Video Article

Multiplexed Fluorometric ImmunoAssay Testing Methodology and Troubleshooting

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Abstract

To ensure the quality of animal models used in biomedical research we have developed a number of diagnostic testing strategies and methods to determine if animals have been exposed to adventitious infectious agents (viruses, mycoplasma, and other fastidious microorganisms). Infections of immunocompetent animals are generally transient, yet serum antibody responses to infection often can be detected within days to weeks and persist throughout the life of the host. Serology is the primary diagnostic methodology by which laboratory animals are monitored. Historically the indirect enzyme-linked immunosorbent assay (ELISA) has been the main screening method for serosurveillance. The ELISA is performed as a singleplex, in which one microbial antigen-antibody reaction is measured per well. In comparison the MFIA is performed as a *multiplexed* assay. Since the microspheres come in 100 distinct color sets, as many as 100 different assays can be performed simultaneously in a single microplate well. This innovation decreases the amount of serum, reagents and disposables required for routine testing while increasing the amount of information obtained from a single test well. In addition, we are able to incorporate multiple internal control beads to verify sample and system suitability and thereby assure the accuracy of results. These include tissue control and IgG anti-test serum species immunoglobulin (αIg) coated bead sets to evaluate sample suitability. As in the ELISA and IFA, the tissue control detects non-specific binding of serum immunoglobulin. The αIg control (Serum control) confirms that serum has been added and contains a sufficient immunoglobulin concentration while the IgG control bead (System Suitability control), coated with serum species immunoglobulin, demonstrates that the labeled reagents and Luminex reader are functioning properly.

Video Link

The video component of this article can be found at https://www.jove.com/video/3715/

Protocol

1. Explanation of the MFIA Procedure (Figure 1)

1. The MFIA requires Charles River's antigen coated polystyrene microspheres (beads), test sera, labeled reagents (BAG, SPE) and buffers (Primary Diluent, Assay buffer).
2. The reagents are added stepwise to the wells of 96-well filter-bottom microtiter plates.
3. The MFIA is performed as a heterogeneous test meaning incubations are followed by filter-wash steps to remove unbound serum constituents or labeled reagents. Wash solution added to plate wells is removed by aspiration through well filter-bottoms, which retain the beads.
4. The MFIA assays are performed at room temperature (27°C+/-2°C).
5. Antigen-antibody complexes formed during the test serum incubation step are detected by incubations with biotinylated goat anti-species conjugates (BAG) followed by R-phycoerythrin-labeled streptavidin (SPE).
6. In the assay reader, the beads pass one at a time through a detector where they are exposed to two lasers. One laser excites the internal dyes that identify the bead's color set, which corresponds to an assay; the other excites the phycoerythrin reporter dye captured during the assay. A predetermined number of beads are read per assay and the intensity of phycoerythrin fluorescence is reported as a Median Fluorescence Index (MFI).

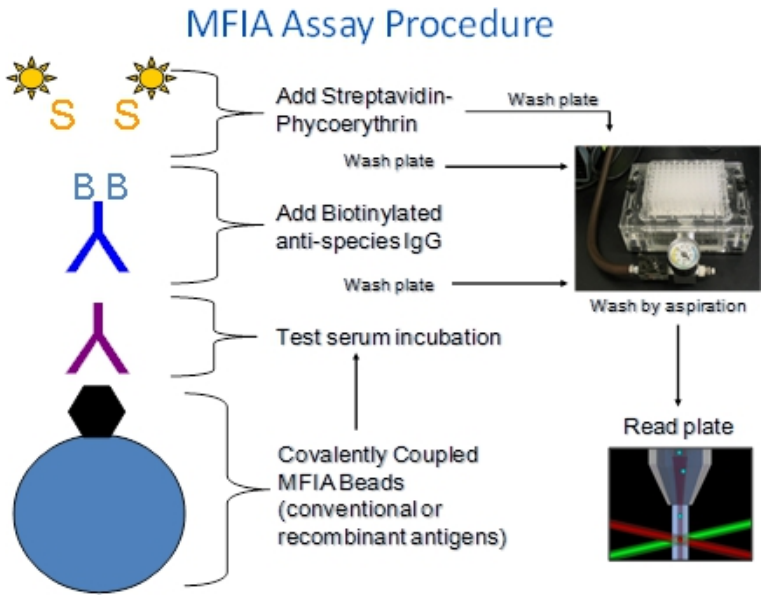

Figure 1. MFIA Procedure. The xMAP-based MFIA is a suspension microarray which utilizes color-coded polystyrene 5.6 micron beads to which antigens (or controls) are covalently linked. Since the beads come in 100 distinct color sets, as many as 100 different assays can be performed in a single well Assay steps are performed in filter-bottom microtiter plates so that beads can be washed by aspiration on a vacuum manifold. Reactions are read with the Luminex xMAP 100 fluorometer. The intensity of phycoerythrin fluorescence is reported as a median fluorescence index (MFI)

2. Before Getting Started Please Note the Following:

1. The MFIA beads are light sensitive
    1. Limiting the amount of direct light exposure is crucial. The normal amount of light exposure that occurs during routine testing is acceptable but prolonged exposure can lead to photobleaching of the beads which render them unreadable by the Assay reader.
    2. It is crucial to the success of the assay that the MFIA beads are always vortexed into suspension and then sonicated (10-30 seconds) prior to use.
    3. The Assay reader gathers information from single beads only. Aggregated bead clusters can lead to longer read times.
2. Be sure to keep the test plate covered with the plate lid during all incubation steps to avoid evaporation of assay solutions.
3. Personal Protective Equipment required: Laboratory coat, gloves and eye protection should be worn at all times while working in a laboratory setting.

3. Reagents

1. Table 1 includes most of the materials necessary to establish an in-house MFIA laboratory. Additional or duplicate items may be necessary depending on your specific needs. Please note that this inventory excludes reagents purchased from Charles River (MFIA beads, controls and supplemental reagents). Several commercial sources of biotinylated conjugates and streptavidin tagged phycoerythrin are available. However the reagents available from Charles River have been titrated to yield the optimal signal to noise score with our reagents, using alternative vendors or reagent lots is not recommended.

Table 1. CR-RADS uses a BioPlex Suspension Array Reader from BioRad and automated microplate washer from BioTek. Equivalent instrumentation is available from alternate vendors.

Item	Vendor	Catalog Number
Equipment		
Suspension array reader	*BioRad	171-000205
96-well microplate washer	BioTek	ELX50/8FMW
Ultrasonic cleaner/bath	Cole Palmer	EW0884900
Analog vortex mixer	VWR	58816-121
-20°C freezer	Various	
4°C refrigerator	Various	
12-channel pipettor, 20-200µl	VWR	83009-718
Orbital plate shaker	VWR/Lab-Line	57019-600

Single-channel pipettors, 20-200µl, with tips	VWR	83009-732
Single-channel pipettors, 2-20µl, with tips	VWR	83009-726
Single-channel pipettors, 100-1000µl, with tips	VWR	83009-736
Vacushield vent device	VWR	55095-006
Vacuum pressure pump	VWR	54908-037
Vacuum system waste reservoir	VWR	80200-640
Disposables		
96-well polystryrene plate	Fisher Scientific	14-245-145
MultiScreen HTS-BV plate, 1.2µm filter, styrene	Millipore	MSBV N12 50
15ml conical tubes (polypropylene)	Sarstedt	62554.002
50ml conical tubes (polypropylene)	Sarstedt	62547.004
Serum vials	Sarstedt	72694.007
Aluminum foil	VWR	89079-068
1L bottles, sterile	VWR	28199-246
0.22µm bottle-top filters	VWR	28199-307
Reagent reservoirs (100ml)	VWR	82026-356
5ml, 10ml, 25ml pipets for dispensing liquid reagents	VWR	
Reagents		
PBS, pH 7.4 1 BSA, powder (pouches)	Sigma	P3813
ProClin 300	Sigma/Supelco	48912-U
Uncoated microspheres	Luminex	varied based on type

4. Reagent Preparation

1. Two buffers are required for the MFIA procedure. Primary Diluent is available from Charles River and contains proprietary blocking agents to decrease non-specific protein interactions. Assay Wash buffer is made at your facility with commercially available reagents.
2. Assay Wash buffer: PBS/ 1% BSA pH 7.4 is available as a powder from Sigma-Aldrich.
    1. Removal of particulates by filtration is critical as these particulates can lead to clogs in the assay reader. Filter the buffer through a 0.2micron bottle-top filter unit into sterile, labeled containers.
    2. BAG and SPE are diluted to their 2X working concentration in Assay Wash buffer. Note: SPE is light sensitive and should have limited light exposure.

5. Sample Preparation

1. Assemble your testing materials, reagents and disposables.
    1. Multi- and single-channel micropipettes and tips
    2. 96-well low protein binding microtiter plates (serum dilutions) and filter-bottom plates (MFIA assay)
    3. Collect blood specimens according to your standard procedure. Allow the blood specimens to clot fully by holding them at room temperature for at least 30 minutes before centrifugation and serum removal. Undilute serum should be vortexed briefly to mix the serum components prior to sampling.
    4. The final testing dilution for the MFIA is 1/50. It is necessary to make a 2X (1/25) sample of your test serum for the assay. Dilute the undiluted test sera by adding 1 part serum to 24 parts MFIA Primary Diluent. Organization of your test serum in a 96-well microtiter plate greatly reduces the transfer time of your samples to the test plate and is highly recommended.

6. Test Plate Preparation

1. Pre-wetting the 96-well Test plate is required to insure proper, even well evacuation. Even if the entire plate will not be used this step is initially required, you will not have to add subsequent reagent or wash buffer to the empty wells.
    1. Some labs run less than a full 96-well plate by sealing off the unused wells on the test plate and plan to re-use these wells at a later date. We do not recommend using this method since it may affect the proper filtration of the test plate.
2. Proper test plate aspiration is crucial to the success of the MFIA assay. Evacuation of the sample wells should take approximately 5-10 seconds. Aspirating the well contents too quickly can lead to bead aggregation and slow read times at the end of the assay.
3. Blot the underside of the test plate with paper towels after every wash step to ensure liquid drainage has stopped. Failure to blot the test plate can lead to liquid reagents wicking out of the wells during incubation and result in failing Goat anti-species and/or Species IgG bead scores.

These beads are designed to score within a pre-determined MFI range when the appropriate amount of primary serum and labeled reagents are added to each well.

7. MFIA Bead Suspension Preparation

1. It is crucial to the success of the assay that the beads are always vortexed and sonicated prior to use. The assay reader gathers information from single beads only, bead aggregates or clumps can lead to longer read times.
2. Vortex the stock coupled bead suspension (typically 10 ± 5 seconds)
3. Resuspend the beads followed by sonication in a sonicator bath for 10-20 seconds.
4. Dilute the 20X stock suspension to a 2X working bead suspension in Primary Diluent. Dispense 50µl of the 2X bead suspension to each assay well of the pre-wet Test Plate.

8. Addition of Test and Control Sera to the Test Plate

1. Pipette 50µl of each 2X Test and Control serum to the Test Plate based on your predefined plate map. Secure lid to the plate with an elastic band or aluminum foil and incubate the Test Plate for 60minutes on an orbital shaker, in darkness at room temperature.
    1. The shaker speed should be greater than 400 but less than 700rpm. It is critical that the beads be kept in suspension to allow serum antibodies access to all surfaces of the coupled antigens. Failure to keep the beads suspended will result in low IgG bead control scores and the assay will need to be repeated.

9. Washing the Test Plate

1. Aspirate the well contents using a vacuum manifold. Evacuation of the sample wells should take approximately 5-10 seconds.
2. Blot the underside of the test plate with paper towels after every to ensure liquid drainage has stopped.
3. Resuspend the beads in 50µl of Assay Wash buffer. It is important that the beads are not allowed to dry out during the assay process.

10. Adding BAG and SPE to the Test Plate

1. Dispense 50µl of 2X working dilution BAG to all wells containing MFIA beads. Secure the lid to the plate with an elastic band or aluminum foil. Incubate the Test Plate for 30minutes with shaking as above, in darkness at room temperature.
    1. After this incubation wash the Test Plate and resuspend the beads with Assay Wash buffer as previously described following serum addition.

2. Dispense 50µl of 2X working dilution SPE to all wells containing MFIA beads. Secure the lid to the plate with an elastic band or aluminum foil. Incubate the Test Plate for 30minutes with shaking as above, in darkness at room temperature.
    1. After this incubation wash the Test Plate and resuspend the beads with 125µl of Assay Wash buffer and shake the plate for ˜two minutes to resuspend the beads prior to placing into the assay reader.

11. Reading the Test Plate

1. Place the test plate into the assay reader within 10 minutes of resuspending the beads. In the event of a power failure or other incident, the Test Plate can be stored at room temperature in darkness up to 12 hours until ready to read.
    1. Observe the first sample well to verify that the selected bead panel matches the bead profile in the test wells. If there are beads falling outside their designated 'bead region' on the protocol display, an incorrect profile selection has been made.
    2. If the beads have been properly resuspended they should fill-in their specified 'regions' indicated in the assay reader software.
    3. If the beads are aggregated they will fill in their regions at a slow rate. You can remove the test plate from the reader and manually resuspend the wells. Triturate each well with a multi-channel pipettor 3-4 times to assist in breaking up the bead aggregates. Replace the test plate into the reader and continue.

12. Representative Results

1. A minimum number of beads are read per assay and the intensity of phycoerythrin fluorescence is reported as a Median Fluorescence Index (MFI) ranging from 0 to 32,667. After comparing counts of 25, 50 or 100 beads per well we saw no statistical difference and routinely count 25 beads per agent per sample well. Examine the results report for errors, such as inadequate bead counts or failing IgG / anti-IgG bead scores. These beads are designed to 'score' within a pre-determined MFI range when the appropriate amount of primary serum and labeled reagents are added to each well. Sample wells with errors, (low bead counts) or failing IgG control bead scores should be repeated to acquire valid results.
2. Export the results to an Excel worksheet. For our data interpretation each assay is assigned:
    1. A Tissue Control (TC) Test: For most rodent MFIA assays, the tissue control is an extract of wild-type baculovirus-infected insect cells.
    2. An Assay Cutoff: This value is the minimum fluorescence expected and is adjusted for each assay to maximize diagnostic accuracy. For most assays, the fluorescence cutoff is 3000.

3. Net Median Fluorescence Index (MFI) is calculated for each assay (except tissue and IgG control tests) by the following formula. In other words, the specific assay signal is the total assay MFI minus the TC (background) MFI This value is then divided by 1000 which simply sets the range of Net MFI between 0-32 instead of 0-32,667.
   Net MFI = ($MFI_{Antibody\ Assay}$ - $MFI_{Tissue\ Control}$) / 1000
   Net MFI and TC MFI are converted to scores by comparison to the Assay Cutoff specific for each antigen.

	Mouse Test Sera						Serum Controls					
Well:	**A1**	**B1**	**C1**	**D1**	**E1**	**F1**	**A2**	**B2**	**C2**	**D2**	**E2**	**F2**
Antigen	Serum #1	Serum #2	Serum #3	Serum #4	Serum #5	Serum #6	Mouse-1 High	Mouse-1 Low	Parvo Mid	NMS	NRS	Diluent only
SEND	0	0	5	-1	0	0	11	4	0	0	0	0
PVM	0	0	1	0	0	0	13	6	0	0	0	0
MHV-A59	0	0	22	0	0	0	12	5	0	0	0	0
MVM	0	0	15	0	0	0	17	3	25	0	0	0
MPV-1	0	0	0	0	0	0	15	5	24	0	0	0
MPV-2	0	0	0	0	0	0	10	3	2	0	0	0
MPV-5	0	0	0	-1	0	0	21	13	1	0	0	0
NS-1	0	0	0	0	0	0	1	0	4	0	0	0
rMNV	23	23	0	23	0	0	16	9	0	0	0	0
GDVII	0	0	5	-1	0	0	10	5	0	0	0	0
REO-3	0	0	7	0	0	0	15	4	0	0	0	0
wBAC	0	0	0	1	0	0	0	0	0	0	0	0
Mouse IgG	7	7	6	6	7	5	6	7	6	7	8	7
GAM IgG	15	15	14	15	13	12	14	15	14	15	0	1

Figure 2. Representative MFIA data for test sera with all IgG controls passing.

4. 12.4 MFIA Interpretation
   1. Test Plate results should only be interpreted if the System-Suitability Control and Serum Control (IgG / anti-IgG beads) results meet the acceptance criteria listed in Table 2. Failure of these two IgG control beads can indicate several procedural errors such as improper dilution or insufficient volume conjugate or SPE, improper test serum dilution or use of serum from an immunocomprimised animal.

Table 2

Acceptance Criteria for Assay Controls		
Control	**Acceptable Result**	
	Score	**Classification**
High Range Immune Serum	≥ 4.5	Positive
Low Range Immune Serum	≥ 1.5	≥ Borderline
Non-immune Serum	< 2.5	≤ Borderline
Diluent	< 2.5	≤ Borderline
Ig Bead Set*	≥ Cutoff/1000	Pass

*Bead set coated with species-specific anti-test serum immunoglobulin (Ig): failing scores for this sample suitability control could result from the addition of insufficient sample, too high a sample dilution, incorrect species or testing serum from an immunodeficient host.

2. If any of the Test Plate Assay Controls fail except the High Range Immune Serum, the results are not acceptable and must be repeated. Passing Low range Serum control scores are critical since it demonstrates the detection sensitivity level of the test plate.
3. The Goat anti-species IgG bead score is based on the amount of primary test serum added to the assay. If this control bead fails but the species IgG passes (Well B1, Fig.3) then it indicates that enough testing reagents (BAG, SPE) were added to the test well and it is a serum related issue. If the amount of testing reagents is insufficient in a particular well both the IgG and anti-IgG control beads will fail (Well E1, Fig.3).

	Mouse Test Sera						Serum Controls					
Well:	A1	B1	C1	D1	E1	F1	A2	B2	C2	D2	E2	F2
Antigen	Serum #1	Serum #2	Serum #3	Serum #4	Serum #5	Serum #6	Mouse-1 High	Mouse-1 Low	Parvo Mid	NMS	NRS	Diluent only
SEND	0	0	5(3)	1	0	0(2)	11	4	0	0	0	0
PVM	0	0	1	0	0	0(2)	13	6	0	0	0	0
MHV-A59	0	0	16(3)	0	0	0(2)	12	5	0	0	0	0
MVM	0	0	0	0	0	0(2)	17	3	25	0	0	0
MPV-1	0	0	0	0	0	0(2)	15	5	24	0	0	0
MPV-2	0	0	0	0	0	0(2)	10	3	2	0	0	0
MPV-5	0	0	0	2	0	0(2)	21	13	1	0	0	0
NS-1	0	0	-2(3)	0	0	0(2)	1	0	4	0	0	0
rMNV	12(5)	0	0	1	0	0(2)	16	9	0	0	0	0
GDVII	0	0	0	1	0	0(2)	10	5	0	0	0	0
REO-3	0	0	0	0	0	0(2)	15	4	0	0	0	0
wBAC	5	0	3	1	0	2	0	0	0	0	0	0
Mouse IgG	7	7	6	6	2	5	6	7	6	7	8	7
GAM IgG	15	3	14	15	4	12	14	15	14	15	0	1

Figure 3. Representative MFIA test results with failures (indicated in orange). Wells A1, C1 and F1 indicate a TC (tissue reactive) failure where the TC score is represented in parenthesis. Wells B1 and E1 illustrate the two types of IgG internal control failures, insufficient test antibody (B1) or test reagents (E1) BAG / SPE respectively.

4. If Test Plate Assay Control results are satisfactory, individual assay scores are classified as shown in Table 3. It is critical to confirm any borderline or positive finding by an alternative test method.

Table 3

MFIA Score Classification			
Score			**Classification**
TC	**Net**	**TC + Net***	
≥ 2	< 0.5	< 2.5	Negative (-)
	≥ 0.5	≥ 2.5	TC Reaction (T)
< 2	< 1.5		-
	1.5 ≤ X < 2.5		Borderline (B)
	≥ 2.5		Positive (+)

*A classification of negative can still be determined with a non-zero TC score provided the TC + Net score (= Ag score) is negative

Discussion

The MFIA testing process is highly efficient, requiring less equipment and smaller sample and reagent volumes than traditional singleplex tests. The functionality of the multiplex system gives the user the flexibility to screen simultaneously for multiple strains or serotypes of common agents in laboratory rodents (i.e. Coronavirus, parvoviruses etc.). This also enables us, to design customized bead panels based on the area of interest (ie. specific virus family) and is adaptable to screening other types of biomolecules including cytokines and other biomarkers. In addition, it allows us to incorporate several internal control assays to verify sample and system suitability and thereby assure the accuracy of results. These include tissue control and IgG anti-test serum species immunoglobulin (αIg) coated bead sets to evaluate sample suitability. A tissue control bead detects non-specific binding of serum immunoglobulin and the αIg bead control confirms that serum has been added and contains a sufficient immunoglobulin concentration. Another control bead, coated with serum species immunoglobulin, demonstrates that the labeled reagents and assay reader are functioning properly. Other commercially available multiplex format serologic assays (ie. MicroArrays, ImmunoComb) may not offer this same level of result confirmation.

The ability to narrow down the possible source of assay failure prior to retesting can help an investigator save on time and materials. Critical aspects of the MFIA should be confirmed before repeating a failed sample. Since the assay is conducted at ambient temperature it should be verified that the laboratory temperature is approximately 27°C±2°C, higher temperatures can lead to lower than expected scores for controls and samples. Washing is perhaps the most critical step in the assay. Insuring that the test plate is properly washed, blotted and resuspended can eliminate the vast majority of sampling errors due to low bead count (due to aggregated beads) or insufficient reagent addition (lost by wicking out of test plate filter bottom). We routinely count 25 beads per assay (agent) and have found no statistical difference in the results by counting higher number of beads which will also lead to a longer read time for the plate.

We have performed comprehensive validation studies of MFIA on several species of commonly used laboratory animals (Mouse, Rat, Hamster, Guinea Pig and Rabbit) to demonstrate diagnostic accuracy, reproducibility, and ruggedness by testing large numbers of known positive and negative serum samples, and comparing their ELISA, IFA and MFIA results. The detection limits (i.e., standard immune serum titration endpoints) of MFIA were comparable to, and in some cases surpassed, those of corresponding ELISA. Diagnostic specificity, measured with

SPF rodent sera, exceeded 99%; the overall correspondence between ELISA and MFIA performed on known positive and known negative sera was greater than 95%. In summary, these results proved that multiplex MFIA is a good alternate to the singleplex ELISA, and is suitable for its intended use, i.e. in routine serosurveillance of laboratory animal colonies.

Disclosures

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Acknowledgements

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