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Technical Sheet

Qualification of EZ-Spot™ Dried-Blood-Spot (DBS) Samples for Rodent Serology

Serology is used in the surveillance of lab animal colonies for adventitious infections by viruses and other invasive, immunogenic microbial pathogens. Modern serologic immunoassay techniques, such as the Luminex® xMAP®-based multiplexed fluorometric immunoassay (MFIA®) developed at Charles River,[4] are rapid, inexpensive and highly sensitive, permitting pathogen-specific antibodies to be detected within 1 to 3 weeks of infection and indefinitely thereafter.

Small rodents, including mice and rats, are frequently euthanized for blood collection to ensure that the volume of serum obtained is adequate to perform large serologic panels as well as confirmatory repeat testing. The dried blood spot (DBS) is an alternative to serum that has long been used for neonatal screening[3] and is increasingly popular for bioanalytical testing of rodents in pharmacologic studies.[1]

EZ-Spot is the Charles River system for the collection, storage, shipment and processing of DBS samples for serologic testing. A drop of blood, readily collected from a conscious mouse or rat, is applied to one of the four circumscribed spots on an EZ-Spot card labeled with the sample ID. DBS are allowed to dry at room temperature for an hour; cards are then placed in plastic bags with desiccants for storage at 4 °C or shipment at ambient temperature. In the Charles River serology laboratory, serum immunoglobulins are eluted from a portion of each DBS for primary screening by MFIA® and repeat testing, typically by IFA.

For research colonies maintained in individually ventilated and static microisolation cages, serology is usually performed on sentinels exposed to colony infections by routine transfers of soiled bedding. A limitation of this indirect approach to surveillance is that certain pathogens are transmitted inefficiently or not at all via soiled bedding.[2] Moreover, the ability of microisolation cages to control the spread of infection frequently keeps the percentage of cages with actively infected rodents low, thereby increasing the risk that the pathogen dose in pooled bedding will be insufficient to infect and cause seroconversion in sentinels.

Minimally invasive survival blood collection with EZ-Spot facilitates direct serologic testing of immunocompetent colony animals to:

- Supplement (or replace) sentinel surveillance;
- Verify positive sentinel findings;
- And, in conjunction with PCR, identify and preserve uninfected cages containing unique rodent models.

Moreover, antemortem blood collection with EZ-Spot saves labor and is consistent with the goals of the 3Rs to reduce and refine the use of animals in biomedical research.

As is our standard practice prior to introducing a new assay method, we have carried out an extensive qualification study of the suitability of EZ-Spot samples for serosurveillance by MFIA®. The study results presented herein show that the overall performance characteristics and diagnostic accuracy of MFIA® on EZ-Spot DBS and serum samples are equivalent.

The Study

Immune and nonimmune EZ-Spot DBS serum sample pairs from mice and rats were tested by MFIA®. The immune (known-positive) samples were prepared from rodents naturally or experimentally infected with one or more pathogens; the nonimmune (known-negative) samples were derived from barrier-raised immunocompetent, specific pathogen-free (SPF) mice and rats. MFIA® results are presented as median fluorescence intensity (MFI) in thousands or as net and tissue control (TC) scores calculated according to the formulae shown in the footnotes of Table 1.

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Analytical Specificity and Sensitivity

Mouse and Rats Assessment-Plus MFIA® panels (comprising 29 and 23 assays, respectively) were performed on serial dilutions of (typically monospecific) immune EZ-Spot DBS eluates and paired serum samples. Dilutions ranged from the standard test dilution (STD) to a 3,125-fold dilution. For summarizing results, assay-antiserum combinations were classified as homologous or heterologous if the average net score for serum at the STD was ≥ 4.5 or < 1.5, respectively. An assay-antiserum combination was classified as borderline if the average net score at the STD was between 1.5 and 4.4. As summarized in Table 1, there were no positive MFIA® reactions (i.e., those having net scores ≥ 2.5) with 20 DBS eluates and paired sera at the STD in 471 heterologous MFIA®. Heterologous net scores were well below the negative cutoff score of 1.5, averaging 0.13 for DBS eluates compared to 0.12 for sera. Thus, immune DBS eluates did not react nonspecifically in heterologous MFIA®. The titration curves for homologous MFIA® of immune DBS eluates and sera shown in Figure 1 run in parallel, with serum net scores being on average slightly higher than those for DBS eluates. The strong correlation between DBS eluate and serum net scores is supported by the linear regression curves and analyses presented in Figure 2.

Diagnostic Specificity (DSp), Sensitivity (DSn) and Reproducibility

Two analysts each performed triplicate Assessment-Plus MFIA® panel runs of a common set of samples consisting of 32 DBS-serum pairs. The set was equally divided between samples from rats and mice, and between immune and nonimmune samples.

DSp (Table 2): None of the 2,496 MFIA® of 16 nonimmune DBS eluates and paired sera in the 6 runs had positive net scores $\geq$ 2.5. Net and TC scores were consistently nominal for DBS eluates as well as the sera.

DSn (Table 3): One or more of the 16 immune samples was positive in 24 (of 29) mouse and 23 (of 23) rat MFIA®. Of the 720 immune sample-assay combinations expected to yield positive results, 714 (99.2%) of the sera and 702 (97.5%) of the DBS eluates gave positive net scores. Thus, the high DSn and reproducibility of net scores for MFIA® with immune sera were replicated with DBS eluates

Sample Suitability (Table 4): Standard internal controls to assess sample suitability included microbeads coated with a tissue control (which is an extract of either uninfected host cells or a related microorganism) and Anti-Test Species IgG (which is an affinity-purified rabbit or goat IgG that is made specific to IgG of the species being tested by absorption with IgG from related species). As noted, the tissue control (TC) detects nonspecific binding of the serum immunoglobulin indicated by an MFI $\geq$ 1,500. The TC background for DBS eluates and paired sera were the same and substantially below the upper acceptable limit of 1,500 (although, as expected, background was higher for immune versus nonimmune samples and for rat versus mouse samples). The Anti-Test Species IgG control determines whether a sufficient concentration of the correct IgG species was added to a test well; the IgG concentration is considered to be insufficient if the MFI is below a minimum of around 8,000. Anti-Test Species IgG control MFI for DBS eluates were uniformly above the lower acceptance limit, with MFI averages and standard deviations identical to those for serum samples.

Correlation of Net Scores: Linear regression analysis of net scores by sample and assay demonstrated a strong correlation between average net scores for sera and paired DBS eluates (Figure 3) and for assays of DBS eluates carried out by the two analysts (Figure 4). For both comparisons, R^2 exceeded 0.96 and slopes were near 1.

Discussion

This study to qualify the use of EZ-Spot DBS for serologic testing was undertaken because of the advantages of the DBS sample type in comparison to serum; these advantages include simpler, less invasive antemortem sample collection, elimination of the steps, reagents and equipment required for preparing serum, and the ability to ship DBS safely in envelopes or small boxes at ambient temperature (rather than shipping serum in leak-proof vials in Styrofoam containers with dry ice or cold packs). Easier antemortem blood collection for DBS facilitates direct sampling of colony animals to supplement sentinel monitoring, verify positive sentinel findings and identify and cull cages of infected animals.

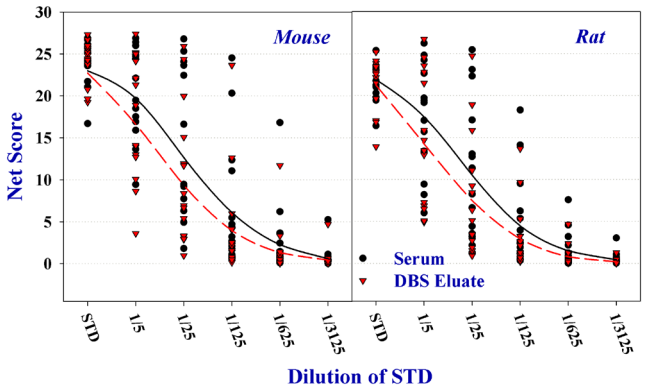
The analytical performance, diagnostic accuracy and reproducibility of MFIA® with DBS and serum samples were compared using immune samples from naturally and experimentally infected mice and rats, and nonimmune samples from SPF barrier-reared rodents. MFIA® of heterologous immune and nonimmune DBS eluates were uniformly negative with the same low background signals as with serum samples. The titration curves for MFIA® of homologous immune DBS eluates and paired sera were comparable and there was strong correlation between DBS eluate and serum net scores. The MFIA® results for triplicate runs of preparing and testing DBS eluates by each of 2 analysts showed a DSp (diagnostic specificity) of 100% and DSn (diagnostic sensitivity) of 98%, versus 99% for serum. Additionally, there was strong correlation between MFIA® net scores for immune DBS eluates and matching sera, and between the immune DBS eluate net scores obtained by the 2 analysts. In summary, this study shows that the results of MFIA® of DBS are analytically and diagnostically equivalent to those with serum; therefore, the EZ-Spot DBS is a suitable alternative to serum for serologic testing.

Table 1. Heterologous MFIA® of Immune EZ-Spot DBS Eluates and Paired Serum Samples at the Standard Test Dilution

Matrix	Species	Assay Count	Net Score	±	SD	% Positive
Serum	Mouse	267	0.08	±	0.21	0.0%
	Rat	204	0.17	±	0.37	0.0%
	Total	**471**	**0.12**	**±**	**0.30**	**0.0%**
DBS Eluate	Mouse	267	0.11	±	0.22	0.0%
	Rat	204	0.14	±	0.30	0.0%
	Total	**471**	**0.13**	**±**	**0.25**	**0.0%**

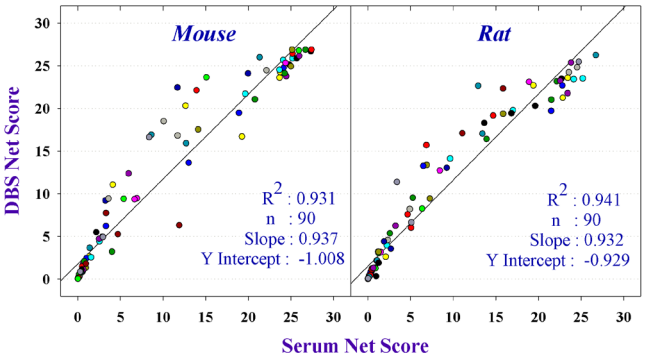
The Assessment Plus mouse and rat MFIA® panels comprise 29 and 243 assays, respectively. Net scores presented in this and subsequent tables and figures were calculated as follows: 1) Net median fluorescence intensity (MFI) = MFI Antigen – MFI Tissue Control; 2) Net Score = IF Net MFI ≤ Positive Cutoff MFI Then Net MFI/Cutoff MFI * 3 ELSE (Net MFI-Cutoff MFI)/1000 + 3.

Figure 1. Endpoint Titrations of Immune EZ-Spot DBS Eluates and Paired Serum Samples by Homologous MFIA®



Circles denote the net scores for 15 rat and 15 mouse homologous assay-antiserum combinations. The triangles show the net scores for paired DBS eluates. The solid and dashed lines represent the average for all homologous immune assay-serum and -DBS eluate combinations. STD is the standard test dilution.

Figure 2. Correlation of Immune EZ-Spot DBS Eluate and Serum Net Scores by Homologous MFIA®



The linear regression analysis, including calculation of the correlation coefficient R^2, slope and Y-intercept values, was performed in SigmaPlot™ 11.0 (Systat Software).

Table 2. Diagnostic Specificity of MFIA® with Nonimmune EZ-Spot DBS Eluates and Paired Serum Samples

Samples		# of MFIA				Average ± Std Dev	
Species	**#**	**Panel**	**Runs**	**Total**	**Matrix**	**Net Score**	**TC Score**
Mouse	8	29	6	1,392	Serum	0.02 ± 0.05	0.05 ± 0.02
					DBS	0.04 ± 0.06	0.05 ± 0.02
Rat	8	23	6	1,104	Serum	0.07 ± 0.18	0.06 ± 0.02
					DBS	0.01 ± 0.12	0.13 ± 0.05

DBS eluate and serum sample pairs were prepared from barrier-reared, immunocompetent SPF rodents. Assessment Plus mouse and rat MFIA® panels comprised 29 and 23 microbial antibody assays, respectively. All specimens were tested in 6 separate runs, with 3 runs being performed by each of 2 analysts.

Table 3. Diagnostic Sensitivity of MFIA® with Immune EZ-Spot DBS Eluates and Paired Serum Samples

Samples		# of MFIA				Net Score (Avg ± Std Dev)	Positive	
Species	**#**	**Positive**	**Runs**	**Total**	**Matrix**		**#**	**%**
Mouse	8	29	6	1,392	Serum	20.8 ± 3.4	324	100%
					DBS	18.0 ± 4.4	314	97%
Rat	8	23	6	1,104	Serum	20.7 ± 2.7	390	98%
					DBS	18.3 ± **3.5**	394	98%
Total	**16**	**47/52**	**6**	**720**	**Serum**	**20.6 ± 3.0**	**714**	**99%**
					DBS	**18.4 ± 3.9**	**708**	**98%**

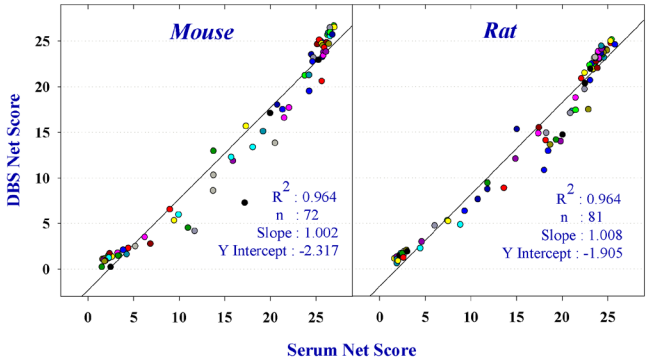
Paired DBS eluate and serum samples were prepared from naturally and experimentally infected rodents. The samples were positive for 24 (of 29) mouse and all 23 rat MFIA®. All specimens were tested in 6 separate runs, with 3 runs being performed by each of 2 analysts.

Table 4. Internal Sample Suitability Control MFI Signals for EZ-Spot DBS Eluates and Paired Serum Samples

Internal Control Beads # Test/ species		MFI/1000 (Average ± Std Dev)			
	Species:	Mouse		Rat	
	Sample Set	Serum	DBS Eluate	Serum	DBS Eluate
TC (96)	SPF	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
	Immune	0.3 ± 0.2	0.4 ± 0.2	0.8 ± 1.7	0.8 ± 1.2
Anti-Test Species IgG (48)	SPF	22.5 ± 0.9	22.5 ± 0.7	12.2 ± 0.8	11.9± 0.7
	Immune	23.4 ± 0.6	22.8 ± 1.3	12.4 ± 0.9	12.4 ± 0.9

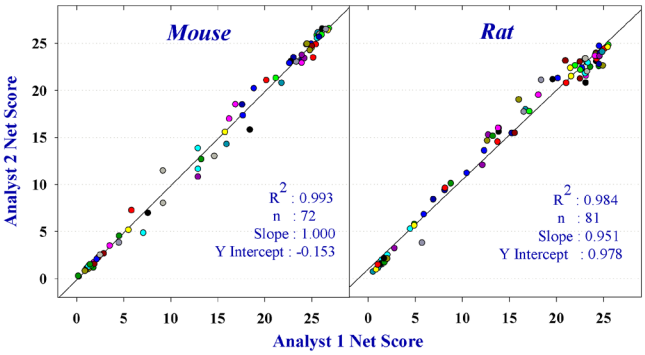
MFIA® internal controls include microbeads coated with a tissue control (TC), which is an extract of either uninfected host cells or a related microorganism; and Anti-Test Species IgG, which is an affinity-purified rabbit or goat IgG that is made specific to IgG of the species being tested by absorption with IgG from closely related species. Both controls demonstrate sample suitability. The TC detects nonspecific binding of the serum immunoglobulin indicated by an MFI ≥ 1,500, while the Anti-Test Species IgG control determines whether a sufficient concentration of the correct IgG species has been added to the test well. The IgG concentration is considered to be insufficient if MFI in thousands is below the minimum acceptable value of 8.

Figure 3. Correlation of Immune EZ-Spot DBS Eluate and Serum MFIA® Net Score Averages from 6 Runs of 8 Immune Sample Pairs per Species



A total of 8 pairs of DBS eluates and serum samples per species were tested in 6 runs. Each dot represents a comparison of the average net scores for a DBS eluate and its paired serum f sample-assay combination. The linear regression analysis, including calculation of the correlation coefficient R^2, slope and Y-intercept values, was performed in SigmaPlot™ 11.0 (Systat Software).

Figure 4. Correlation of Analyst MFIA® Net Score Averages from 3 Test Runs per Analyst of 8 Immune EZ-Spot DBS Eluates per Species



A total of 8 DBS eluates per species were tested in 3 separate runs by each of 2 analysts. Each dot represents a comparison of the average net scores obtained by the 2 analysts for a sample-assay combination.

References

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3. Mei J, Alexander R, Adam B, Hannon W. 2001. Use of filter paper for the collection and analysis of human whole blood specimens. *J Nutr* **131**:1631S–1636S.

4. Wunderlich M, Dodge M, Dhawan R, Shek W. 2011. Multiplexed fluorometric immunoassay testing methodology and troubleshooting. *J Vis* Exp **58**:3715.