

Multiplexed Fluorometric ImmunoAssay™

September 2007

At Charles River Research Animal Diagnostic Services, ensuring the quality of animal models used in biomedical research is our highest priority. To accomplish this goal, we have developed a number of diagnostic testing strategies and methods to determine if animals have been exposed to adventitious infectious agents. Infections of immunocompetent animals are generally transient, yet antibody responses to infection usually develop within days to weeks of exposure and for prolonged periods thereafter. Since the presence of antibodies to a specific etiologic agent is evidence that the animal has been exposed to that agent, antibody immunoassays are the primary method of microbiological quality control and disease surveillance in laboratory animals. In addition, a single serum sample can be tested for antibodies to a panel of microbial agents by immunoassays that are inexpensive, rapid, sensitive, and specific.

The indirect enzyme-linked immunosorbent assay (ELISA) has been the primary screening method for viral serosurveillance because of its sensitivity and ease of automation, and the indirect fluorescent antibody (IFA) assay has been the principal method of confirming positive or unexpected results. ELISA and IFA assays are performed as *singleplexes* in which one microbial antibody-antigen reaction is measured per well.

The importance of high throughput screening to the biopharmaceutical industry has encouraged development of technologies for performing multiple assays in a single well or *multiplexing*. One such technology, the Luminex® Multi-Analyte Profiling system, utilizes suspended microspheres comprising 100 differently colored bead sets. As each bead set can have a unique reactant coupled to its surface, up to 100 different assays can be simultaneously performed in a single microtiter well. We call this diagnostic test the **Multiplexed Fluorometric ImmunoAssay™ (MFIA™)**.

MFIA™ reactions are performed in much the same way as the indirect ELISA or IFA, except that antigen-antibody complexes are demonstrated by incubation with a fluorophore labeled reagent, phycoerythrin, which allows the assays to be read in a modified flow cytometer. Essentially, the antigen-coupled beads pass in single file through a dual laser fluorometer, which first determines the bead's dye color (e.g., which antigen the bead represents) and the intensity of phycoerythrin fluorescence (e.g., the assay result). This efficient technology can be used for multiplexing microbial antibody serologic tests for rodents and other laboratory animals. Since up to 100 different assays can be simultaneously performed in each sample well by attaching different antigens to each of the 100 differentially colored bead sets, we are able to include all necessary diagnostic antigens and tissue controls (TC), plus additional assay internal controls in each sample well. Results for each sample well are reported from the

flow cytometer as the median fluorescent intensity (MFI) for each bead set (antigen) within the well. Since MFI values range from 0 to 32,667, a formula was developed to convert raw MFI results to integer values that roughly correspond to previously reported ELISA scores; thus, an MFIA™ score of 1 is negative, 2 is equivocal, and 3 or higher is generally positive.

To ensure that MFIA™ performs at the high level that customers have come to expect from our ELISA, a comprehensive validation study was performed. For the validation study, a large number of previously characterized positive and negative serum samples were run side-by-side by ELISA, IFA, and MFIA™. Information from this analysis was used to calculate diagnostic sensitivity and specificity. Additionally, assays were performed at multiple times, by multiple technicians, over multiple days to evaluate assay ruggedness, reproducibility, and robustness. The validation study demonstrated that the diagnostic sensitivity and specificity of the MFIA™ is comparable to the indirect ELISA and IFA. Further, the day-to-day and technician-to-technician variation in MFIA™ results is minimal. A comprehensive MFIA™ validation report will be placed on the Charles River Research Animal Diagnostic Services website in the future.

We believe that MFIA™ testing is an excellent alternative to traditional ELISA testing: MFIA™ offers similar sensitivity and specificity to ELISA while allowing for additional sample controls, faster sample throughput, and a smaller sample volume. Thus, we will begin using the MFIA™ as our primary serologic testing method while continuing to provide confirmatory testing by ELISA, IFA, hemagglutination inhibition (HAI) or Western blot (WIB). Due to the extensive assay development and validation, and the rigorous in-house testing that has been performed, we expect the transition from ELISA testing to MFIA™ testing to be transparent to our customers. Following is a list of frequently asked questions and their answers. If you have additional questions, please do not hesitate to contact Charles River's Technical Services at 1.800.338.9680 or comments@crl.com.

• Why is Charles River Research Animal Diagnostic Services changing their primary serology testing method?

At Charles River, we are continuously evaluating new technologies that can provide similar or better results in a more timely manner to our customers. Additionally, MFIA™ allows us flexibility to further refine and expand our assay capabilities by performing supplementary internal controls and adding antigens upon development or when necessary.

• Will ELISA testing still be available?

Yes, we believe that both ELISA and MFIA™ provide excellent results and we will continue to produce and use ELISA plates for diagnostic testing where appropriate.

• What MFIA™ panels are available?

The Research Animal Diagnostic Services Serology Department will provide multiplexed MFIA™ testing in the following panels:

Species/Profiles	Agents Included
Mouse	
Parvovirus	MPV-1, MPV-2, MVM, NS-1
Prevalent	Parvovirus Profile and MHV, MNV, TMEV, EDIM
Tracking	Prevalent Profile and SEND, PVM, REO, MPUL
Assessment	Tracking Profile and LCMV, MAV, ECTRO, K, POLY
Assessment Plus	Assessment Profile and MTLV*, MCMV, HANT, ECUN, CARB
Rat	
Parvovirus	RPV, H-1, KRV, RMV, NS-1
Prevalent	Parvovirus Profile and SDAV, RTV
Tracking	Prevalent Profile and SEND, PVM, REO, MPUL
Assessment	Tracking Profile and LCMV, MAV, TMEV
Assessment Plus	Assessment Profile and HANT, ECUN, CARB
Rabbit	
Tracking	ECUN, CARB, TREP**
Assessment	Tracking Profile and CPIL, PI-1, PI-2, REO, ROTA, LCMV
Assessment Plus	Assessment Profile and AV
Hamster	
Assessment	SEND, SV5, PVM, REO-3, LCMV, ECUN

* IFA is the primary test; no alternative test available.

**Tested by alternative method.

• How do you control for non-specific binding of antibodies?

Approximately two years of research and development went into developing the antigens and reagents for MFIA™ testing and in validating the assays for diagnostic sensitivity and specificity and for robustness, ruggedness, and repeatability. The proprietary reagents that Research Animal Diagnostic Services developed for MFIA™ testing limit problems associated with non-specific antibody binding.

• Will there be any additional charge for this new testing?

No, there will be no change in the pricing of our current serology panels, only the technology behind the panels will be changing.

• How much serum will I need to submit for serology testing and what sample preparation do I have to perform?

We need 50µl of a 1:5 diluted serum sample for any of the rodent MFIA™ panels; however, additional confirmatory testing for positive or unexpected results by ELISA, IFA, HAI, or WIB will require a total of 150µl of a 1:5 diluted serum sample.

• How long will it take to get my results?

The turn around time for results should be within 48-72 hours of sample receipt. Data is also available online via ILIMS. In case of positive or unexpected results additional time may be needed for confirmatory testing by ELISA, IFA, HAI, or WIB.

• How do I submit samples?

A completed sample submission form should accompany all samples. Please call 1.800.338.9680 to arrange for provision of a free shipper. Ship samples by overnight delivery to:

Serology Laboratory
Charles River
251 Ballardvale Street
Wilmington, MA 01887

A Sample Submission Label should accompany all submissions made to Charles River to insure accurate and prompt delivery to the intended destination. Please cut out, fill in, and adhere the label to the outside of your shipping package. Do not place the label in the location used by overnight courier.

• Will there be any additional charge for confirmatory testing of positive or unexpected MFIA™ results and how will confirmatory testing be performed?

Confirmatory testing will be performed free of charge using alternative serologic testing techniques such as ELISA, IFA, HAI, or WIB, where appropriate.

• Will MFIA™ reagents be available for customers to purchase?

Yes, MFIA™ reagents are available as the diagnostic testing panels listed above.

• Will ELISA reagents for rodent microbial antibody screening still be available?

Yes, we will continue producing ELISA plates and other ELISA reagents for Charles River customers; however, MFIA™ will be the primary testing method for mouse, rat, and rabbit serum samples that are submitted for microbial antibody testing.

Figure 1

Figure 1: A comparison of MFIA™ and ELISA for the limit of detection of rodent anti-viral antibodies. For this study, positive control serum was serially two-fold diluted and the results are reported as the reciprocal of the dilution factor on the x axis. The MFIA™ and corresponding ELISA scores are reported on the left and right y-axes, respectively. This study indicates that the limit of detection of MFIA™ and ELISA for rodent anti-viral antibodies is comparable.

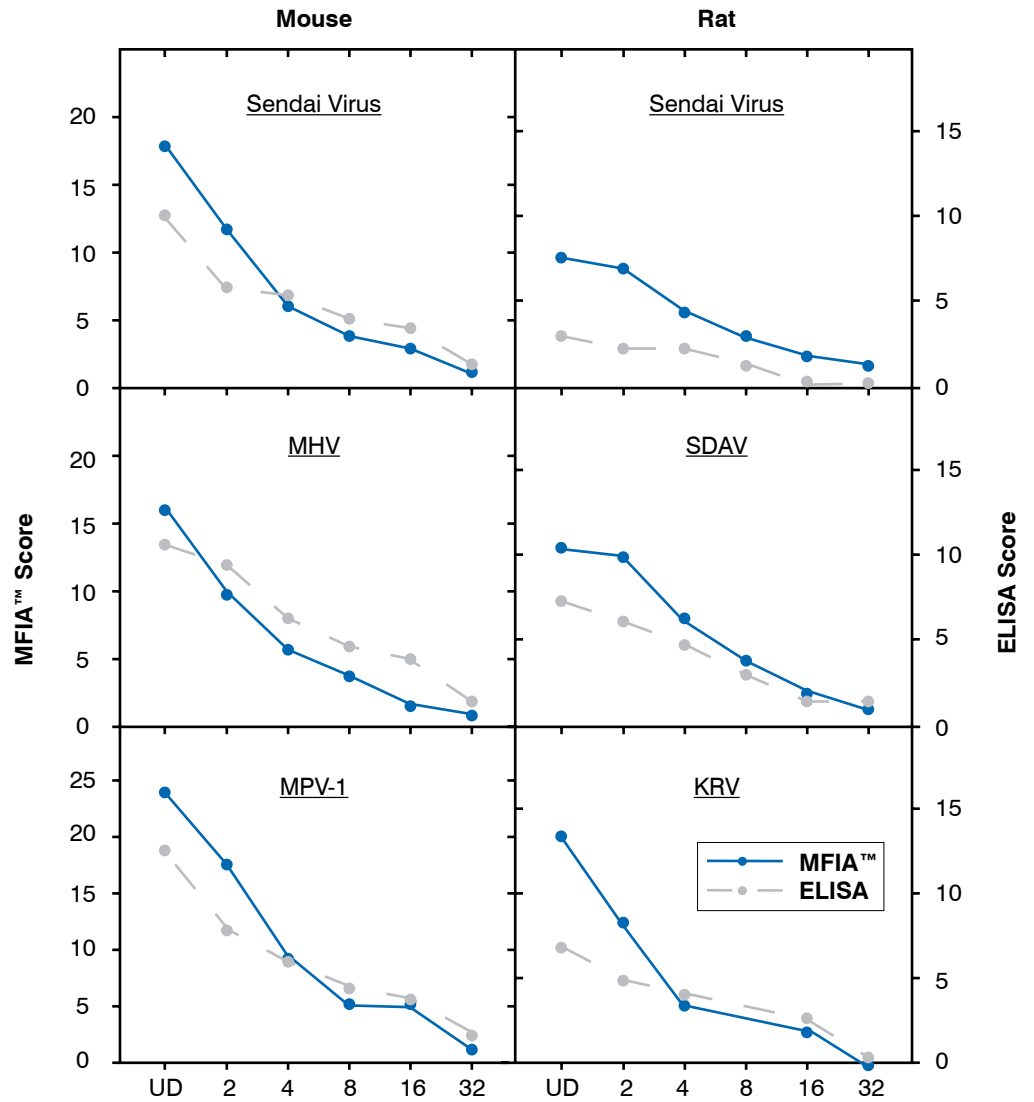


Figure 2

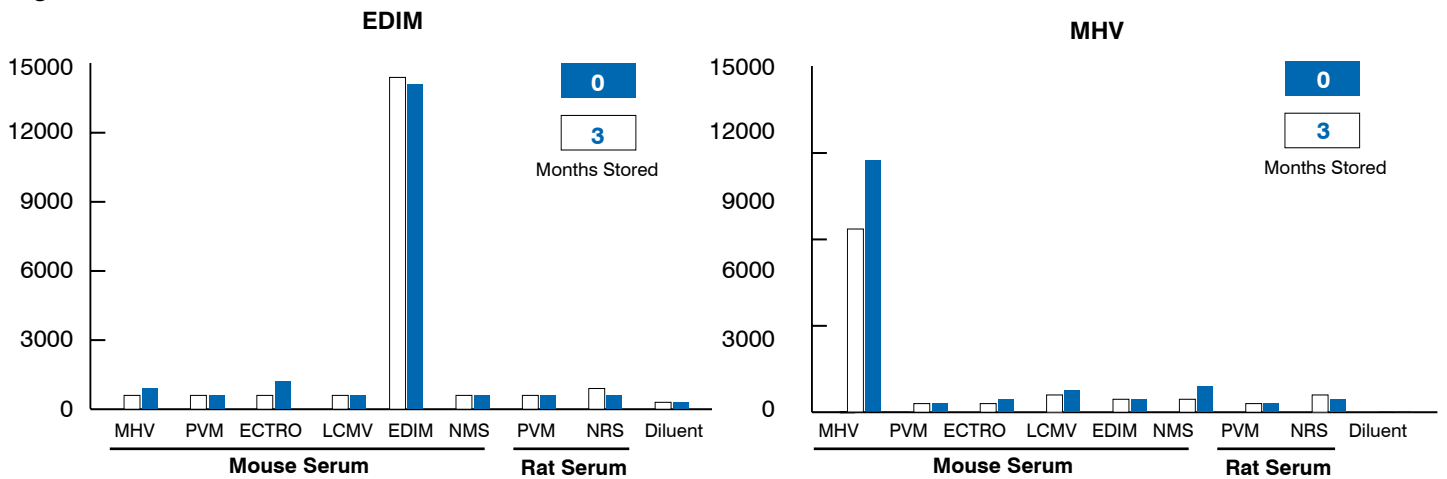


Figure 2: Selectivity of MFIA™ beads after preparation and storage at 4°C for three months. This study demonstrates that covalently attached antigens are not exchanged between different bead sets during long term storage of multiplexed assay panels. This study also demonstrates that there is very little non-specific cross reactivity among antigens, i.e. that the antigens are selective.

Table 1

Classification	Mice		Rats		All	
	#	%	#	%	#	%
True Negative	2496	100.0%	2182	98.8%	4678	99.4%
Borderline	0	0.0%	3	0.1%	3	0.1%
Nonspecific	0	0.0%	0	0.0%	0	0.0%
False Positive	0	0.0%	23	1.0%	23	0.5%
# of Assays	2496		2208		4704	

Table 1: Evaluation of MFIA™ diagnostic specificity with a large number of known negative rodent serum samples: this study demonstrates that MFIA™ is highly specific and that MFIA™ results are comparable to those that we have come to expect from ELISA.

Table 2:

		MFIA™		
			+	-
E L I S A	+	#	154	5
		%	22.9%	0.7%
	-	#	4	509
		%	0.6%	75.7%

of Assays: 672
% Agreement: 98.7%

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E L I S A	+	#	154	5
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	-	#	4	509
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of Assays: 672
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Table 2: Comparison of MFIA™ and ELISA classifications of pathogen-free and microbial antibody positive rodent sera. This data shows that there is almost 99% agreement between MFIA™ and ELISA results for mouse serum samples, indicating that there is excellent agreement between the two assays. For rat serum samples, there was 94% agreement between MFIA™ and ELISA, which is very good; however, based on IFA assays that were performed on serum samples where MFIA™ and ELISA disagreed, we believe that the MFIA™ was detecting the early stages of infections before the samples became ELISA positive (i.e. MFIA™ might be slightly more sensitive, at detecting early infections, than ELISA).