Notes for Establishing Study Protocols for Anti-Environmental Factor Antibody Assays

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Environmental factors, especially intestinal microbes and their toxins, may contribute to the development of autoimmune diseases. The immune system plays a key role in protecting the host against these environmental factors while it is simultaneously affected by a variety of exogenous environmental factors (infectious bacteria and viruses), drugs (immunosuppressants and immunostimulants), and intrinsic factors (genetics, stress, gastrointestinal disorders, and immunosenescence associated with aging). Currently, this concept has been expanded to include other diseases such as cancer, Alzheimer's, and Type 2 diabetes.

Chondrex, Inc. provides useful antibody ELISA kits against environmental factors as immune function evaluation tools. However, several considerations must be addressed before establishing study protocols. Here we would like to share these considerations to help users better choose kits and interpret results.

1. BACTERIA COMPONENT COMPOSITION MAY DIFFER DUE TO THE SOURCE OF BACTERIA

Bacteria component composition, especially expressed components on cell surfaces, varies among bacteria, even among sub-strains of bacteria. For example, the peptidoglycan-associated proteins in outer membranes are expressed at different ratios in different strains of *E. coli* K12 (Figure 1) (1). Anti-bacterial antibodies are elicited by many different species of bacteria inherently present in their host or due to inoculation or immunization of cultured bacteria. This suggests that the anti-bacterial antibody levels in samples, such as serum or fluid, may differ with bacteria species and that the antibody assay results against bacteria and their toxins may differ in each study.

Figure 1. Expressed major outer membrane proteins (Peptidoglycan-associated protein) in *E. coli* K12 and other strains.

Table 2. Distribution of major outer membrane proteins in various *E. coli* strains

Strain	Mating type	Relative amounts of major outer membrane proteins (percentage of total major outer membrane protein)[a]			
		a	*b*	*c*	*d*
A. *E. coli K12 strains*					
PC 0221	F^-	7	6	48	39
AB 1859	F^-	12	7	37	44
PC 1349	F^-	10	16	23	51
D 21	F^-	7	24	26	43
P 400	F^-	6	35	15	44
PC 2105	F^-	7	37	14	42
PC 0205	F^-	8	42	15	35
PC 0668	F^+	8	10	42	40
JF 404	Hfr H	7	18	40	35
B. *Other E. coli strains*					
B		<2	72	0	26
C		4	5	44[b]	47
J 5		7	24[b]	31	38
K 235		36	10[b]	19	35
MRE 600		3	3	38[b]	56

[a] Cell envelope proteins were separated by gel electrophoresis. The data were calculated from scans of stained gels

[b] These bands have a slightly lower electrophoretic mobility than the corresponding bands of *E. coli* K12

Molec. gen. Genet. 147, 251–262 (1976)

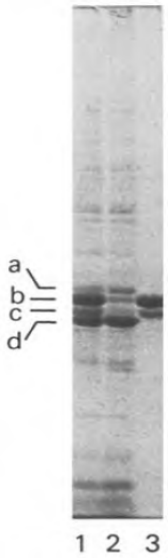

Fig. 1. 1–3. Peptidoglycan-associated protein of strain PC 0205. Cell envelopes (1) were heated in sample buffer without bromophenol blue at 60° C for 30 min. The solubilized proteins (2) were separated from the peptidoglycan fraction (3) by ultracentrifugation

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16928 Woodinville-Redmond Rd NE Suite B-101
Woodinville, WA 98072
Phone: 425.702.6365 or 888.246.6373
Fax: 425.882.3094
www.chondrex.com
support@chondrex.com

2. BACTERIA COMPONENT COMPOSITION MAY DIFFER IN DIFFERENT CULTURE CONDITIONS

Different culture conditions can cause different ratios of expressed bacterial components, even in isolated bacteria. Moreover, the environment that the bacteria live in is constantly changing. Figure 2 shows that in two different culture conditions (1-4 and 5-8), Lipopolysaccharides (LPS), one of the cell membrane components of *E. coli*, from *E. coli* O18 and O6, showed different staining patterns on SDS-PAGE (Figure 2, a). The immunoblot with monoclonal antibodies against LPS showed unique reactivities against LPSs cultured under different conditions (Figure 2, b and c) (2).

Chondrex, Inc. cultures bacterial antigens under common conditions and optimizes conditions for individual bacteria. The antigenicity of the bacterial antigens is validated by ELISA using antiserum and monoclonal antibodies. Therefore, the assay results from the ELISA should be consistent. However, if your studies employ immunization or inoculation of bacteria, the bacterial samples or antigen preparation must be validated for consistent results.

Figure 2. Different culture conditions altering LPS composition and antigenicity against monoclonal antibodies.

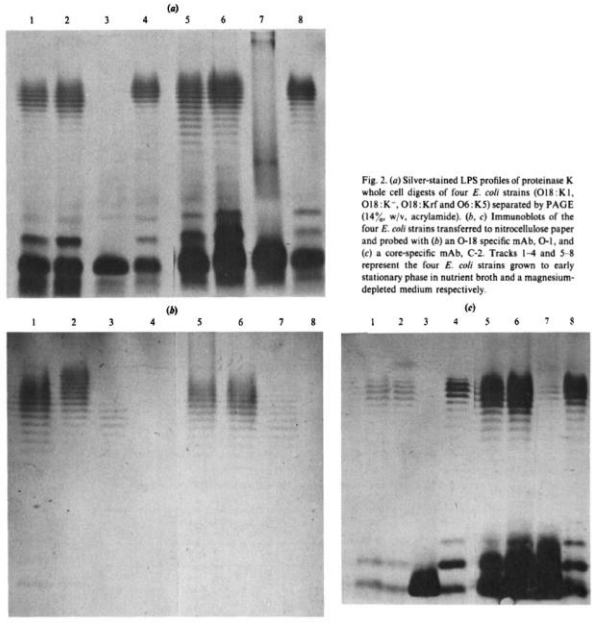

Fig. 2. (*a*) Silver-stained LPS profiles of proteinase K whole cell digests of four *E. coli* strains (O18:K1, O18:K⁻, O18:Krf and O6:K5) separated by PAGE (14% w/v, acrylamide). (*b*, *c*) Immunoblots of the four *E. coli* strains transferred to nitrocellulose paper and probed with (*b*) an O-18 specific mAb, O-1, and (*c*) a core-specific mAb, C-2. Tracks 1–4 and 5–8 represent the four *E. coli* strains grown to early stationary phase in nutrient broth and a magnesium-depleted medium respectively.

Journal of General Microbiology (1991), **137**, 2741–2751.

3. INACTIVATION METHODS

Generally, bacteria must be inactivated in order to be used as an antigen in ELISA. Chondrex, Inc. inactivates bacteria by heating at 100ºC for one hour (denaturing surface proteins) or fixing with 5% formalin for 15 minutes (cross-linking proteins). The antibody reactivities against native antigens may differ from these inactivated non-native antigens.

4. LOCATION OF ANTIGENS IN BACTERIA

The immune response against bacteria is intended to protect the host from infection. Therefore, antibodies against the surface antigens on the outer membranes of bacteria would be the first line of defense against pathogens. Figure 3 shows protein contents in the outer and inner membranes of bacteria. The outer membrane contains less proteins than the inner membrane. In addition, the protein expression in the outer membrane differed in different culture conditions (LB/8, Gc, Gt, and P) (3).

To analyze the protective roles of the immune response, Chondrex, Inc. employs whole bacteria as an antigen, not cell lysate. The presence of antibodies against the surface antigens on the outer membrane of whole bacteria is indicative of the host's defense immune systems. This assay system can exclude many types of highly conserved proteins in the inner membrane of bacteria.

Figure 3. Different protein contents in outer (OM) and inner membranes (IM) from different culture conditions

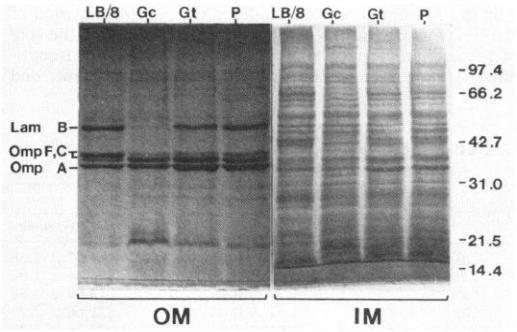

FIG. 2. Outer membrane (OM) and inner membrane (IM) proteins of *E. coli* JF568 electrophoresed on a SDS-polyacrylamide (10%) slab gel. Outer and inner membranes were extracted from cells grown in LB/8, LB/8 supplemented with 5% glucose (Gc), LB/8 supplemented with 10% L-glutamate (Gt), and LB/8 supplemented with 4% phosphate (P). Major outer membrane proteins are indicated on the left, and molecular weight markers (in thousands) are indicated on the right.

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Woodinville, WA 98072
Phone: 425.702.6365 or 888.246.6373
Fax: 425.882.3094
www.chondrex.com
support@chondrex.com

5. BACTERIA COMPONENT HOMOLOGY

Many proteins have conserved structures across bacteria species which may result in cross-reactivity of antibodies against these antigens. Figure 4 shows that monoclonal antibodies against components of *E. coli* cross-reacted to the same protein in other bacteria strains (4). In particular, polyclonal antibodies in serum can react to many of the same antigens in different bacteria. Therefore, the antibody levels may indicate that serum contains not only antigen-specific antibodies but also antibodies cross-reacting to other antigens as well.

6. INDIVIDUAL IMMUNE RESPONSE IN MICE

Mice from the same animal provider can show diverse immune responses when assaying for the same antigens. Figure 5 shows individual mice from the same cage (A-F) having unique antibody levels against several different bacteria and their toxins, even though the mice are supposed to have the same genetic backgrounds and parents (in-house data).

These mice should not have *P. gingivalis* (PG) in their resident microbiota, however several mice indeed showed high antibody levels against PG. This indicates the antibodies in mice may react to antigens in the existing resident bacteria, and these antibodies may cross-react to the antigens with conserved epitopes in PG. Indeed, antibodies against purified PG-LPS from PG showed different specificities in individual mice. Therefore, antibody cross-reactivity may be involved in antibody assay results and such results need to be carefully interpreted.

Along with mice, we assume humans must have a wide variety of antibodies against environmental factors not only in diseased patients, but also in healthy controls, depending on individual location, age, genetic background, and MHC type expressing immune function against bacteria.

Figure 4. Cross-reactivities of monoclonal antibodies against different bacterial species

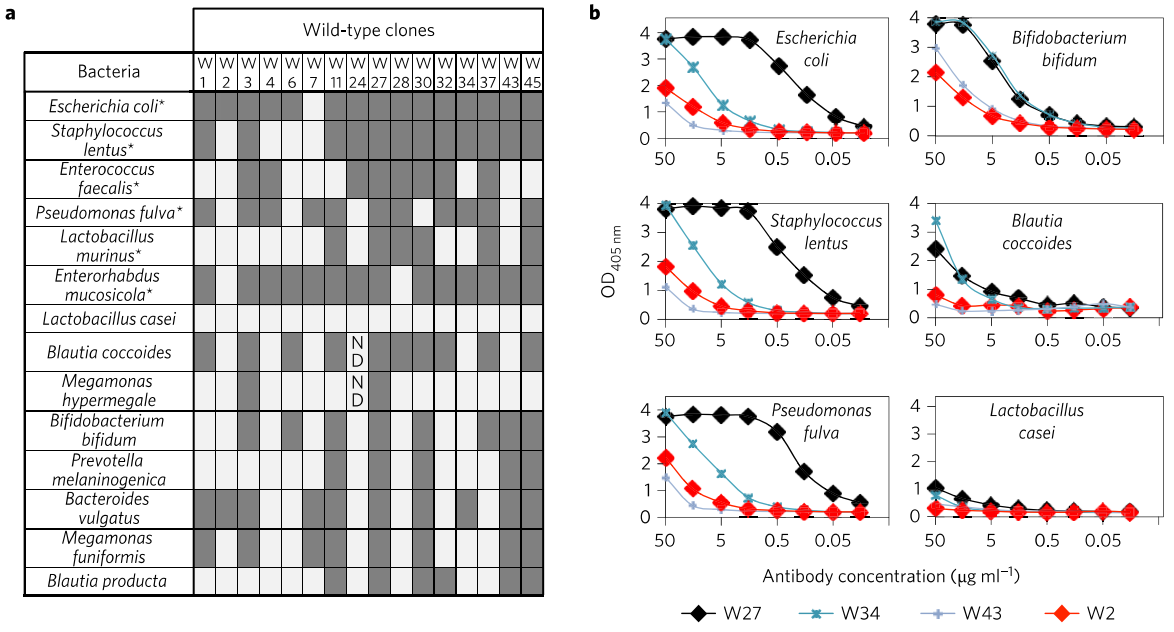

Figure 1 | LP-derived monoclonal W27 antibody is identified as a high-affinity polyreactive IgA. a, The reactivities of 16 monoclonal IgA antibodies against 14 different bacterial strains were evaluated by ELISA assay. Each monoclonal IgA (concentration of 1.4 μg ml^{-1}) was applied to ELISA plates coated with each strain of bacteria. The positive binding was determined by an optical density (OD) of >0.3. Shaded cells, positive binding; open cells, no binding; ND, not determined; *isolated bacteria from mouse faeces. **b**, The relative binding ability of each IgA clone was analysed by ELISA assay with serially diluted monoclonal IgA antibodies. All data in **a** and **b** are representative of at least three independent biological experiments. **c**, Left: Representative fluorescence

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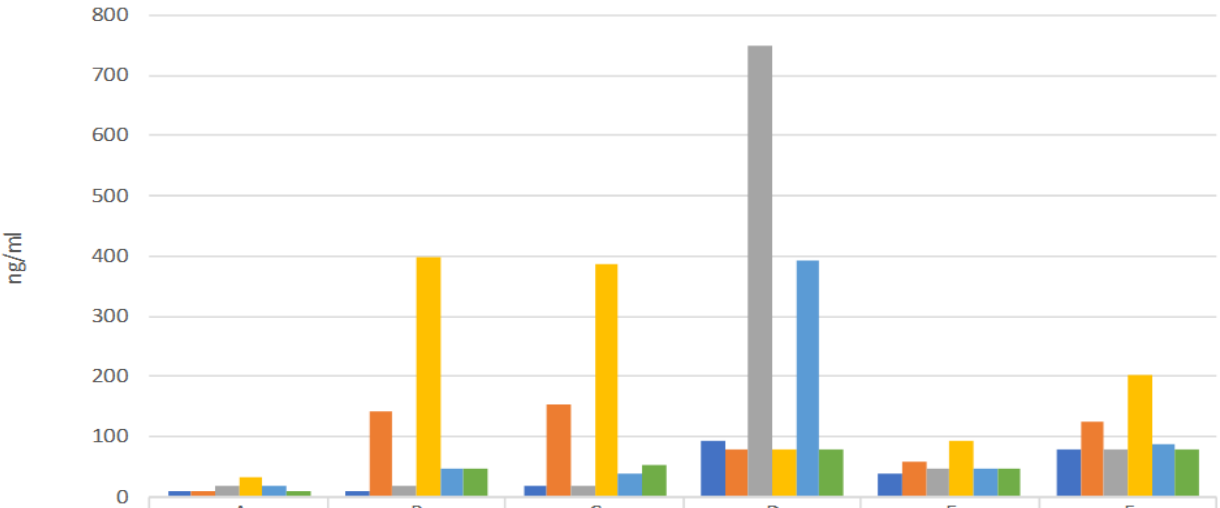
Figure 5. Diverse immune responses of serum anti-bacteria and toxin antibodies in individual cage mate mice (A-F).



	A	B	C	D	E	F
EC-LPS	9.0	9.0	16.9	92.3	38.8	79.3
PG-LPS	9.0	142.6	154.8	79.3	59.5	124.0
PG-PS	16.9	16.9	16.9	749.3	45.8	79.3
E .coli	31.7	397.0	385.9	79.3	92.3	202.9
P.gingivalis	16.9	45.8	38.8	391.5	45.8	85.9
L.Casei	9.0	45.8	52.7	79.3	45.8	79.3

CONCLUSION

Chondrex. Inc. developed antibody assay kits against environmental factors. All of the bacteria and toxins used as antigens in these kits were prepared with consistent protocols and validated by antiserum and monoclonal antibodies. Even considering the complex nature of assaying bacteria and analyzing results as mentioned above, these kits are still useful tools to determine immune function. These antibody assay results demonstrate the abilities of the host defense systems to react to individual bacteria, even despite the cross-reactivities.

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