

1 **Anti-chlamydial effect of plant peptides**

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14 **Abstract**

15 Even in asymptomatic cases of *Chlamydia trachomatis* infection, the aim of the antibiotic
16 strategy is eradication of the pathogen so as to avoid the severe late sequelae, such as pelvic
17 inflammatory disease, ectopic pregnancy, and tubal infertility. Although first-line
18 antimicrobial agents have been demonstrated to be predominantly successful in the treatment
19 of *C. trachomatis* infection, treatment failures have been observed in some cases. Rich source
20 of antimicrobial peptides was recently discovered in *Medicago* species, which act in plants as
21 differentiation factors of the endosymbiotic bacterium partner. Several of these symbiotic
22 plant peptides have proved to be potent killers of various bacteria *in vitro*. We show here that
23 7 of 11 peptides tested exhibited antimicrobial activity against *C. trachomatis* D, and that the
24 killing activity of these peptides is most likely due to their interaction with specific bacterial
25 targets.

26 **Keywords:** Chlamydia, antimicrobial, NCR peptide

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Introduction

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Chlamydia trachomatis is a Gram-negative, obligate intracellular bacterium with a characteristic biphasic life cycle, forming metabolically inactive infectious forms (elementary bodies [EBs]) and metabolically active, non-infectious forms (reticulate bodies [RBs]). Serovars D to K cause urogenital infections that are often asymptomatic, but which can lead to severe complicated diseases [1].

C. trachomatis is of great public health significance because of the impacts of the untreated diseases on human reproduction. Cervicitis and urethritis commonly occur in women and about 40% of the untreated cases progress to pelvic inflammatory disease (PID). Infertility results in 20% of the PID cases, while 18% of the women with this disease experience chronic pelvic pain, and 9% may suffer an ectopic pregnancy [2].

At the individual level, *C. trachomatis* infection can generally be treated effectively with antibiotics, though antibiotic resistance appears to be increasing [2]. At the population level, public health control of the infection is rather problematic. With regard to the severe potential consequences of urogenital *C. trachomatis* infection in women, many countries offer screening. Vaccination, which is currently unavailable, would be the best way to reduce the prevalence of *C. trachomatis* infections, as it would be much cheaper and would have a greater impact on controlling *C. trachomatis* infections worldwide [3]. The development of new antimicrobial agents is required to overcome this problem.

Antimicrobial peptides (AMPs), natural antibiotics produced by nearly all organisms, from bacteria to plants and animals, are crucial effectors of innate immune systems, with different spectra of antimicrobial activity and with the ability to perform rapid killing. To date, more than 800 AMPs have been discovered in various organisms, including 270 from plants. It has become clear in recent years that these peptides are able not only to kill a variety of

54 pathogens, but also to modulate immune responses in mammals. However, their modes of
55 action are poorly understood. In some species these peptides serve as the primary
56 antimicrobial defense mechanism, whereas in others they serve as an adjunct to existing
57 innate and adaptive immune systems [4]. Cationic AMPs interact with negatively charged
58 microbial membranes and permeabilize the membrane phospholipid bilayer, resulting in lysis
59 and the death of microbes [5, 6]. In view of their rapid and broad-spectrum antimicrobial
60 properties, interest has emerged in AMPs as potential antibiotic pharmaceuticals with which
61 to combat infections and microbial drug resistance [7, 8].

62 Most plant AMPs are cysteine cluster proteins. This group includes major plant immunity
63 effectors such as defensins, and also symbiotic peptides, including the nodule-specific
64 cysteine rich (NCR) peptides, which are produced in *Medicago -Sinorhizobium meliloti*
65 symbiosis and provoke irreversible differentiation of the endosymbiont. The NCR family is
66 composed of about 500 divergent peptides in *Medicago truncatula* [9, 10, 11]. Some cationic
67 NCRs have been shown to possess genuine antimicrobial activities *in vitro*, killing various
68 Gram-negative and Gram-positive bacteria highly efficiently [12].

69 In the present study, 7 of the 11 NCR peptides examined displayed dose- and time-dependent
70 anti-chlamydial activity *in vitro*. NCR247 was also demonstrated to bind to the 60-kDa
71 putative GroEL protein of *C. trachomatis* D.

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Materials and Methods

74 *Inoculum preparation*

75 *C. trachomatis* D (ATCC) was propagated on HeLa cells as described earlier [13]. The
76 partially purified and concentrated EBs were aliquoted and stored at -80 °C until use. A mock
77 preparation was prepared from an uninfected HeLa cell monolayer processed in the same way
78 as the infected cells. The titer of the infectious EBs was determined by indirect

79 immunofluorescence assay. Serial dilutions of the EB preparation were inoculated onto tissue
80 culture monolayers and, after a 48-h culture, cells were fixed with acetone and stained with
81 murine monoclonal anti-*Chlamydia* LPS antibody (AbD Serotec, Oxford, UK) and FITC-
82 labeled secondary anti-mouse IgG (Sigma, St. Louis, MO, USA). The number of inclusions
83 was counted under a UV microscope, and the titer was expressed in inclusion forming
84 units/ml (IFU/ml).

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86 *Measurement of in vitro antibacterial activity of NCR peptides*

87 First, the toxicity of the NCR peptides was tested on non-infected HeLa cells in the highest
88 concentration (10 µg/ml) used during our experiments. The toxic peptides were excluded
89 from the further experiments.

90 EBs of *C. trachomatis* D (4×10^4 IFU/ml) were incubated with chemically synthesized
91 mature NCR030 (AFLPTSRNCITNKDCRQVRNYIARCRKGQCLQSPVR pI=10,37);
92 NCR044 (AFIQLSKPCISDKECSIVKNYRARCRCRKGVCVRRRIR pI=10,32); NCR055
93 (VNDCIRIHCKDDFDICIENRLQVGCRLQREKPRCVNLVCRCLRR pI=9,21); NCR095
94 (ELVCDTDDDCLKFFPDNPYPMECINSICLSLTD pI=3,62); NCR137
95 (MTLRPCLTDKDCPRMPPHNIKCRKGHCVPIGKPFK pI=9,7); NCR168
96 (YPFQECKVDADCPTVCTLPGCPDICSFPDVPTCIDNNCFCT pI=3,61); NCR169
97 (EDIGHIKYCGIVDDCYKSKKPLFKIWKCVENVCVLWYK pI=8,45); NCR183
98 (ITISNSSFGRIYWNCKTDKDKQHRGFNFRCSGNCIPIRR pI=10,1); NCR192
99 (MKNCKHTGHCPKMKCGAKTTKCRNKKCQCVQL pI=9,54); NCR247
100 (RNGCIVDPRCPYQQCRRPLYCRRR pI=10,15); or NCR280
101 (MRVLCGRDGRCPKFMCRFTL pI=9,8) (Proteogenix Oberhausbergen, France) at various
102 concentrations (10, 5, 2.5, or 1.25 µg/ml) in sucrose-phosphate-glutamic acid buffer (SPG) for
103 2 h at 37 °C. As control, *C. trachomatis* D was incubated in buffer alone. The time courses of

104 the anti-chlamydial effects of the NCR peptides were tested after incubation periods of 15, 30,
105 60 and 120 min. To quantify the anti-chlamydial effects of the NCR peptides, HeLa cells were
106 seeded in 24-well tissue culture plates with 13-mm cover glasses. After 24 h, the confluent
107 cells were infected with NCR-treated *C. trachomatis* D or the control. After 48 h, the cells
108 were fixed with acetone at $-20\text{ }^{\circ}\text{C}$ for 10 min. Fixed cells on cover glasses were stained by
109 the indirect immunofluorescence method described in “Inoculum preparation” section. The
110 number of recoverable inclusions was counted under a UV microscope, and the titre was
111 expressed in IFU/ml.

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113 *Far-Western blot assay for identification of NCR-binding Chlamydia proteins*

114 Concentrated *C. trachomatis* (2×10^5 IFU) (prepared as described earlier) and a mock
115 preparation were heated at $95\text{ }^{\circ}\text{C}$ for 5 min in sample buffer, and polyacrylamide gel
116 electrophoresis (PAGE) was performed. The proteins were separated on 10% sodium dodecyl
117 sulfate (SDS) polyacrylamide gel in duplicate, and half of the gel carrying the separated
118 proteins of the *C. trachomatis* or the mock samples was blotted onto a polyvinylidene
119 difluoride membrane (SERVA, Heidelberg, Germany). The membrane was blocked overnight
120 at $4\text{ }^{\circ}\text{C}$ with 5% skimmed milk and 0.05% Tween 20 containing PBS. The membrane was
121 probed for 4 h with a buffer [1% bovine serum albumin in PBS with 0.05% Tween 20
122 (PBST)] containing $10\text{ }\mu\text{g/ml}$ NCR247. After washing 3 times with PBST, the filter was
123 incubated with anti-NCR247 rabbit IgG for 4 h and further incubated after washing 3 times
124 with HRP-conjugated anti-rabbit antibody (Sigma). A control lane with separated *C.*
125 *trachomatis* EBs was also incubated with anti-NCR247 and HRP-conjugated anti-rabbit
126 antibody without prior treatment with NCR247 peptide. Following 3 further washings, the
127 colour was developed by using diaminobenzidine tetrahydrochloride (Sigma) with hydrogen
128 peroxide in 10 mM Tris at pH 7.5. The second half of the gel with the separated proteins of *C.*

129 *trachomatis* or the mock preparation was stained with PageBlue Protein Staining Solution
130 (Fermentas).

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132 *Identification of proteins by mass spectrometry*

133 The gel slices containing the polypeptides of the concentrated *C. trachomatis* EBs
134 corresponding to proteins exhibiting NCR247 positivity in the blotting assay were cut out
135 from the gel and analyzed by mass spectrometry. Briefly, protein bands were diced and
136 washed with 25 mM NH₄HCO₃ in 50% (v/v) acetonitrile/water. Disulphide bridges were
137 reduced with dithiothreitol (DTT), and free sulphhydryls were alkylated with iodoacetamide.
138 Proteins were digested with modified porcine trypsin (Promega Madison, WI, USA) for 4 h at
139 37 °C. Samples were analysed on liquid chromatography-tandem mass spectrometry (LC-
140 MSMS) instruments. LC-MSMS raw data were converted into a Mascot generic file with
141 Mascot Distiller software (v2.1.1.0). The resulting peak lists were searched by using the
142 Mascot Daemon software (v2.2.2) against the NCBI non-redundant database without species
143 restriction (NCBI nr 20080718, 6833826 sequences). Monoisotopic masses with a peptide
144 mass tolerance of ±0.6 Da and a fragment mass tolerance of 1 Da were submitted.
145 Carbamidomethylation of Cys was set as a fixed modification, and acetylation of protein N-
146 termini, methionine oxidation, and pyroglutamic acid formation from peptide N-terminal Gln
147 residues were permitted as variable modifications. Acceptance criteria were at least 2
148 individual peptides with a minimum peptide score of 55 per protein.

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150 *Detection of NCR peptide binding to Chlamydia EBs by FACS*

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152 Chlamydia EBs (1×10^6 IFU) were treated with 1 µg of FITC-labelled NCR247 or FITC-
153 labelled NCR035 peptide containing PBS for 2 h at 37 °C. As controls, untreated Chlamydia

154 EBs were used. After 3 times washing with PBS, cells were analyzed with the FACS StarPlus
155 (Becton Dickinson) device.

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Results

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Anti-chlamydial effect of plant peptides.

To determine whether they possess anti-chlamydial activity, 11 NCR peptides (NCR030, NCR044, NCR055, NCR095, NCR137, NCR168, NCR169, NCR183, NCR192, NCR247 and NCR280) were co-incubated individually with *C. trachomatis* EBs at 10 µg/ml for 2 h at 37 °C. Counting of the number of viable *C. trachomatis* inclusions demonstrated that 7 of the 11 peptides (NCR044, NCR055, NCR095, NCR183, NCR192, NCR247 and NCR280) were effective killers of *C. trachomatis in vitro*, while NCR030 and NCR168 displayed weaker activity and NCR137 and NCR169 did not exert an anti-chlamydial effect (Fig. 1A). *C. trachomatis* inclusions were then treated for 2 h with concentrations of the peptides ranging from 1.25 µg/ml to 10 µg/ml (Fig. 1B). NCR044, NCR055 and NCR183 were found to exert the strongest anti-chlamydial activities by reducing the viability to 95%, 78% and 85%, respectively, at 1.25 µg/ml, whereas the other peptides revealed no effect at 1.25 µg/ml concentration. NCR192 and NCR247 had significant anti-chlamydial effects at 2.5 µg/ml concentration. The time course of killing was investigated in the cases of NCR044, NCR055, NCR183 and NCR247 at 5 µg/ml concentration (Fig. 1C). NCR044 elicited the fastest effect, achieving an 80% reduction in the number of viable Chlamydia inclusions after a 15-min co-incubation with *C. trachomatis* EBs. The other three peptides required longer times to attain the killing effect. Of the tested peptides therefore, NCR044 exhibited the strongest anti-chlamydial activity, acting at the lowest concentration and most rapidly.

182 *Identification of the chlamydial ligand responsible for NCR247 binding*

183 Further investigations were carried out with NCR247, which displayed anti-chlamydial
184 activity in the previous tests.

185 To identify the chlamydial ligand responsible for NCR peptide binding, concentrated *C.*
186 *trachomatis* EB preparations and mock control preparations were separated by SDS-PAGE.
187 After blotting, the membranes were probed with synthetic NCR247 peptide and incubated
188 with anti-NCR247 IgG and then with HRP-labeled anti-rabbit antibody. The control lane with
189 Chlamydia EBs was stained with anti-NCR247 IgG and HRP-labeled anti-rabbit antibody
190 without incubation with synthetic NCR247 peptide. The synthetic NCR247 peptide was
191 bound to a 60-kDa protein band in the Chlamydia lysate (Fig. 2A, lane 4). The synthetic
192 NCR247 did not react with the mock lysate (lane 2), and the Chlamydia EB lysate did not
193 react with the HRP-conjugated anti-rabbit antibody (lane 3). The gel slice containing the
194 corresponding polypeptide of the concentrated *C. trachomatis* EBs associated with the
195 synthetic NCR247 peptide was cut out from the gel and analyzed by LC-MSMS. A 60 kDa
196 putative GroEL protein of Chlamydia was indicated by LC-MSMS and confirmed by post
197 source decay analysis (Fig. 2B).

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199 *FACS analysis for the detection of NCR247 binding to the whole C. trachomatis EBs*

200 To show that NCR247 is able to bind not only to the degraded Chlamydia particles but to the
201 native, viable Chlamydia EBs, a FACS analysis was carried out. Fig. 3 reveals that Chlamydia
202 EBs interacted with FITC-conjugated NCR247 peptide. Untreated or FITC-labeled NCR035
203 peptide-treated (this peptide showed no anti-chlamydial effect earlier) Chlamydia EBs did not
204 demonstrate increased fluorescence.

Discussion

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206 *C. trachomatis* is the leading cause of sexually transmitted bacterial diseases in both
207 developed and developing countries, with more than 90 million new cases of genital
208 infections occurring annually. The development of effective new antimicrobial compounds is
209 indispensable if the late severe sequelae of the infections, such as ectopic pregnancy and
210 infertility, are to be avoided [14]. AMPs appear to be potentially promising candidates for this
211 purpose. Although their antimicrobial activity against bacteria, fungi and protozoa has been
212 extensively tested [15], their anti-chlamydial action has not yet been tested. In the present
213 study, therefore, we investigated the *in vitro* activity of 11 NCR peptides against *C.*
214 *trachomatis*. Seven of these peptides exerted significant anti-chlamydial activity at a 10 µg/ml
215 concentration. A number of synthetic NCR peptides from *Medicago truncatula* have been
216 reported to be potent killers of various Gram-negative (*Escherichia coli*, *Salmonella*
217 *Typhimurium*, *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa* and *Xanthomonas*
218 *campestris*) and Gram-positive (*Bacillus megaterium*, *Bacillus cereus*, *Clavibacter*
219 *michiganensis*, *Staphylococcus aureus* and *Listeria monocytogenes*) bacteria, including
220 human/animal and plant pathogens [12]. Furthermore, AMPs were effective against
221 *Staphylococcus epidermidis* in *in vivo* mouse model, and they also displayed anti-
222 inflammatory activity [8].

223 Our LC-MSMS experiment identified the GroEL protein of *C. trachomatis* as the
224 chlamydial ligand of the NCR247 peptide. The GroEL protein is one of the few proteins that
225 have so far been confirmed as relevant in chlamydial pathogenesis; it is also referred to as
226 heat shock protein 60 (Hsp60) [16]. This protein belongs to group I chaperones produced by
227 almost all prokaryotic and eukaryotic cells, which assist as intracellular proteins, in the correct
228 folding of nascent or denatured proteins under both normal and stress conditions [17].
229 Several reports have indicated that molecular chaperones produced by pathogenic bacteria,

230 can function as intracellular, cell surface, or extracellular signals in the course of infection
231 processes [18]. The immune responses to chlamydial GroEL correlate significantly with
232 disease sequelae in humans, and 80 to 90% of patients infected with *C. trachomatis* have
233 antibodies directed against GroEL [19]. The high degree of antigenicity of GroEL in patients
234 implies that the protein is easily accessible to the immune system, perhaps because it is
235 localized on the surface of the chlamydial particles. Early studies on isolated outer membrane
236 complexes from *C. trachomatis* and *Chlamydophila psittaci* EBs had indeed pointed to the
237 possibility that GroEL might be associated with chlamydial membranes [20]. Taken together,
238 GroEL is accessible for the binding of the NCR247 peptide.

239 The present study indicates that certain of the NCR peptides possess substantial *in*
240 *vitro* activity against *C. trachomatis* D. Studies of chlamydial infection in animal models are
241 clearly needed to establish whether they have parallel *in vivo* results and whether these
242 peptides can be useful lead compounds for the development of anti-chlamydial drugs.

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314

315 **Figures**

316

317 Fig. 1. Concentration and time dependences of the anti-chlamydial effects of NCR peptides.

318 *C. trachomatis* at 4×10^4 IFU/ml was incubated with 10 $\mu\text{g/ml}$ of synthetic NCR peptide for 2

319 h at 37 °C (A). *C. trachomatis* EBs were incubated with different quantities of synthetic NCR

320 peptides for 2 h at 37 °C (B) *C. trachomatis* was co-incubated individually with different

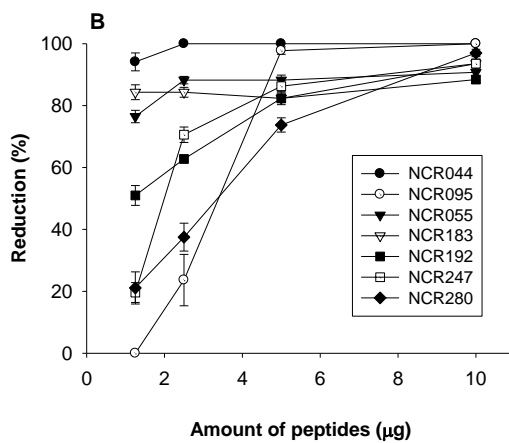
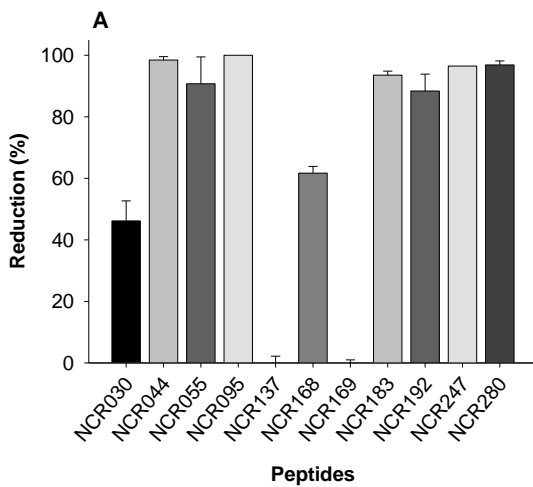
321 NCR peptides (5 $\mu\text{g/ml}$) for 0, 15, 30, 60 or 120 min (C). The infectivity of the NCR peptide-

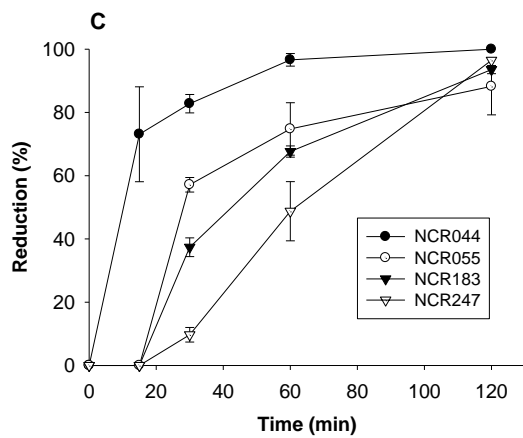
322 treated *C. trachomatis* was determined by inoculating the mixture onto confluent HeLa cells

323 on cover glasses. After a 24-h incubation, the fixed cells were stained with anti-chlamydia

324 LPS antibody and the number of inclusions was counted under a UV microscope. All the data

325 are representative of three separate experiments.



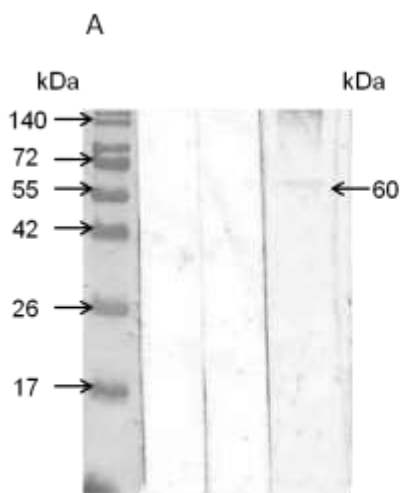


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331 Fig. 2. Interaction of NCR247 peptide and *C. trachomatis* EBs. Far-Western blot analysis of
 332 the chlamydial ligands responsible for NCR247 peptide binding. (A) Concentrated *C.*
 333 *trachomatis* and mock control preparations were separated by SDS-PAGE. After blotting, the
 334 membrane (lane 2,4) was probed with synthetic NCR247 peptide and incubated with anti-
 335 NCR247 IgG and HRP-labelled anti-rabbit antibody. (lane 1- molecular weight marker, lane 2
 336 - mock preparation, lane 4 - Chlamydia EBs lysate). A control lane (lane 3) with separated *C.*
 337 *trachomatis* EBs was also incubated with anti-NCR247 and HRP-conjugated anti-rabbit
 338 antibody without prior treatment with the NCR247 peptide. Identification of the *C.*
 339 *trachomatis* proteins by LC-MSMS (B). Peptide fragments that match the defined protein
 340 sequences are to be found in the Table.



B

D7DCT1 Uniprot ID: D7DCT1_CHLTD Species: CHLTD Name: **60 kDa chlamydia**
 Organism: Chlamydia trachomatis serovar D (strain D-EC) Gear: proEL Existence: Inferred from homology Version: 1
 Protein MW: 56147.4 Protein pI: 5.3 Protein Length: 544

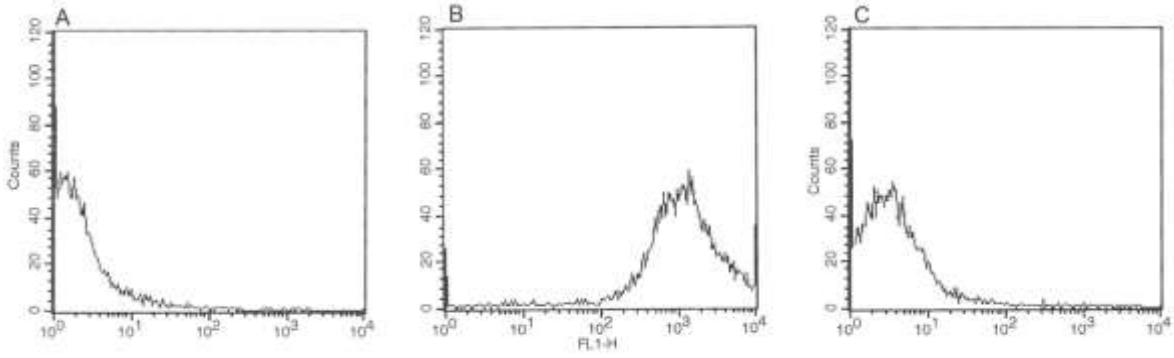
Num	Category	% Cov	Best Dec Score	Best Expect Val
17		56.9	-4.84	0.1e-8

m/z	z	Da	Peptide	RT	Start	End	Score	Expect
643.0646	3	0.040	EVELADKHENMGAQMVK	13.5501	59	75	18.6	0.015
615.7597	2	-0.051	NYTAGANPMDLK	15.3489	106	117	36.5	1.9e-5
911.0558	2	-0.096	EIAQVATISANSDAEIGNLIAEAM(Oxidation)EK	27.2685	143	168	26.1	7.9e-5
957.1251	3	-0.016	NGSITVEEAKGFTIVLDIVEGMFNR	35.5565	172	197	20.3	0.0027
928.9320	2	-0.014	GFETVLDIVEGM(Oxidation)FNR	29.8399	182	197	40.0	2.4e-7
920.9175	2	-0.031	GFETVLDIVEGMFNR	33.6969	182	197	49.1	1.1e-8
1122.8974	3	0.011	GYLSSYFATNPEIQEC(Carbamidomethyl)VEEDALVLIYDKK	14.1365	198	226	22.8	1.6e-5
826.1337	3	0.028	KAM(Oxidation)LEDIAILTGGQLISEELGMK	31.5215	286	308	10.6	1.1e-6
820.7403	3	-0.034	KAMLEDIAILTGGQLISEELGMK	33.2846	286	308	57.2	4.5e-9
1173.6437	3	0.034	AM(Oxidation)LEDIAILTGGQLISEELGMKLENAANLAM(Oxidation)LGK	35.7915	287	310	20.4	7.4e-4
1108.3364	3	0.058	AMLEDIAILTGGQLISEELGM(Oxidation)KLENAANLAMLGK	37.7253	287	319	29.5	1.0e-6
1106.8025	2	-0.0081	AMLEDIAILTGGQLISEELGMK	36.0232	287	308	47.2	5.3e-7
595.2812	2	-0.035	LENAANLAM(Oxidation)LGK	35.5306	309	319	34.9	4.2e-5
665.3398	3	0.0022	EDTIVTEGM(Oxidation)GKEALEAR	14.4267	328	343	19.3	0.0080
763.8327	2	0.0044	QcGh-pyro-GluIEDSSSDYDKEK	11.5751	352	384	18.1	0.0078
524.7479	2	-0.023	VGAATEHEMK	13.1446	381	390	35.2	3.5e-5
861.8609	2	-0.055	DAYTDMLEAGILDPAK	27.8021	485	500	31.1	1.8e-4

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344 Fig. 3. FACS analysis of NCR247 peptide binding to whole *C. trachomatis* EBs. Untreated
345 and unstained Chlamydia EBs (A). FITC-labelled NCR247 peptide treated *C. trachomatis*
346 EBs (B). Chlamydia EBs treated with FITC-labelled NCR035 peptide (C).

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