

Negative Grey Zone Supplemental Testing to Enhance Sensitivity of Chlamydia Enzyme Immunoassays and Nucleic Acid Probe Assays.

National Chlamydia Laboratory Committee
National Infertility Prevention Project (IPP)

Introduction/Background

A major goal of screening programs for *Chlamydia* is to detect as many infections as possible with the limited funds available. Two testing methods commonly used to meet this goal are the Enzyme Immunoassay (EIA) and the Nucleic Acid Probe (NAP) assay. These methods offer affordable cost and high throughput capabilities. However the sensitivities of both the EIA and the NAP are significantly lower than those of the newer nucleic acid amplification assays such as Polymerase Chain Reaction (PCR), Strand Displacement Amplification (SDA), and Transcription-Mediated Amplification (TMA).

It is apparent that replacement of a non-amplified method with an amplified molecular method for screening would result in improved sensitivity for the detection of *Chlamydia* infections. However, the increased cost of performing the same number of tests with an improved, but more expensive method is often prohibitive. One possible strategy to keep costs manageable is to limit the number of tests performed by establishing selection criteria to screen those at higher risk with the more expensive amplified molecular methods. This may result in the performance of fewer tests for the same or only slightly higher cost while detecting more positives, thus actually lowering the cost per infection detected. This strategy may have limited application, however, since the sensitivity and predictive value of selective screening criteria often vary among different populations, selective screening may be logistically difficult in some settings, or selective screening by a non-amplified method may already be occurring.

An alternative to replacement of EIA or NAP methods with a nucleic acid amplification assay would be to improve the sensitivity of the current non-

amplified screening assay. This can be accomplished through the use of supplemental tests on selected specimens yielding borderline negative, or “negative grey zone”, results by the screening method. Use of supplemental testing on a small percentage of specimens which fall within a negative grey zone, but are likely to be false-negative, can significantly increase the sensitivity of the screening test without a large increase in cost. Several methods are available for negative grey zone supplemental testing of non-amplified screening methods. Direct Fluorescent Antibody (DFA), PCR and LCR have been shown to perform well on residual material from EIA specimens⁹. While DFA has not been feasible with NAP specimen remnants, there has been success using a Probe Competition Assay (PCA)⁴, or PCR¹⁵. In addition, TMA has been used as a potential supplemental assay for NAP.

Method Selection

The method used for supplemental testing will depend upon the capabilities of the laboratory, cost requirements, and the desired level of increased sensitivity. Is the equipment available, or will equipment need to be ordered? What is the laboratory’s experience level with the possible supplemental assays? Is training by an experienced laboratory an option? Are any other similar tests performed by the laboratory? Is a reference laboratory available to perform testing? If a laboratory has both DFA and molecular capabilities and experience, then which test fits best into the workflow? How many specimens will need supplemental testing? Is turn-around time a factor?

Factors to Consider in the Selection of a Supplemental Assay:

1. DFA on EIA remnants or PCA on NAP specimens are the least complicated to set up and perform (if FA/PCA capabilities already exist)
2. For DFA, intensive training by an experienced laboratory is recommended if *Chlamydia* DFA is not currently being performed.
3. NATTs have been shown to be more sensitive than DFA on EIA remnants.
4. Throughput can be higher for NATTs than for DFA.

5. Use of automated equipment such as diluters and sequential pipettors may cross-contaminate specimen remnants for molecular amplification testing. Procedural modifications may be necessary.
6. Even if the supplemental assay is in use for routine testing, use for sensitivity enhancement of another method may require additional verification for quality assurance purposes.

Defining the Gray Zone

For optimum cost-effectiveness, the lower limit of the grey zone selected for supplemental testing should be set at a “point of diminishing returns”, which may vary depending on the method used, its performance in a particular laboratory, and the population being tested. The goal is to detect a significant number of additional positives by conducting supplemental testing on as few specimens as possible. Each laboratory must determine the distribution of absorbance values (for EIA) or RLUs (for NAP) of their negatives specimens, and select a grey zone, which will identify a manageable number of specimens for supplemental testing. Each laboratory should also evaluate possible grey zones in terms of the yield of positives identified through this expanded testing. Often, the optimum grey zone will be fairly obvious when the distribution of absorbance’s/RLUs is evaluated, and the yield of positives for different grey zones is determined.

Factors to Consider in Selecting Grey Zone Lower Limit:

1. What is the maximum percentage of specimens that can be tested by a supplemental assay, and still is cost-effective?
2. How much of an improvement in sensitivity is desired?
3. Gender differences may exist, both in the percentage of specimens falling within a specified gray zone, and in the likelihood that those specimens will be positive.

Data: EIA

A variety of studies evaluating the use of EIA with supplemental DFA have been published (TABLE). In the studies reviewed, negative grey zones ranged from 30% to 70% of the cutoff OD, and percent of the total specimen results falling within the grey zone ranged from <0.5 % to >12.0 %. Increases in sensitivity ranged from 1.3% (in a study with a 30% GZ and 1.0% of total specimens tested) to over 20% (70% GZ and 12.4% of total specimens). Fewer studies have been published on the use of amplified molecular methods as supplemental tests. Data from these studies, and from unpublished reports, show an average sensitivity increase of over 10%, by testing an average of less than 5 % of total specimens.

Several studies have been conducted at the Wisconsin State Laboratory of Hygiene (WSLH) to determine the feasibility of using grey zone supplemental testing to improve the sensitivity of the Syva/Behring MicroTrak EIA routinely used for *Ct* screening. Specimens yielding results within a 50% negative gray zone (NGZ) were evaluated for the presence of *Ct* elementary bodies by centrifugation and DFA testing²⁹. It was determined that supplemental DFA testing on a small percentage of specimens could identify a significant number of positive specimens, which would otherwise be missed by EIA. Based on the results of this study, routine supplemental DFA testing of all *Ct* EIA specimens yielding NGZ results was initiated at WSLH. From October 1995 through February 1997, only 635 (1.0%) of 61,950 endocervical EIA specimen results fell within the 50% gray zone and required supplemental testing by DFA, with 143 (22.5%) found to be positive³⁵. These DFA-positive NGZ EIA specimens accounted for 5.4% of all positives detected during this time period. This improved sensitivity, with minimal additional costs for the supplemental DFA testing, has reduced the laboratory testing costs from \$154.21 per infection detected to \$148.38. In a subsequent study it was demonstrated that supplemental testing using PCR is slightly more sensitive, and more cost-effective than DFA³⁶. Therefore, PCR has replaced DFA as the supplemental test used to enhance the sensitivity of the EIA routinely used at WSLH.

Data: NAP

Several studies have been published on supplemental grey zone testing of Gen-Probe PACE 2 and PACE 2C NAP assays. A collaborative study by five state public health laboratories (CO, WY, UT, MT, and SD) was conducted to determine the performance characteristics of the Gen-Probe PCA as a supplemental test for NAP⁴. In this study, 0.4% of specimens initially tested by PACE 2 and 0.9% of those tested by PACE 2C fell within a negative grey zone of 200 RLUs to the cut-off. The supplemental PCA assay was found to be positive in 35% of PACE 2 specimens and in 12% of PACE 2C specimens.

In a recent study conducted by the Colorado Public Health Laboratory it was demonstrated that PCR can be used as a supplemental test using residual Gen-Probe PACE NAP specimens¹⁵. Although simple dilutions of small aliquots of Gen-Probe transport medium into Roche Amplicor transport medium produces inconsistent results, the use of a DNA purification device (Microcon 100, Amicon, Inc.) yields consistent results with end-point dilution studies using purified elementary bodies. When the technique of Microcon 100 purification coupled with PCR testing was performed on Gen-Probe PACE specimens, positive results were obtained for 86% of negative grey zone specimens testing positive by PCA, and for 7% of negative grey zone specimens, who were also negative, by PCA. It is estimated that routine use of PCR as a negative grey zone supplemental test for PACE NAP would increase detection of positives by 5-10%.

Recommendations:

Recommendation: Negative Grey Zone Supplemental Testing

Laboratories using EIA or NAP as their routine *Chlamydia* screening assay should evaluate and, if possible, implement negative grey zone supplemental testing to enhance the sensitivity of their testing procedures.

Recommendation: Method Selection

Due to the increased sensitivity, nucleic acid amplification techniques (NAATs) are preferable if the laboratory has the capability to perform the assays. However, DFA (for EIA) or PCA (for NAP) may be used if amplified techniques are not available, and if the laboratory has experience with these methods.

Recommendation: Selection of Appropriate Grey Zone

The grey zone lower limit must be empirically determined in each laboratory prior to performing supplemental testing. The range of absorbance's or RLU values of specimens selected for supplemental testing should be chosen that will maximize the number of additional positives detected and minimize the proportion of the negative specimens that require testing.

Recommendation: Specimen Processing and Testing Performance

Specimens must be processed, and assays performed, according to the manufacturers' specifications and recommendations wherever possible. Because supplemental testing of specimen residuals is not an intended use for some assays, in-house verification will be required for quality assurance purposes.

Recommendation: Result Reporting

There is the option of reporting both the results of the screening assay and the supplemental test, or reporting only a final interpretation. If both results are reported to the clinician, they should be reported together, along with a final interpretation. Non-reactive grey zone EIA or NAP results may be reported as "equivocal", "indeterminate" or other appropriate wording. A phrase explaining the supplemental testing, such as "a supplemental" or "a second, more sensitive assay was performed" may be added to explain the additional result.

Recommendation: Additional Studies

Additional studies should be conducted to evaluate the use of nucleic acid amplification assays NAATs for negative grey zone supplemental testing to enhance performance of EIA and NAP assays. In addition, economic analyses

should be conducted to evaluate these strategies as alternatives to using amplified molecular assays as routine screening tests.

TABLE. Studies Evaluating Negative Gray Zone *Chlamydia trachomatis* Supplemental Testing.

Screening Assay Author ^{Ref}	Supplemental Assay	Gray Zone	Total No. in Study	Negative Gray Zone					Estimated Increased Sensitivity
				No.	%	No. Tested	True Positive		
							No.	%	
Abbott EIA									
Abraham ¹	DFA	50%	22,064	892	4.0%	292	86	29.5%	9.5%
Hallander ¹⁶	Blocking Ab	40%	12,864	79	0.6%	79	31	39.2%	5.7%
Kellogg ¹⁷	Blocking Ab/DFA	70%	341	31	9.1%	31	6	19.4%	25.0%
Kellogg ¹⁸	Blocking Ab/DFA	70%	4,000	202	5.1%	202	34	16.8%	12.7%
Krepel ²¹	Blocking Ab/DFA	40%	61,744	1,076	1.7%	1076	217	20.2%	14.2%
Newhall ²⁵	Blocking Ab	0.04 OD	4,553						14.5%
Schwebke ³¹	DFA (2nd Spec)	70%	990	50	5.1%	50	7	14.0%	10.0%
Williams ³⁴	PCR	0.05-0.09 OD		44		44	12	27.3%	
Williams ³⁴	DFA	0.05-0.09 OD		44		44	8	18.2%	
Syva/Behring EIA									
Beebe ²	Repeat EIA/DFA	30%	4,571	31	0.7%	27	12	44.4%	3.1%
Beebe ³	DFA	50%	6,039	62	1.0%	43	22	51.2%	8.9%
Canas ⁵	DFA	30%	617	7	1.1%	7	4	57.1%	
Chan ⁶	DFA	30%	6,022	58	1.0%	58	7	12.1%	1.3%
Dean ⁹	DFA/PCR/LCR	50%	6,571	230	3.5%	230	29	12.6%	7%/5%/1%
Faur ¹⁰	DFA	30%	18,495	502	2.7%	502	195	38.8%	10.5%
Fonseca ¹³	DFA	30%	13,283	94	0.7%	94	21	22.3%	5.6%
Ferrero ¹²	DFA	50%	1,190	57	4.8%	57	2	3.5%	5.4%
Ferrero ¹²	PCR	50%	1,190	57	4.8%	57	4	7.0%	10.3%
Marrs ²²	DFA	30%	2,053	8	0.4%	8	7	87.5%	6.8%
McCarthy ²³	Blocking Ab	50%	15,069	319	2.1%	319	105	32.9%	
McCarthy ²³	DFA	50%	15,069	319	2.1%	319	80	25.1%	
Newhall ²⁵	DFA	0.10 OD	4,553						10.1%
ND DOH ²⁶	DFA	30%	>15,000	76	<0.5%	76	7	9.2%	
ND DOH ²⁷	DFA	30%	5,154	48	0.9%	48	15	31.3%	5.3%
ND DOH ²⁷	DFA/LCR	30%	5,154	48	0.9%	48	29	60.4%	9.8%
Ostergaard ²⁸	DFA/PCR	70%	8,716	253	2.9%	253	56	22.1%	11.6%
Pfister ²⁹	DFA	50%	43,422	567	1.3%	567	276	48.7%	8.0%
Region III ³⁰	PCR	70%	10,658	1,319	12.4%	1319	128	9.7%	20.2%
Spokane Co. ³²	DFA	45%	9,162	259	2.8%	259	64	24.7%	12.6%
Univ of WA ³³	LCR	30%	27,634	107	0.4%	105	28	26.7%	
WI State Lab ³⁵	DFA	50%	61,950	635	1.0%	635	143	22.5%	5.4%
WI State Lab ³⁶	DFA	50%	5,427	33	0.6%	33	11	33.3%	7.7%
WI State Lab ³⁶	PCR	50%	5,427	33	0.6%	33	14	42.4%	9.7%
Gen-Probe PACE 2 NAP									
Beebe ⁴	PCA	200 RLU	25,081	100	0.4%	100	35	35.0%	
Kluytmans ²⁰	PCA	40%	302	6	2.0%	6	3	50.0%	8.3%
Newhall ²⁵	PCA	200 RLU	4,553						7.2%
Region III ³⁰	PCR	70%	13,427	214	1.6%	214	101	47.2%	17.4%
Gen-Probe PACE 2C NAP									
Beebe ⁴	PCA	200 RLU	10,938	100	0.9%	100	12	12.0%	

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