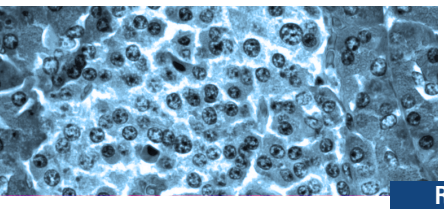
charles river



RESEARCH MODELS AND SERVICES

Summary Report of Non-Human Primate Tuberculosis MFIA® Qualification Protocol (PR-462)

Purpose and Background:

Tuberculosis (TB) is a fatal disease of New and Old World monkeys whose chief causative agent is *Mycobacterium tuberculosis*. Because of its ability to spread rapidly not only through lab animal colonies, but potentially to their human caretakers as well, diligent animal colony health surveillance is critical. Animals in breeding colonies are periodically tested using the tuberculin skin test (TST) and/or the Primagam® blood assay. However, these tests lack desirable sensitivity, specificity, efficiency, and/or throughput. To assist in routine colony health surveillance, Charles River Research Animal Diagnostic Services (RADS) has developed an assay on the Multiplexed Fluorometric Immunoassay® (MFIA®) platform [1,2] to detect *M. tuberculosis* antibodies in serum [3–6].

The MFIA® [1,2] is a serologic assay in which an array of uniquely-colored microbeads is coated with specific antigens and act as the solid phase of the immunoassay. Each unique color can be used to bind a unique antigen, thus multiple antibody determinations can be achieved using a single sample in a single test well. When a serum sample is added to a well containing the antigen-coated microbeads, specific antibodies to any of the specific antigens, if present, will bind to the antigen on the microbeads. After a washing step, a biotinylated anti-species immunoglobulin is added. If a specific antibody is bound in the first step, the biotinylated anti-species immunoglobulin will be bound. A final addition of a fluorescent reporter molecule, streptavidin-phycoerythrin conjugate, is performed, then the microbead contents of the wells are placed in a fluorometer. This device then analyzes each bead, identifying the unique color of each one, and simultaneously measures any fluorescent signal. These data are compiled and analyzed by computer and a Mean Fluorescent Index (MFI) is calculated for each antigen on each serum sample. An MFI cutoff value is established and samples are classified as either negative, borderline, or positive based on the MFI.

The purpose of this qualification is to perform MFIA® on *M. tuberculosis* (TB) antibody positive and negative sera, and to demonstrate that the TB-Plex MFIA® is sensitive and specific. We have used a MagPlex magnetic bead-based multiplex panel with seven recombinant TB antigens/assays in the current study. Antibody levels were examined in the non-human primate (rhesus or cynomolgus) serum or plasma samples from cohorts of well characterized specific pathogen-free (SPF) colonies at three different facilities including Charles River Laboratories. Additional samples from macaques of various TB exposures (experimentally infected, natural infection, cohorts of infected animals) were also evaluated [3].

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Materials and Methods

The recombinant antigenic proteins coupled to the MagPlex magnetic microspheres are listed in Table 1.

Table 1.

Antigens	
ESAT6/CFP10 FP	HSPX
ESAT6	RV3881
CFP10	RV3841
RV2875	

The MFIA procedure was described elsewhere [2] and performed according to the online manual. For each assay, the net median fluorescence intensity signal (MFI) was calculated by subtracting the tissue control (TC) from the antigen (AG) MFI. In the following tables and graphs, the results are presented as Net MFI/1,000 (or Net MFI in thousands); in this study, values of < 1.5 and ≥ 3 were classified as negative and positive, respectively; net signals between these cutoffs were called equivocal/indeterminate.

Standard positive assay control (immune sera) was antiserum or antiserum pools that were collected from naturally infected non-human primates and/or humans, and/or mice experimentally inoculated with recombinant TB proteins. Standard negative assay control sera (non-immune) was serum pools collected from TB antibody-free non-human primates.

Briefly, analytical sensitivity (limit of detection - LOD), and diagnostic sensitivity and specificity of the TB-Plex assay were evaluated. The LOD comparison between two technicians was done by the evaluation of serially diluted TB-Plex High and midrange positive assay controls. The controls were serially diluted two-fold into standard assay diluent (Primary Diluent-2). A total of six dilutions were analyzed beginning undiluted and ending at 1/32. The titration data points represent the average for all seven TB-Plex panel agents with assay net MFI/1,000 reported.

Assay sensitivity and reproducibility was measured by analyzing sixteen known positive test samples by two technicians (Tech01 and Tech02) performing three different MFIA runs on different days for a total of six runs. In addition, assay specificity was measured by analyzing sixteen known negative test samples in all six runs.

Antibody levels were examined in a total of 786 macaque serum or plasma samples from historically known TB-negative colonies at three different North American facilities including Charles River Laboratories.

Results Summary

Assay Cutoffs and Interpretation Guidelines

Due to the complexity of the TB immune response several hundred negative macaque serum samples were evaluated (before performing this study) to aid in developing scoring/interpretation criteria for the TB-Plex assay. Based on this screening of TB-positive and negative sera, assay cutoffs were determined and the following interpretation guidelines were developed. TB antigens/assays were divided into two groups based on the reactivity profiles (Table 2).

Table 2. TB-Plex antigens/assays were categorized into two groups for interpretation purposes.

Group	Antigen
1	ESAT6/CFP10 FP
	ESAT6
	CFP10
	RV2875
	HSPX
2	RV3881
	RV3841

Table 3. TB-Plex Interpretation guidelines using group 1 and 2 MFIA assay/antigen (AG) scores.

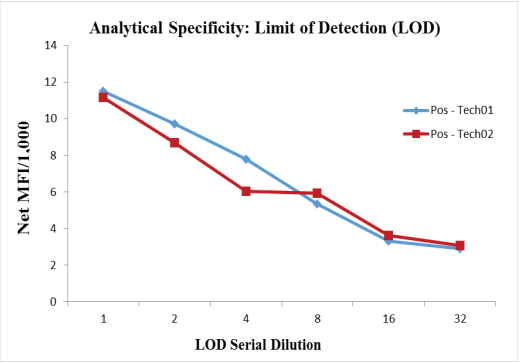
Assay(s)	Criteria	Interpretation
Combination of Group 1 AGs	Scores ≥ 3 on multiple AGs	Positive
Any Group 1 AG and one or more Group 2 AGs	Scores ≥ 3 on multiple AGs	Positive
Combination of Group 1 and 2 AGs	Score = 2 on multiple AGs	Indeterminate
Both Group 2 AGs	Scores ≥ 3 on multiple AGs	Indeterminate
Individual Group 1 AG	Scores ≥ 3	Indeterminate
Individual Group 2 AG	Any score	Negative

Samples were designated as positive or negative based on the scores from individual assays and guidelines in Table 3.

Limit of Detection (LOD)

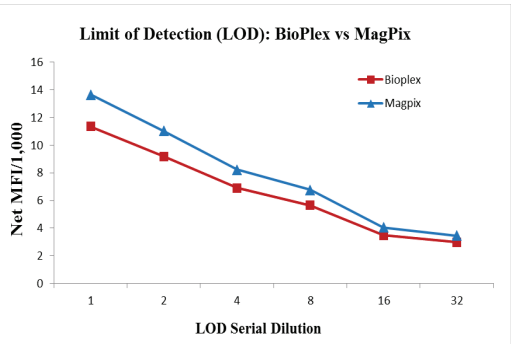
To assess the analytical sensitivity of the TB-Plex assays, control sera were titrated and scores between two technicians were compared. The graphs for analytical performance in Figure 1 show MFIA® endpoint titration curves of serially diluted TB-Plex positive assay controls. Average assay scores for all seven assays were plotted against serum dilutions. LOD was further assessed by comparing results obtained from both Bio-Plex and MagPix instruments (Figure 1).

Figure 1. MFIA® LOD Titration of TB-Plex Control Sera



Six serial two-fold dilutions of TB assay controls were analyzed by TB-Plex MFIA®. The titration data points represent the average for all seven TB-Plex panel agents with assay net MFI/1,000 reported.

Figure 2. MFIA® LOD Titration of TB-Plex Control Sera

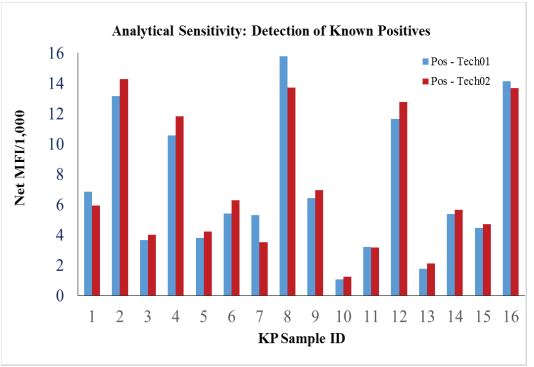


Six serial two-fold dilutions of TB assay controls were analyzed by TB-Plex MFIA® on both Bio-Rad Bio-Plex and Luminex MagPix instruments. The titration data points represent the average for all seven TB-Plex panel agents with assay net MFI/1,000 reported.

Diagnostic Sensitivity, Specificity, and Repeatability

To determine TB-Plex assay sensitivity 16 known positive antiserum samples were analyzed using a Bio-Plex instrument. These samples consisted of non-human primate TB+ sera, mouse monospecific antiserum, or a pool of both antiserum types. Not all TB-Plex panel members (antigens) were expected to score positive for each known positive sample and therefore only antigen-specific reactions will be considered for calculations of sensitivity and specificity. Each individual agent (antigen bead) of the seven-member TB-Plex panel is considered an individual assay. Sensitivity by KP Sample (Figure 3) and by Tech (Table 4) was calculated comparing the total number of expected positive reactions to those of both Tech01 and Tech02 for each of their three test runs.

Figure 3.



Sixteen known immune sera were tested in three separate runs by two technicians each. Sample Net MFI/1,000 of < 1.5 and ≥ 3 were classified as Negative and Positive, respectively. The values shown represent the average for all seven TB-Plex panel assays with net MFI/1,000 reported.

Table 4.

Sample Status	Analyst	Net MFIA/1000			
		AV	Total AV	stDev	%CV
Positive	1	7.3	7.1	0.4	5%
	2	6.8			
Negative	1	0.0	0.0	0.0	
	2	0.1			

Sixteen known antibody-positive and 16 known antibody-negative sera were tested in three separate runs by two technicians each. Sample Net MFI/1,000 of < 1.5 and > 3 were classified as Negative and Positive, respectively. Displayed is the Net MFI/1000 score, averaged for all TB-Plex assays, for three runs performed by both Tech01 and Tech02. The average scores (AV) were similar between two technicians.

Specificity was calculated comparing the total number of expected negative reactions for the 16 known negative samples to those of both Tech01 and Tech02 for each of their three test runs. The known negative samples were taken from non-human primate colonies historically TB-negative based on TST results. Data was analyzed from the Bio-Plex instrument (Table 5).

Table 5.

	Sensitivity	Specificity
	Positives	Negatives
#	292	666
Total Assays Tested	294	672
%	99.3%	99.1%

Sixteen known antibody positive and negative sera were tested in three separate runs by two technicians each. Total Tested = # of Sample X Assay X Runs. Each individual agent (bead) of the seven-member TB-Plex panel is considered an individual assay. The number of total positive assays (294) are less than the number of total negative assays (672) because all seven assays were not positive for each KP sample. Sample NetMFI/1,000 of < 1.5 and > 3 were classified as Negative and Positive, respectively.

Field Testing

We conducted a multisite evaluation of the TB-Plex bead panel to further evaluate diagnostic specificity. Each site analyzed known negative samples (previously tested negative by TST analysis) using a Bio-Plex instrument: site A (n=372), site B (n=227), and site C (n=187). Results from all sites were combined to calculate the overall diagnostic specificity of the TB-Plex assay.

Table 6.

Lab	# Tested	# Positives	% Specificity
A	372	1	99.7%
B	227	0	100.0%
C	187	0	100.0%
Total	**786**	**1**	**99.9%**

Samples were classified as negative or positive following interpretation guidelines (Table 2).

Conclusion

Tuberculosis (TB) in nonhuman primates is highly contagious and often produces rapid disease. Identification of animals infected with TB in a timely manner is therefore critical. This study was undertaken to develop a multiplexed and higher throughput assay than the currently used tuberculin skin test (TST) and Primagam® blood assay. Evaluation of over 1,000 negative and positive macaque samples determined the appropriate individual TB-Plex antigen cutoffs and facilitated the development of the scoring and interpretation guidelines for the panel.

Results from the qualification of the TB-Plex panel with seven antigen-coupled beads show a very high sensitivity, specificity, and reproducibility. The overall diagnostic sensitivity and specificity of the TB-Plex bead panel were found to be > 99% (Tables 5 and 6). In conclusion, we have validated a blood-based multiplex panel that is highly sensitive and specific for routine screening of *M. tuberculosis* in non-human primates based on their antibody profiling. The data demonstrates that the TB-Plex assay can be used for routine monitoring of non-human primate colonies and can be performed in a user friendly and high-throughput format.

References

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