Comparison of Charles River PCR Rodent Infectious Agent (PRIA) Panels to Standard Diagnostic Methodologies

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I. Introduction

The polymerase chain reaction (PCR) technique utilized at Charles River to detect infectious agents is the fluorogenic 5' endonuclease TaqMan® PCR from Life Technologies. In addition to the primers used in standard gel-based PCR, this method incorporates a TaqMan® probe that hybridizes to a sequence between those targeted by the primers. The probe is labeled with two dyes, a fluorescent reporter dye and a quencher dye that masks the reporter dye signal while the probe is intact. During specific amplification, the Taq polymerase digests hybridized probe, releasing the reporter dye and generating a sequence-specific signal [2; 4].

Why have we chosen to develop and deploy TaqMan® assays rather than the standard gel-based PCR tests?

- TaqMan® assays are typically **more specific** than gel-based tests because a positive signal is generated only when the probe (as well as the primers) hybridizes to the targeted microbial genomic sequence. In addition, test wells are sealed after sample and control addition and do not need to be opened to read results. This closed system reduces amplicon contamination of the lab that can lead to false-positive reactions.

- TaqMan® assays have been reported to be up to one-hundred times **more sensitive** than corresponding gel-based PCR[1; 3]. The reasons for this include the sensitivity of the fluorometers used to measure the reporter dye signal, the high number of cycles that can be run because probe does not bind to nonspecifically amplified product and the small size of TaqMan® PCR amplicons.

- "Real Time" TaqMan® PCR are quantitative. For many agents, we are able to use the quantity of microbial genome in the specimen to distinguish active infection from low-level environmental contamination.

PCR rodent infectious agent (PRIA) panels are performed on the OpenArray® platform, also from Life Technologies. OpenArray® assays are spatially multiplexed TaqMan® PCR. Each OpenArray® assay is performed in separate duplicate "holes" within the 64-hole subarray to which a sample is added. This avoids the low sensitivity and specificity pitfalls of standard PCR multiplexing when primers for different agents are mixed together in the same well.

Because we continue to use the fluorogenic PCR technique on the OpenArray®, we did not need to develop all new tests for this platform. Rather, for many agents we were able to transfer, to the OpenArray®, 96-well TaqMan® assays used and refined over many years. Thus, we have been able to keep the important benefits of TaqMan®technology in PRIA OpenArray®.

The data in this report provide evidence that:

- The detection limits (LOD) of TaqMan PCR carried out in PRIA OpenArray®match those of corresponding 96-well assays.

- PRIA OpenArray® assays clearly distinguish between positive and negative samples.

- The diagnostic sensitivity and specificity of PRIA OpenArray® assays for a broad range of infectious agents (e.g., viruses, bacteria and parasites) and standard diagnostic methodologies (including serology, microbiology and parasitology) are comparable.

II. Performance of PRIA OpenArray® TaqMan® PCR

A. Analytical Performance

1. Detection limits in comparison to corresponding 96-well assays

The LOD (or analytical sensitivity) is the smallest amount of assay target (in this case a microbial genomic sequence) that can be consistently distinguished from background. The LODs of quantitative TaqMan® PCR in the OpenArray® and 96-well formats were compared by testing serial 10-fold dilutions of positive template control (PTC) containing from 100 and 0.1 copies of the targeted microbial gene sequence per 5 μL. As shown in Figure 1, the sensitivities of OpenArray® and corresponding 96-well TaqMan® PCR were comparable and $\leq$ 10 PTC copies, despite the OpenArray® assays' small reaction volume of 30 nL per hole, versus 25 μL per well of a 96-well plate.

2. Signal-to-noise ratios

In addition to LOD, the diagnostic accuracy of assay results is dramatically affected by the separation between the signals given by positive and negative samples, and the variation in the background signal given by negative samples. This is commonly referred to as the signal-to-noise ratio. Based on our many years of experience developing TaqMan® PCR, we have been able to design OpenArray® assays that give consistently absent or low Δ Ct values for negative samples and high Δ Ct values easily distinguished from background for positive specimens (Figure 3).

B. Diagnostic Performance in Comparison to Standard Diagnostic Methodologies

To evaluate the accuracy of the PRIA OpenArray® assays, we compared PRIA results to those obtained by standard diagnostic methodologies including serology, microbiology and parasitology. This comparative testing was performed on 86 mice and 60 rats from conventional colonies enzootically infected with a variety of viral, bacterial and parasitologic pathogens, and approximately one thousand specific-pathogen-free (SPF) mouse, rats and hamsters sampled from Charles River breeding colonies this year and last. As shown in Tables 1 and 2 for conventional and SPF rodents, respectively, there was a high degree of correspondence between the prevalent pathogenic and opportunistic microorganisms detected by PRIA and those found by standard methodologies. Overall, the percent positive by PRIA was comparable to the prevalence by the standard methodologies, thus demonstrating that PRIA OpenArray® assays are sensitive and specific. For 5 of 9 agents, the percentages of conventional mice that were positive by serology were higher than by PCR. This is expected, as for most agents the active phase of infection in an enzootically infected colony is limited to the 6 to 10 week-old age group. It should be noted that fur swabs, the optimal sample type for fur mites, were not collected; consequently, the percentage of animals that were fur mite PCR-positive was artificially low.

III. Discussion

The PRIA panel assays (and PCR in general) are an accurate and reliable substitute for standard diagnostic methodologies. In comparison to microbiology and parasitology, PRIA testing offers the key benefit of avoiding live animals shipments, which are expensive as well as hazardous during hot weather. Noninvasively collected fecal specimens can be used to detect most prevalent pathogens and opportunists, as many are shed from the gastrointestinal tract; fur swabs can be tested for ectoparasites. Thus, PCR testing can be performed directly on animals in quarantine or on study. Because of its exquisite sensitivity, the PCR can be used to test highly pooled specimens representing many cages. This is particularly helpful when attempting to corroborate unexpectedly positive sentinel findings (often positive viral serology results) for colonies housed in microisolation cages where the prevalence of actively infected animals is often quite low.

IV. References

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3. **Guo Y, Cheng A, Wang M, Shen C, Jia R, Chen S, Zhang** N. 2009. Development of TaqMan MGB fluorescent real-time PCR assay for the detection of anatid herpesvirus 1. Virol J 6:71.

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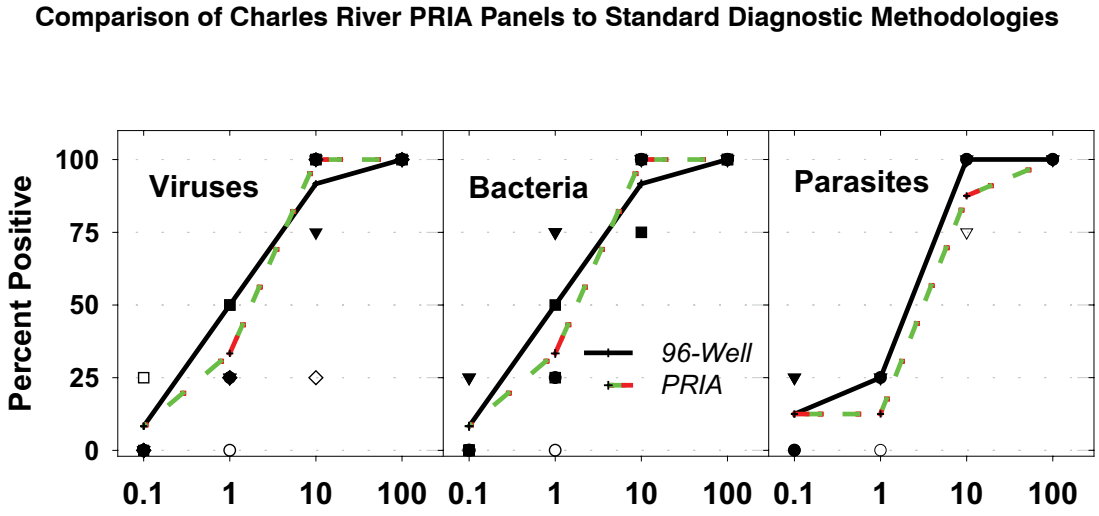

Figure 1.

Comparison of limits of detection (LOD) of quantitative Charles River TaqMan® PCR PRIA assays in the OpenArray® and 96-well formats. The viral tests compared include MHV (✷), MNV (▼), MPV-NS1 (■), and TMEV (◆); bacterial tests include β Strep group G (✷), *Mycoplasma pulmonis* (▼), and *Pasteurella pneumotropica*; the parasite assays are for fur mites (✷) and pinworms (▼). Filled and empty symbols are for 96-well and PRIA assays, respectively. The lines connect the average percent positive for each infectious agent.

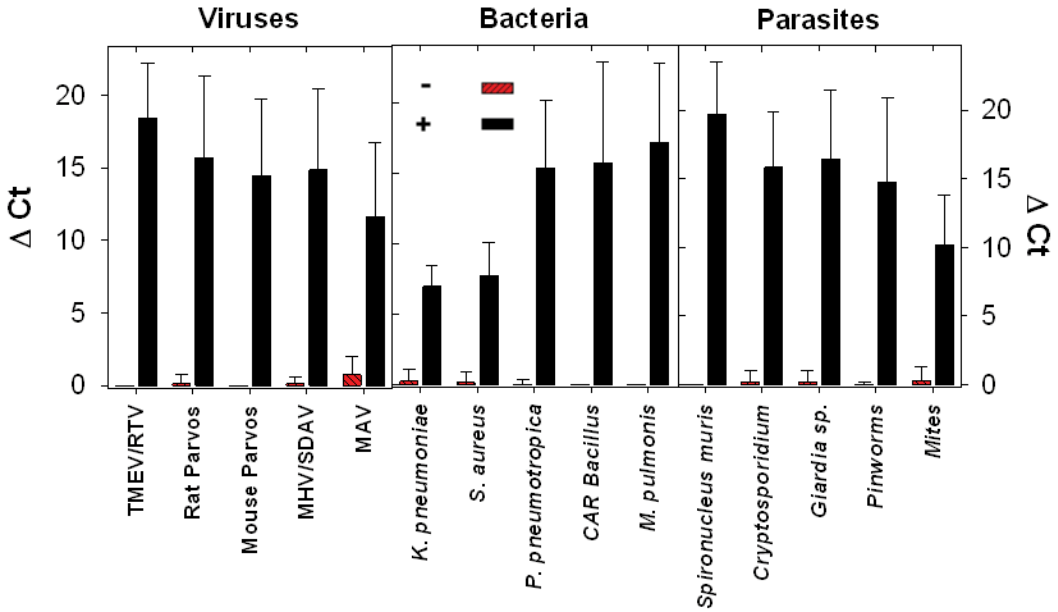

Figure 2.

Charles River PRIA OpenArray® TaqMan® PCR testing of conventional mice and rats (n=146): Comparison of positive (+) and negative (-) signals. The 146 animals comprised 86 mice and 60 rats derived from conventional, enzootically infected rodent breeding colonies that typically supply animals to pet shops. Animals were equally divided among 3 age groups, including 3-4 weeks old, 6-10 weeks old and retired breeders. The assay results are shown as the change in the cycle threshold (Δ Ct) for the test specimens from a base Ct, or Ct0. The bar represents a standard deviation. Nucleic acid was extracted from pooled specimens of feces, and nasopharyngeal and bronchial washes. The percentage of samples classified as positive by the assays varied from 16% (for CAR bacillus) to > 99% (for *S. muris*).

Table 1. Comparison of the microbial pathogens and opportunists detected in conventional mice and rats (n=146) by PRIA OpenArray® PCR to those found by standard diagnostic methodologies[A]

		% Positive	
Standard	**Agent**	**Standard**	**PRIA**
Microbiology	*C. rodentium*	0%	0%
	K. oxytoca	0%	0%
	B. bronchiseptica	6%	9%
	C. kutscheri	7%	21%
	K. pneumoniae	4%	43%
	P. aeruginosa	28%	27%
	S. aureus	10%	26%
	Strep. Pneumoniae	0%	0%
	Beta Strep. sp	0%	0%
	Beta Strep. sp. - Group G	0%	0%
	Beta Strep. sp. - Group B	0%	1%
	Salmonella sp.	0%	0%
	Helicobacter sp.	NT[B]	100%
	S. moniliformis	NT[B]	28%
	Microbiology subtotal	**5%**	**10%**
Serology	MAV	32%	57%
	MNV	0%	0%
	MHV/SDAV	95%	62%
	Mouse Parvos	86%	62%
	Rat Parvos	79%	27%
	TMEV/RTV	85%	93%
	CAR Bacillus	13%	16%
	M. pulmonis	53%	49%
	P. carinii	NT[B]	8%
	Serology Subtotal	**65%**	**46%**
Parasitology	Mites[C]	55%	26%
	Pinworms	17%	28%
	Giardia sp.	16%	29%
	Spironucleus muris	NT[B]	68%
	Parasitology Subtotal	23%	28%
	Grand Total	29%	24%

[A] The 146 rodents comprised 86 mice and 60 rats derived from conventional, enzootically infected rodent breeding colonies that typically supply animals to pet shops. Animals of each species were equally divided among 3 age groups, including 3-4 weeks old, 6-10 weeks old and retired breeders. Nucleic acid was extracted from pooled specimens of feces, and nasopharyngeal and bronchial washes. The percentage of samples classified as positive by the assays varied from 16% (for CAR bacillus) to > 99% (for *S. muris*).

[B] Although not tested (NT) by a standard methodology, the positive PRIA results were confirmed by alternative PCR or DNA sequencing.

[C] Fur swabs, the recommended sample type for mites, were not collected.

Table 2. Comparison of the microbial pathogens and opportunists detected in conventional mice and rats (n=146) by PRIA OpenArray® PCR to those found by standard diagnostic methodologies[A]

		% Positive	
Standard	**Agent**	**Standard**	**PRIA**
Microbiology	*Bordetella bronchiseptica*	0%	0%
	Beta Strep. sp.	0%	0%
	Beta Strep. sp. - Group B	16%	37%
	Beta Strep. sp. - Group G	0%	1%
	K. oxytoca	3%	5%
	P. aeruginosa	3%	4%
	Salmonella sp.	0%	0%
	Staph. aureus	0%	0%
	Strep. pneumoniae	52%	54%
	Campylobacter jejuni[B]	0%	1%
	Campylobacter spp.[B]	KP C	53%
	Helicobacter spp.[B]	KP C	100%
	S. moniliformis	KP C	100%
	Microbiology Subtotal D	**9%**	**11%**
Serology	Mouse Parvoviruses	0%	0%
	Rat Parvoviruses	0%	0%
	MHV/SDAV	0%	0%
	MNV	0%	0%
	TMEV/RTV	0%	0%
	ROTA-A	0%	0%
	MAV	0%	0%
	CAR bacillus	0%	0%
	M. pulmonis	0%	0%
	P. carinii	0%	0%
	Serology Subtotal	0%	0%
Parasi-tology	Pinworms	0%	0%
	Giardia sp.[B]	25%	67%
	Parasitology Subtotal	0%	1%
Grand Total		6%	5%

[A] Rodent species comprise mice, rats and hamsters tested in 2010 and 2011. Approximately one thousand animals were tested including roughly equal numbers of mice and rats and fifty hamsters.

[B] *Campylobacter, Helicobacter* and *Giardia* PCR results are for HAMSTERS ONLY. Result for mice and rats were all negative.

[C] The hamster colonies are known-positive (KP) for campylobacters and helicobacters.

[D] The microbiology subtotal does not include PCR results for agents not detected by an alternative methodology, e.g., the campylobacters and helicobacters are not included in the microbiology subtotal.