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Technical note

A multiplexed assay to detect antimicrobial peptides in biological fluids and cell secretions

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ABSTRACT

Mucosal tissues represent the front line in defense against potential pathogens, and one means by which mucosa provide protection is via the secretion of antimicrobials which can interfere with potential pathogens as well as recruit and modify the responses of immune cells. Here we describe adaptation of ELISA assays to microsphere format, facilitating simultaneous quantification of antimicrobial peptides including elafin, MIP3α, HBD2, HBD3, SLPI, RANTES, SDF1, lactoferrin, LL-37, and HNP1-3. The multiplexed assay exhibits excellent reproducibility, shows linearity over a two order of magnitude concentration range for most analytes, is compatible with biological fluids such as cervicovaginal lavage fluid, and presents significant cost and sample savings relative to traditional ELISA assays.

1. Introduction

Mucosal tissues are susceptible portals of entry for many human pathogens that are a major cause of infectious disease worldwide (Woodrow et al., 2012). The female reproductive tract (FRT) alone is vulnerable to infection from over 20 sexually transmitted pathogens including *N. gonorrhoeae*, *C. trachomatis*, HSV and HIV among others (Wira et al., 2011a, 2011b). Development of prevention and treatment methods for sexually transmitted infections remains a challenging area of research.

The mucosal epithelium of the gastrointestinal, upper respiratory, nasopharyngeal, mammary, lacrimal, and FRT tracts can actively combat infection by pathogens by secreting antimicrobial peptides as well as cytokines and chemokines that act as anti-microbial agents (reviewed in McGhee and Fujihashi, 2012). Collectively, these antimicrobial factors have a wide range of effects; RANTES, SDF1, HNP1-3, HBD2-3, SLPI and elafin are effective against HIV-1, the causative agent of AIDS, as well as bacteria, fungi, and other enveloped viruses (Schneider et al., 2005; Wiesner and Vilcinskas, 2010). Importantly, antimicrobial peptides are often multifunctional, performing anti-pathogen as well as chemotactic and other necessary homeostatic functions such as recruiting effector cells directly or indirectly by stimulating chemokine or cytokine production (Yang et al., 2004). Additionally, secreted AMPs can perform their functions while sparing essential commensal flora, such as *L. crispatus* (Wira et al., 2011a, 2011b).

The detection and quantification of antimicrobial factors in biological fluids are important for identification and characterization of a variety of anti-pathogenic effects, and can be used to elucidate the cellular origin of these factors. The most common method of analyzing antimicrobial in secretions from the FRT and other mucosal tissues is by ELISA (Fahey et al., 2005). Although this method performs reasonably well, it requires a large sample size, can measure only

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one factor at a time, and is relatively costly. Further, despite the availability of multiplex assays for some cytokines and chemokines, to the best of our knowledge, no platform has been developed that is dedicated to measuring antimicrobials in biological fluids and cell secretions.

We report the development of a customized multiplex microsphere assay that permits simultaneous detection of multiple antimicrobials from FRT-derived secretions that are known to inhibit HIV. Our method performs comparably to or better than ELISA, is multi-factorial, economical, and most significantly, has greatly reduced sample volume requirements. While we show the advantages of a multiplex assay for measuring antimicrobial agents found in the FRT, this method could easily be applied to identifying antimicrobial agents found in a variety of biological fluids including saliva, stool, and in the mucosal linings of the respiratory and intestinal tracts.

2. Materials and methods

2.1. Antibodies and standard curve analytes

Capture and detection antibodies, as well as antimicrobial factor standards were sourced as described in Table 1. In some cases, special requests were made to manufacturers to supply the antibodies in the absence of carrier protein, typically bovine serum albumin (BSA), in order to facilitate microsphere conjugation.

2.2. ELISA assays

High binding polystyrene 96 well plates (Corning) were incubated with 100 μl of 5 μg/ml of capture antibody in phosphate buffered saline (PBS) overnight at 4 °C. The plates were washed three times with 200 μl of PBS 0.05% Tween-20 (PBS-T) and blocked with 100 μl of PBS 1% BSA for 1 h at room temperature. The plates were washed three times with 200 μl of PBS-T and were incubated with analyte at the manufacturer's recommended concentrations and buffer conditions for 2 h at room temperature. The plates were washed three times and incubated with detection antibody at the recommended concentration and buffer condition for 1 h at room temperature. After detection, 100 μl of Strep-HRP diluted 1:200 into PBS (R&D Systems) was incubated for 30 min at room temperature. The plates were washed three times with 200 μl of PBS-T and 150 μl of ABTS one-step substrate (Thermo Scientific) was added and incubated for 30 min at room temperature. The absorbance at 405 nm was measured using a UV/Vis spectrophotometer (Molecular Devices) at 25 °C.

2.3. Preparation of capture antibody-conjugated microspheres

A customized multivariate microsphere assay was developed using a panel of capture antibodies coupled to carboxylated magnetic fluorescent microspheres (MagPlex-C Microspheres, Luminex Corp.) in an adaptation of a previously described method (Brown et al., 2012). A total of 1 million carboxylated microspheres were covalently coupled to 5 μg capture antibody using a two-step carbodiimide reaction. The antibodies used are listed in Table 1. Microspheres were washed by centrifugation and magnetic separation, then activated by resuspension in 80 μl of 100 mM monobasic sodium phosphate, pH 6.2, followed by the addition of 10 μl of 50 mg/ml N-hydroxysulfosuccinimide (24520, Pierce) in deinonized water and 10 μl of 50 mg/ml 1-ethyl-3-[3 dimethlyaminopropyl]carbodiimide-HCl (77149, Pierce) in deinonized water. This reaction mixture was mixed end-over-end on an inverter for 20 min at room temperature. Activated microspheres were then washed three times in 150 μl of phosphate buffered saline (PBS), resuspended in 100 μl of PBS, and incubated with 5 μg capture antibody, in a final volume of 500 μl of PBS, on an inverter for 2 h at room temperature. Finally, coupled microspheres were washed with 500 μl of PBS and resuspended in 250 μl of PBS-TBN (PBS, 0.1% BSA, 0.02% Tween 20, 0.05% Sodium Azide, pH 7.4). After either 30 min at room temperature or an overnight incubation at 4 °C in PBS-TBN, microspheres were washed with 500 μl PBS to remove blocking buffer and resuspended in 150 μl of PBS-TBN. The coupled microspheres were counted on an automated cell counter (TC10, Biorad) and stored at 4 °C in the dark for up to 12 months.

2.4. Preparation of standard curves and buffer selection

Recombinant human or human-derived antimicrobial peptides were sourced commercially as described in Table 1. Standard curves were generated using the manufacturer's recommended concentration ranges for the standard whenever it had been shown to include the dynamic range of fluorescence detection on the Luminex FlexMap-3D. For those where noticeable saturation could be observed at high concentrations, the maximum concentration was decreased. Standard curves were serially diluted 2-fold in PBS-T.

Several of the ELISA assays adapted to the microsphere format utilized proprietary buffers, necessitating buffer

Table 1
Reagents used in ELISA and microsphere assays.

Assay	Capture Ab	Analyte	Detection
Trappin/Elafin	842342, R&D Systems	842344, R&D Systems	842343, R&D Systems
CCL20/MIP3α	840316, R&D Systems	840318, R&D Systems	840317, R&D Systems
HBD-2	capture Ab from kit 900-K172, Peprotech	analyte from kit 900-K172, Peprotech	detection Ab from kit 900-K172, Peprotech
SLPI	MAB1274, R&D Systems	890149, R&D Systems	AB-260-NA, R&D Systems
CCL5/RANTES	840216, R&D Systems	840218, R&D Systems	840217, R&D Systems
CXCL12/SDF-1	840931, R&D Systems	840933, R&D Systems	840932, R&D Systems
HBD-3	AHP1802, AbD Serotec	PHP211, AbD Serotec	AHP1802B, AbD Serotec
HNP1-3	HM2058B, Hycult Biotech	HC4014, Hycult Biotech	detection Ab from kit HK317-02
Lactoferrin	HM2173B, Hycult Biotech	HK329, Hycult Biotech	HP9034B, Hycult Biotech
LL-37	HM2070B, Hycult Biotech	61302, AnaSpec	HM2071B, Hycult Biotech

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