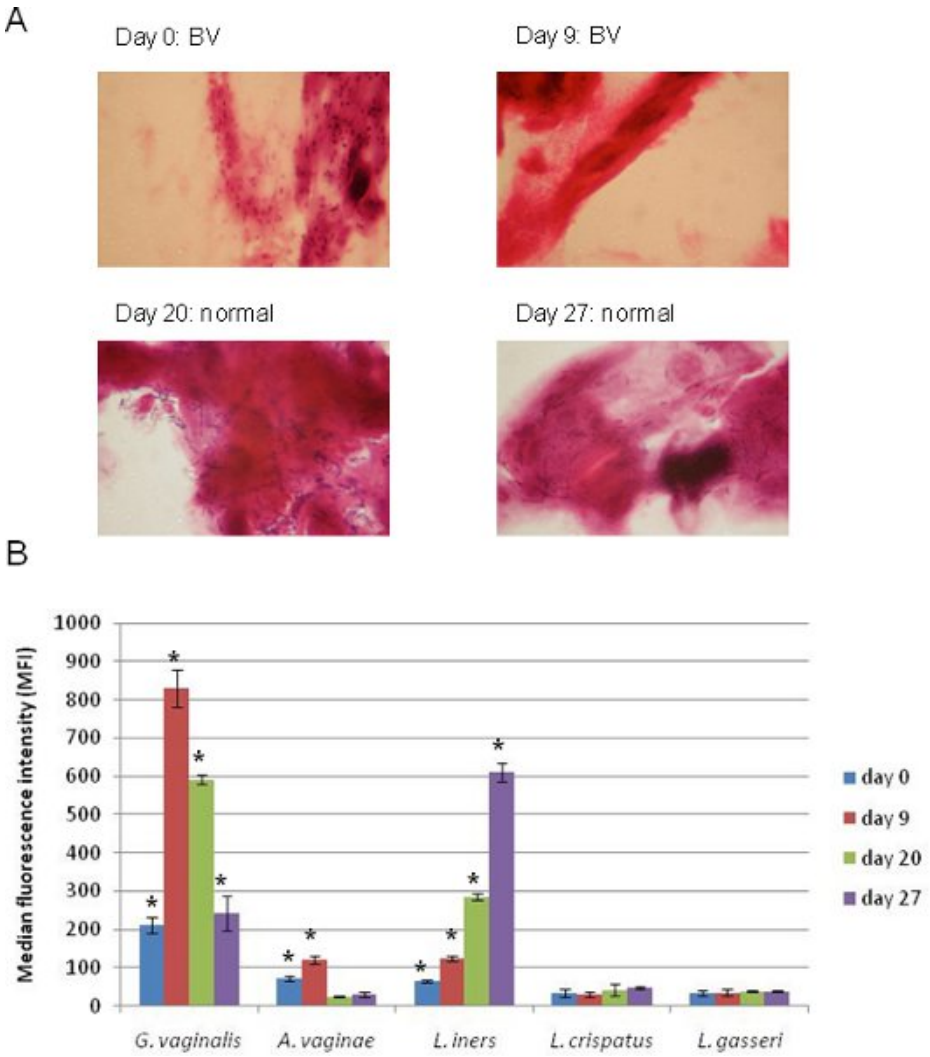

Figure 4. Application of a 5-plex Luminex array to BV diagnosis in sequential samples taken from the same individual. (A). Gram-stained slides showing the traditional diagnostic used to assess each sample for BV. (B). Application of a 5-plex Luminex array prepared and executed as described in this protocol to the same four samples shown in (A). Bacterial targets whose MFI signal was significantly positive by our definition (one-tailed student's *t*-test, $p<0.05$) are indicated by an asterisk (*).

Discussion

The specificity of signal generation is of critical importance; you must be confident that the signal observed truly reflects the detection of amplicon generated from that organism. Software such as PrimerPlex (Premier Biosoft) can help to design probes that will hybridize efficiently, but they may or may not cross-hybridize to non-target species. When universal PCR primers are used as described in this protocol, it is important to keep in mind that the amplicon generated represents all of the organisms present in the sample. It is important, therefore, to analyze potential probes for the possibility of cross-hybridization by comparing the sequences suggested by the probe design software to the cpnDB, a database of *cpn60* UT sequences[16]; probes that match multiple organisms are to be avoided if possible. Furthermore, when potentially specific probes are identified, we have taken an approach to probe validation in which a complex, artificial template is prepared corresponding to the cloned *cpn60* UT of a number of organisms associated with the microbiota of interest[13]. Amplicon is then generated from the complete "mixed panel", including the target template, and the signal is compared to that generated from a second template comprised of the mixed panel that does not contain the target organism's *cpn60* UT. Probes that generate a high signal-to-noise ratio (where the signal is generated from the complete panel and the noise is generated from the panel without the target template) are selected for inclusion in the final assay. We have typically analyzed 4-5 possible probes for each target organism before selecting one for inclusion in the final Luminex array.

The definition of a positive result is an issue to be considered. We have found that different probes have inherently different signal-to-noise ratios, so we decided on an approach whereby a positive result is defined by a very simple statistical test: the student's *t*-test (one-tailed). A positive result is defined by comparing the MFI of the sample to the "no template" control, and when the MFI is significantly higher than the control at $p < 0.05$, the result is considered to be positive for that organism. Normally two independently generated amplicons are each tested in duplicate. Although the PCR and T7 exonuclease steps create about 64.5 µl of single-stranded PCR product (step 2.3) and only 17 µl of this is used for hybridization (step 3.2), sufficient for 3 hybridization assays, we normally analyze duplicates from two amplifications to give an inherent technical replicate without using an excessive number of wells.

An aspect of this assay that we have investigated is its semi-quantitative nature. The MFI signal from a given probe correlates well to the qPCR-determined abundance of a given organism (Spearman rank correlation coefficient $\rho = 0.4686$, $p < 0.0001$)[13], but the dynamic range is limited; the signal tends to saturate at higher target organism levels, as expected since amplicon is analyzed after end-point PCR of 40 cycles. Because it is semi-quantitative, trends in organism abundance can be monitored and patterns observed (keeping the caveat in mind regarding signal saturation at higher abundances). For example, Figure 4 shows a profile of the vaginal microbiota of an individual over time as it changes from BV to normal. As this occurs, the signal corresponding to *G. vaginalis* (a BV-associated organism[17]) increases and then wanes, while over the course of the monitoring time the signal for *L. iners* increases. Corresponding Gram stains, the current "gold standard" for BV diagnosis, show a pattern whereby Gram positive bacilli are initially absent but are detectable at later time points. The Luminex method both identifies the organisms present in the sample and gives some information as to their relative abundances. This type of information could potentially be used to monitor samples of any type for undesirable trends in the abundances of particular organisms and can provide this data for a large number of organisms simultaneously.

Importantly, this method is not limited to the profiling of vaginal microbiota and can reasonably be applied to any environment in which the detection and partial quantification of a range of target organisms is desirable. When applying the method to other environments, it is important to consider the possibility of PCR inhibition occurring, as inhibitors of PCR commonly co-purify with DNA from many environments[18]. Where PCR inhibition is an issue, the sample can either be diluted or further purified in order to generate sufficient signal for this assay.

Disclosures

No conflicts of interest declared.

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