



# DEVELOPMENT OF CCP3, A MORE SENSITIVE CYCLIC CITRULLINATED PEPTIDE ELISA

Rufus W. Burlingame<sup>1</sup>, Carlos A. von Muhlen<sup>2</sup>, Andrea L. Piette<sup>1</sup>, Walter L. Binder<sup>1</sup>

<sup>1</sup>INOVA Diagnostics, Inc., San Diego, CA; <sup>2</sup>PUC School of Medicine, Porto Alegre, Brazil

## ABSTRACT

**Objective:** Develop a third generation peptide for an ELISA that is more sensitive for detecting antibodies in rheumatoid arthritis (RA) patients than the current commercial second generation anti-CCP ELISA, while maintaining high specificity. Manufacture the test in a color-coded, breakaway well, ELISA format.

**Methods:** ELISA plates are coated with a cyclic peptide in which the amino acid citrulline is present at key parts of the sequence. Sera from 156 RA patients, 166 blood donors, 113 rheumatic disease controls and 87 infectious disease samples were tested on the second and third generation CCP ELISAs.

**Results:** For RA patients, the sensitivity of the anti-CCP3 test is 74%, 115 out of 156 RA patients were positive. This is a 5% increase in sensitivity over the second generation anti-CCP ELISA, which was 69%, or 107 out of 156. The specificity of CCP3 was 96% in the 366 control samples, compared to 98% for the second generation CCP ELISA. All 7 samples from the control group that were positive on the second generation CCP ELISA were also positive for anti-CCP3.

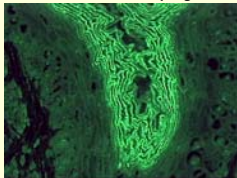
**Conclusion:** The new anti-CCP3 ELISA<sup>Patent Pending</sup> is 5% more sensitive than the current anti-CCP ELISA and it shows very high specificity in healthy volunteers and disease control groups. This increase in sensitivity may aid rheumatologists trying to diagnose RA patients early in the course of their disease.

## METHODOLOGY

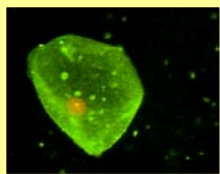
The INOVA QUANTA Lite™ CCP3 ELISA<sup>Patent Pending</sup> is a semi-quantitative immunosay that detects autoantibodies against CCP3 (Cyclic Citrullinated Peptide 3). These autoantibodies are markers for Rheumatoid Arthritis.

CCP3 is coated onto each well of a 96 well ELISA plate. Diluted patient samples are incubated in the wells for 30 minutes, followed by a 30 minute incubation with an anti-IgG conjugate. Bound antibodies are quantitated with a colorimetric substrate.

**Fig. 1**  
Anti-Keratin on Rat Esophagus



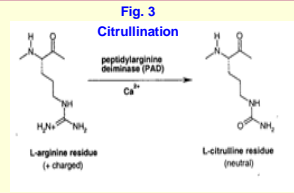
**Fig. 2**  
Anti-Perinuclear Factor



## INTRODUCTION

Rheumatoid Arthritis (RA) is one of the most common systemic autoimmune diseases. Recently, a new diagnostic test for RA, anti-CCP (Cyclic Citrullinated Peptide) was introduced. It has been known for many years that anti-perinuclear autoantibodies, also called anti-keratin, are found in people with RA<sup>1,2,3</sup> (see Figures 1 and 2). It was discovered that these antibodies recognize an epitope that contains the deiminated form of arginine called citrulline<sup>4</sup> (Figure 3). A circular peptide containing citrulline called CCP was found to be better at discriminating RA patients from other patients than either the perinuclear antibody test or the test for RF<sup>5</sup>. Additionally, RA patients with anti-CCP may progress to a more severe disease than those who do not have anti-CCP<sup>6</sup>.

Recent studies found some RA patients who were positive for perinuclear factor or anti-keratin, but negative for anti-CCP<sup>7</sup>, suggesting that the current CCP ELISA could be made more sensitive. It is important for disease management to diagnose and treat people with RA as early as possible<sup>8</sup>. Thus, we tested numerous peptides, both citrullinated and non-citrullinated, searching for those that detected antibodies in more RA patients than the current second generation CCP ELISA. Only citrullinated peptides showed strong reactivity with RA patients. We discovered a peptide that showed increased sensitivity in detecting antibodies in patients with RA, while keeping very high specificity in control patients with rheumatic and infectious diseases.



## Precision and Reproducibility

The within run variation for the CCP3 ELISA was measured in a number of ways and was excellent, averaging 2.9% for all positive samples.

Between run variation was measured by running samples on 6 separate occasions. The results below are the averages of 2 low, 2 negative and 5 high samples.

	Low	High	Negative
Unit	32	106	6
%C.V.	4.5%	3.4%	5.5%

## SENSITIVITY AND SPECIFICITY

### Clinical Sensitivity of CCP3 ELISA

RA Samples	CCP ELISA		Clinical Sensitivity
	+	-	
N=156			
CCP3	105	10*	CCP2 = 69%
ELISA	2**	39	CCP3 = 74%

### Clinical Specificity of CCP3 ELISA

All Controls	CCP ELISA		Clinical Specificity
	+	-	
N=366			
CCP3	7*	9**	CCP = 98%
ELISA	0	350	CCP3 = 96%

\* 9 and 6 of the discrepant samples were positive on RF IgM and RF IgG, respectively.

\*\* Both discrepant samples were high negative on the CCP3 ELISA with values of 18 and 19 EU.

\*Five of these double positive samples have both IgM and IgA RF, two other markers of RA.

\*\*Six of these were positive for either IgM, IgA, or IgG RF, or for anti-citrullinated flagrin, other markers of RA.

### Relative Sensitivity and Specificity of CCP3 ELISA

All Samples	CCP ELISA		Relative Sensitivity	Percent Agreement
N=522	+	-	= 98%	
CCP3	112	19*	Relative Specificity	= 96%
ELISA	-	2**		
		389	= 95%	

\* Ten of these patients have RA, 6 of the remaining are positive for RF, a marker antibody for RA

\*\* These 2 patients have RA, and were high negative, 18 and 19 EU, on the CCP3 ELISA

## REFERENCES

- Wener MH: Rheumatoid Factors. Manual of Clinical Laboratory Immunology, NR Rose et al eds, American Society for Microbiology Press, 961-972 (2002).
- Vincent C et al: Immunoblotting detection of autoantibodies to human epidermis flaggrin: a new diagnostic test for rheumatoid arthritis. J Rheumatol 25:838-846 (1998).
- Serre G et al: Flaggrin (keratin) autoantibodies. Autoantibodies, JB Peter and Y Shoefeld, eds, Elsevier Science B.V. 271-276 (1996).
- Vossenaar ER and Van Venrooij WJ: Anti-CCP antibodies, a highly specific marker for (early) rheumatoid arthritis. Clin Applied Immunol Rev 4: 239-262 (2004).
- Schellekens GA et al: The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic Citrullinated peptide. Arthritis Rheum 43:155-163 (2000).
- van Jaarsveld CHM, et al: The prognostic value of the antiperinuclear factor, anti-citrullinated peptide antibodies and rheumatoid factor in early arthritis. Clin Exp Rheum 17: 1689-697, (1999).
- Saraux A et al: Value of antibodies to citrulline-containing peptides for diagnosing early arthritis. J Rheumatol 30:2535-2539 (2003).
- Kavanaugh A and Keystone EC: The safety of biologic agents in early rheumatoid arthritis. Clin Exp Rheumatol 21: (supplement) S-203-S-208 (2003).

## CONCLUSIONS

- The third generation CCP3 ELISA<sup>Patent Pending</sup> has an overall sensitivity of 74% for RA patients, a 5% increase from the current second generation CCP ELISA.
- The clinical specificity of the CCP3 ELISA in a group of patients with lupus, scleroderma and hepatitis C compares favorably with current ELISA at 96%.