UNIVERSITÀ DEGLI STUDI
DI MILANO – BICOCCA

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Dipartimento di Biotecnologie e Bioscienze

Dottorato di ricerca in Biologia,
XXIV ciclo

Development of high-throughput chemiluminescent immunoassays for the detection of *Clostridium difficile* and *Chlamydia trachomatis*.

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Anno Accademico 2010/2011

Dottorato in Biologia, XXIV ciclo

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Il lavoro presentato in questa tesi è stato realizzato presso i laboratori di DiaSorin S.p.A. Italia e DiaSorin Inc. a Stillwater in Minnesota (USA), sotto la supervisione del Dott. Andrea Dal Corso e Kim Paulsen Ph.D.

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INTRODUCTION

Luminescence spectrometry has become a firmly established and widely employed branch of analytical chemistry applied to qualitative and quantitative analytical detection. Bioluminescence and chemiluminescence (CL) present many advantages including low limits of detection (for highly luminescent molecules) and applicability to complex samples without using hazardous labels (e.g., radioisotopes) (Baeyens WRG, et al. 1998).
The aim of my work was to develop high-throughput chemiluminescence immunoassays, working on the automated LIAISON® system. In particular, this thesis will deals with the creation of 2 semi-quantitative immunoassays to diagnose bacterial infections caused by:
1- *Clostridium difficile (in human stool specimens)*
2 - *Chlamydia trachomatis (in human serum specimens).*

Clostridium difficile

HISTORY

Clostridium difficile is a major cause of healthcare-associated infections, the prevalence and severity of which has dramatically increased since the early 2000's when the surge in morbidity and mortality rates occured.
Clostridium difficile is a gram-positive, endospore-forming, anaerobic, gastrointestinal pathogen that is leading cause of nosocomial infections in developed nations. The bacterium is transmitted by the fecal-oral route and can readily colonize person with suppressed microflora as a result of antibiotic usage.
Originally named *Bacillus difficilis*, *Clostridium difficile* was first described in the mid-1930s (Voth & Ballard. 2005). This bacterium typically has been considered a nuisance causing minor amounts of disease and delaying patients discharge from the hospital by a few days. Typically, *Clostridium difficile* has been difficult to remove from a hospital setting because of its spore production that allows the bacterium to survive longer in this environment.
In 1978, *Clostridium difficile* was identified as the primary cause of psuedomembranous colitis and was proven to be a primary isolate from the feces of humans undergoing clindamycin therapy (Voth & Ballard. 2005).
At the beginning of the 21st century the prevalence of *Clostridium difficile* - associated disease (**CDAD**) in hospitals seemed to change and the first inklings of these changes started to appear in reviewed journals. This bacterium is now starting to garner more press worldwide and has started to raise concerns because of increased prevalence of CDAD in more and more countries. The financial impact of CDAD on the healthcare system is substantial, for the European Union it can be estimated to be €3000 million/ year and $1.1 billion/year in the USA. These values are expected to almost

double over the next four decades. In North America, increasing rates of CDAD have been reported in Canada and the USA since March 2003, involving a more severe course, higher mortality, increased risk of relapse and more complications (E. J. Kuijper. et al. 2006).

CLINICAL FEATURES

Clostridium difficile associated disease (CDAD)

Clostridium difficile manifests itself most readily as *Clostridium difficile* associated disease. Onset of symptoms usually occurs in 5-10 days after antibiotic use, but ranges from 1 day to up to 10 weeks after antibiotics are stopped (Oldfield. 2004).

The extremes of the ranges (1 day and 10 weeks) for onset of symptoms are rare. Clinical symptoms include watery diarrhea, fever, loss of appetite, nausea, and abdominal pain/tenderness (CDC *Clostridium difficile*: Information for healthcare providers. 2005). This disease is the only nosocomial diarrhea from the Clostridium family that should readily be screened for in hospital settings (Heimesaat, Gransow, Leidinger, & Liesenfeld. 2005).

Once the bacterium is in the intestinal tract it causes disease by secreting toxins. These toxins cause the problems associated with infections from Clostridium difficile, and also provide the means to more readily detect the disease (Voth & Ballard. 2005). These toxins lead to more problems and contribute to a higher virulence of the bacterium.

The other complications of the disease are asymptomatic colonization, diarrhea, pseudomembranous colitis, sepsis, toxic megacolon, colonic perforation and death (CDC *Clostridium difficile*: Information for healthcare providers. 2005). Other conditions associated with the bacteria that cause more severe problems are white blood cell count of >20,000 or less than 1500 cells/mm cubed, abdominal pain associated with a preexisting condition or post-operatively, or the presence of bowel wall thickening and ascites (determined by CT or abdominal radiography) (McEllistrem, Carman, Gerding, & Zheng. 2005).

Asymptomatic colonization is believed to be as high as 5% in normal adults. Some reports from long term care facilities set the disease carrier rate higher, but the numbers vary depending on the facilities pooled (Simor et al. 2002). Other reports show that this rate in adults may be as high as 20% of the people cultured (Oldfeild. 2004). This rate seems high compared to other published reports, but it is mentioned here to show the diversity of CDAD carrier rates. A person may carry the bacteria in their colon and not exhibit any symptoms, and then in 2 months spontaneously defecate the remaining bacteria and not be a carrier.

Pseudo-membranous colitis

One of the symptomatic signs of CDAD is the presence of psuedo-membranous colitis. The detection of pseudo-membranes is a highly specific indicator of CDAD and almost pathognomonic of the disease (Bouza, Munoz, & Alonso. 2005).
Pseudomembranous colitis appears in less than 25% of the discharges of CDAD, and is characterized by white-yellowish plaques present anywhere in the colon (Bouza et al. 2005). Pseudomembranous colitis is a good sign that the disease is present, but as stated does not always occur and would require a very invasive test to prove that it is present.

Perforated Colon and Toxic Megacolon

Some manifestations of *Clostridium difficile* in advanced stages are toxic megacolon and perforated colon. Toxic megacolon is a dilated colon with abdominal distention that is usually manifested in Crohn's disease and ulcerative colitis patients (Nayer, Vetrivel, McElroy, Pai, & Koerner. 2005). The usual symptoms consistent with toxic megacolon are hemorrhagic and necrotic colitis from caecum to lower sigmoid colon sparring of the rectum. Toxic megacolon can become very dangerous and can lead to bowel surgery to repair the damage. In CDAD patients toxic megacolon is rare, but it is a recognized complication (Nayar, Vetrivel, McElroy, Pai & Koerner. 2005). Perforated colon is another advanced stage of CDAD causing perforations in the wall of the colon.

Septicemia

Septicemia occurs when bacteria enter the bloodstream and cause an infection. This condition is very serious and if not treated can cause death. The treatment for septicemia is antibiotics. This condition, when discussing CDAD, can be caused by the disease or the treatment for septicemia can lead to an infection with *Clostridium difficile*.

Death

The increase in deaths of patients that have been diagnosed with CDAD is a growing concern in Canada, United States, United Kingdom and other countries. McDonald and his colleagues reviewed the mortality from this disease for the year 2000 and 2003. They found that although the number of deaths had risen from 8000 in the year 2000 to 15000 in the year 2003, the

percent mortality did not rise significantly in the years 2000 to 2003 (McDonald, et al. 2006). Researchers have begun to notice that death is becoming a more common occurrence among patients that have Clostridium difficile. Death occurs in CDAD patients when the bacteria become too much for the body to recover from the disease or the treatment is ineffective. It is feasible that patients with *Clostridium difficile* infection could have other illnesses that predispose them for death.

EPIDEMIOLOGY

Risk factors

Clostridium difficile associated diarrhea risk factors are important to understand and to be able to diagnose and treat hospitalized patients that may be susceptible to contracting the disease. Six conditions that can lead to CDAD are: history of antibiotic use, anti-neoplastic agents, age > 60 years, gastrointestinal surgery, enemas or stool softeners, and enteric feedings, especially post pyloric (Oldfield. 2004). All of these conditions in some way demonstrate the destruction of the normal commensal flora that exists in normal human large and small intestines. The two most prominent risk factors that have received the most attention are the use of antibiotics and age.

Antibiotics

Some antibiotics are more likely to cause *Clostridium difficile* than others, such as Clindamycin, but even Metronidazole and Vancomycin (somministered as a treatment) have been proven to be a cause of the disease (Oldfield. 2004). A study conducted in Paris, France by Blot and his colleagues determined that chemotherapeutic agents could cause CDAD (Blot, Escande, Besson, Barbut,Granpeix & Asselain. 2003).

Age

A majority of the literature examined suggests that age is an important factor causing CDAD. An older patient is more likely to contract the disease (McDonald et al. 2006). The age factor is compounded by the fact that older people are more likely to be hospitalized. Increasing a person's stay-time in hospital increases the likelihood that the person will be introduced to the bacteria.

The other extreme, neonate and pediatric patients, have a low prevalence for CDAD (Tang, Roscoe, & Richardson. 2005). The interesting fact about neonates is that they tend to have higher colonization of the bacteria, as high

as 64%, in their bowels, but the prevalence of CDAD in neonates is low (Tang et al. 2005).

Geographical distribution

An article by the Canadian medical association journal reported that too many hospitals in Montreal are battling outbreaks of *Clostridium difficile* (Eggertson & Sibbald. 2004). This report has been followed by other reports from Canada attempting to define the CDAD problem and why it is occurring. Another researcher from the Netherlands discusses outbreaks occurring in his country and refers to similar problems that the United Kingdom (UK), Canada, and the United States (US) are experiencing (Brierley. 2005). The CDAD problem is also reported by a researchers in Sweden, England, Belgium, France and Netherlands indicating that these countries are also experiencing an increasing prevalence of CDAD (Noren. 2005) (E.J. Kujper, et al. 2006). These reports suggests that the prevalence of this disease is increasing and according to published reports occurs more frequently in developed countries.

Pre-existing medical conditions

Another factor considered for the epidemiology of CDAD is pre-existing medical conditions. Similar to the age factor, pre-existing complications from other diseases appear to create conditions for contracting CDAD. Any medical condition that requires a person to be placed on antibiotics and to have a prolonged stay in the hospital will increase a persons risk for an infection with Clostridium difficile. The risk of developing CDAD as a hospital outpatient is about 7.7 discharges per 100,000 patients, while the 10 inpatient risk rate can be as high as 25 to 60 discharges per 100,000, depending on the antibiotics used (Mylonakis, et al. 2001).

One pre-existing condition that is believed to cause CDAD is the use of proton pump inhibitors (PPI). PPI are drugs that attempt to reduce the amount of acidity in the stomach. One such drug is Nexium®. Some authors believe that this class of drugs has contributed to the increasing prevalence of CDAD (Dial, Alrasadi, Manoukian, Huang, & Menzies. 2004), (Kazakova, et al. 2006). Other researchers have noted PPI use, as a cause of increase in CDAD discharges, is heavily confounded by length of hospital stay and age of the patient (Pepin et al, 2005b). Other researchers found that PPI use did not significantly cause increases in CDAD (Loo, et al. 2005). In an effort to look into community acquired CDAD, researchers are continuing to look at gastric acid and suppressive agents (such as PPI) as reasons why more persons that have no history of being in a hospital might be contracting CDAD (Dial, Delaney, Barkun, & Suissa. 2005).

More research into this topic needs to be performed to make an accurate assessment of the affect of PPI on CDAD prevalence.

C. difficile BACTERIUM AND METHOD OF ACTION

Fig.1: *Clostridium difficile* bacterium photo (download from http://www.sciencephoto.com/media/12105/view).

Clostridia are motile bacteria that are ubiquitous in nature and they are especially prevalent in soil. Under the microscope after Gram staining, they appear as long drumsticks with a bulge located at their terminal ends (fig.1). *Clostridium difficile* cells are Gram positive, while its spores are Gram negative. Clostridium shows optimum growth when plated on blood agar at human body temperatures (Ryan KJ, Ray CG. 2004). When the environment becomes stressed, however, the bacteria produce spores that tolerate the extreme conditions that the active bacteria cannot. First described by Hall and O'Toole in 1935, "the difficult clostridium" was resistant to early attempts at isolation and grew very slowly in culture (Ryan KJ, Ray CG. 2004).
C.difficile is an inhabitant of the human intestine, but normally behaves as a commensal bacterium without causing disease of any significance. Antibiotics, especially those with a broad spectrum of activity, cause disruption on normal intestinal flora. *C. difficile* is resistant to most antibiotics. It flourishes under these conditions. It is transmitted from person to person by the fecal-oral route. Because the organism forms heat-resistant spores, it can remain in the hospital or nursing home environment for long periods of time. It can be cultured from almost any surface in the hospital. Once spores are ingested, they pass through the stomach unscathed because of their acid-resistance. They change to their active form in the colon and multiply (fig. 2).

Surface layer proteins (SLPs), which decorate the pathogen's surface, are involved in adherence to the human intestinal epithelium and are thought to be a critical step in gut colonization (Calabi E., et al. 2002). The primary effect of Clostridium difficile on the human body is the production of two toxins produced during bacterial secretion. These toxins secreted by *Clostridium difficile* are labeled toxin A and toxin B and their production is a cell-density dependent process (Lee A.S., at al. 2005).

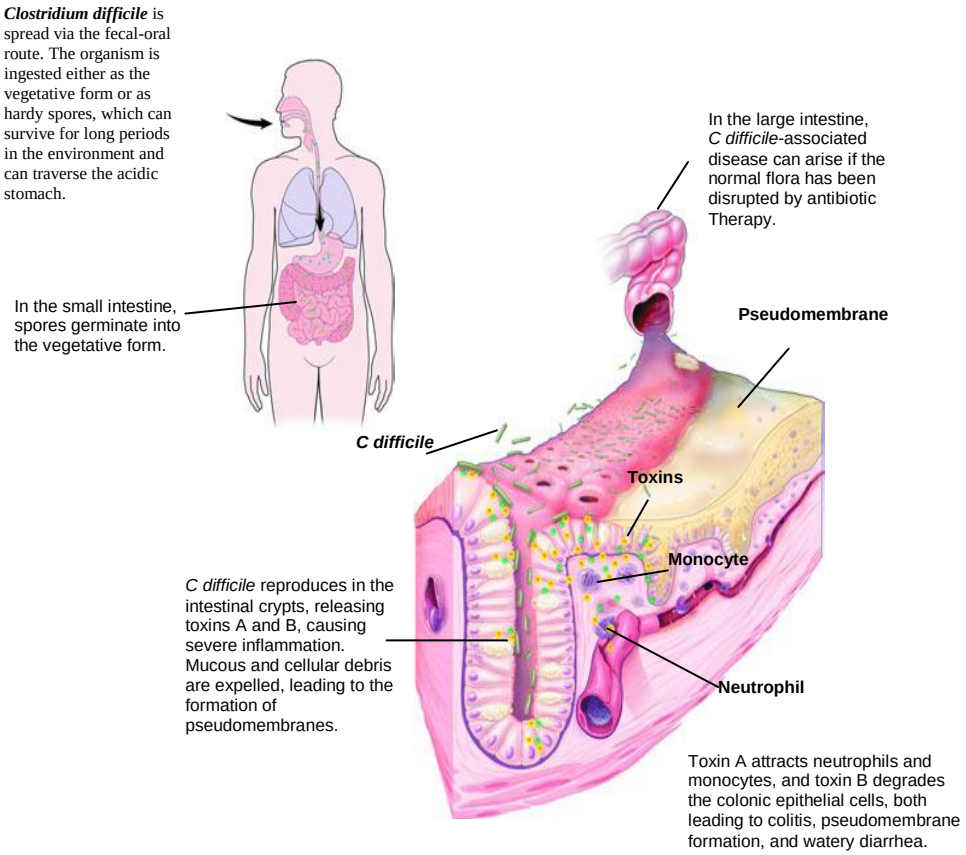


Fig. 2: Pathogenesis of *C. difficile*-associated disease (Rebecca H, et al. 2006).

Toxins A and B structure and function

Toxin A is considered an enterotoxin and cytotoxin and toxin B is a cytotoxin (McDonald, et al. 2006), (Gerding. 2005). These toxins are considered the main virulence factors for the disease. The enterotoxin (toxin A) causes disease by increasing the release of water, which will lead to an increase in the release of electrolytes. This increase in the release of both electrolytes and water leads to the diarrhea portion of the disease. The cytotoxin (both A and B) acts by killing neighboring cells that will in turn cause the inflammation and mucosal damage (fig. 3) associated with the disease.

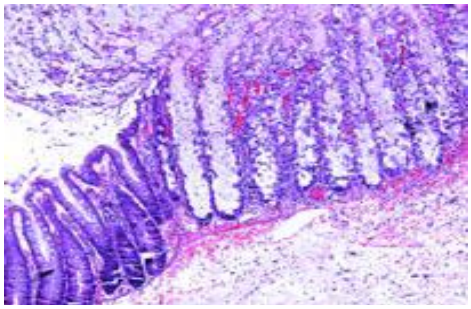


Fig. 3: "Photomicrograph of a hematoxylin and eosin stain of the case patient's colonic mucosa just on the edge of the pseudomembranous lesions. Intact mucosa appears to the left with normal architecture of deep crypts and villi. On the right, destruction of the mucosa with severe flammatory response extending deep into the lamina propria and expulsion of mucous and cellular debris from the crypts into the lumen of the large intestine, giving the appearance of a volcanic eruption. It is this expulsed material that forms the pseudomembranes." (Rebecca H, et al. 2006).

Similar to other members of the large clostridia family of toxins, TcdA and TcdB target the Rho/Ras superfamily of GTPases by irreversible modification through glucosylation (Jank T., Aktories K. 2008; Voth, D.E.; Ballard J.D. 2005). Since GTPases are key cellular regulatory proteins, their permanent inactivation causes disruptions in essential cell signaling pathways that are critical for transcriptional regulation, apoptosis, cytoskeleton integrity and eventually colonic epithelial cell barrier function (Jank, T. et al. 2007) (fig. 3). Binding of TcdA/B via the RBD to epithelial cells induces receptor-mediated endocytosis, permitting entry of the endosome-encapsulated toxin into the cytoplasm. Once internalized, the toxins require an acidic endosome for transport to the cytosol. A decrease in endosomal pH is thought to induce a conformational change, resulting in exposure of the hydrophobic membrane insertion (MI) domain and insertion of the N-terminus (catalytic domain and cysteine protease domain) into and

through the endosomal membrane via pore formation (Jank T., Aktories K. 2008) (fig. 4). Recently, Reineke J. et al. (2007) showed inositol hexakisphosphate (InsP6) from the host cell induces the autocatalytic cleavage of the N-terminal region at the cysteine protease (CP) site, liberating the N-terminal glucosyltransferase (GT) domain into the cytosol while the remaining portions of the toxin is left in the endosome. Upon cleavage, the GT domain is capable of transferring glucose residues from UDP-glucose to Rho-GTPases (Just I., et al. 1995), locking the important cell signaling mechanism in an inactive conformation. Inhibition of Rho-GTPases causes a series of cascading effects, including disruption of actin cytoskeleton and tight junction integrity in human intestinal epithelial cells. Collectively, these events lead to increased membrane permeability and loss of barrier function (Hecht G., et al. 1992), fluid accumulation, diarrhea, inflammation, and a massive influx of neutrophils and other members of the innate immune response (Laffler, et al. 2009).

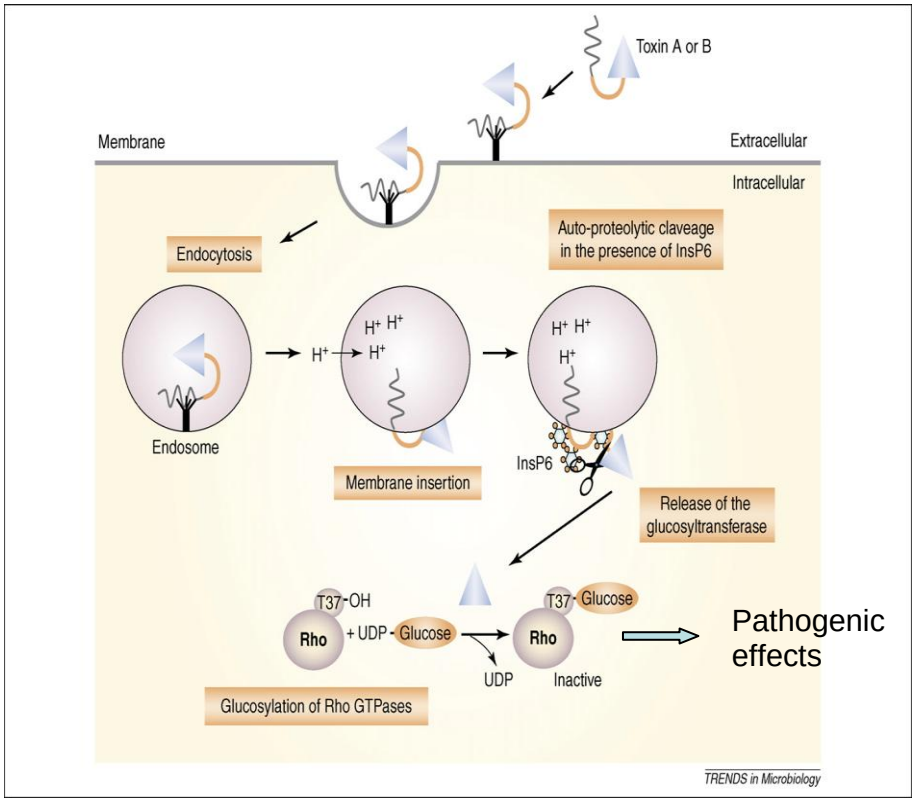


Fig. 4: Mechanism of action of toxin A and toxin B (fig. modified from Jank T., Aktories K. 2008).

Clostridium difficile toxins synthesis is growth phase-dependent and it is regulated by various environmental signals (Lee A.S. & Song K.P. 2005). TcdA and TcdB (Toxins A and B genes) are single-polypeptide chain, high-molecular weight exotoxins (308 kDa and 269 kDa, respectively) organized into multi-domain structures (Albesa J.D., et al. 2010). The toxin genes tcdA and tcdB are located in the 19.6 kb *Clostridium difficile* pathogenicity locus (PaLoc), which also includes three accessory genes, tcdR, tcdC and tcdE. TcdR has been shown to act as an alternative factor that mediates positive regulation of both the toxin genes and its own gene. The tcdA, tcdB and tcdR genes are transcribed during the stationary growth phase. The tcdC gene, however, is expressed during exponential phase. This expression pattern suggested that TcdC may act as a negative regulator of toxin gene expression. TcdC is a small acidic protein without any conserved DNA-binding motif. Like other members of the large clostridial toxin family, TcdA and TcdB are organized as modular domains with each domain performing a distinct function (fig. 5A). The C-terminal region of TcdA/B is responsible for toxin binding to the surface of epithelial cells possibly via multi-valent interactions with putative cell-surface carbohydrate receptors (Dingle, T., et al. 2008). Structural studies of this cell receptor binding domain (RBD) from TcdA and TcdB revealed a β-solenoid fold (Albesa, J.D., et al. 2010) with seven carbohydrate binding sites identified for receptor binding in TcdA (Greco, A., et al. 2006). While the C-terminal region of TcdA has been shown to bind various oligosaccharides, including the trisaccharide α-Gal-(1,3)-β-Gal-(1,4)-β-GlcNac (Krivan H.C., et al. 1986), the native human ligand has not been positively identified.

In the fig. 5B is shown the Domain structure of clostridial glucosylating toxins. The biologically active glucosyltransferase A-domain (bold) is located at the N terminus (amino acids 1–543) and has been crystallized recently. The DXD motif, which is involved in Mn2+ coordination, is located in this domain. The C terminus of the clostridial glucosylating toxins, which consists of polypeptide repeats, is involved in receptor binding (B-domain, bold). A fragment of the C-terminal repeats of toxin A has been crystallized, showing a solenoid-like structure. The C-domain (bold) is involved in processing and cutting of the toxin. The cysteine protease C-domain can be characterized by the catalytic triad consisting of Asp587, His653 and Cys698 (DHC). Alternatively, the DXG (D1665) motif was suggested to be part of an aspartate protease domain, possibly involved in processing of the toxins. In the middle part of the protein is a short hydrophobic region (residues 956–1128), which might be involved in pore formation and delivery of the catalytic domain into the cytosol (D-domain, bold) (Jank T., Aktories K.2008).

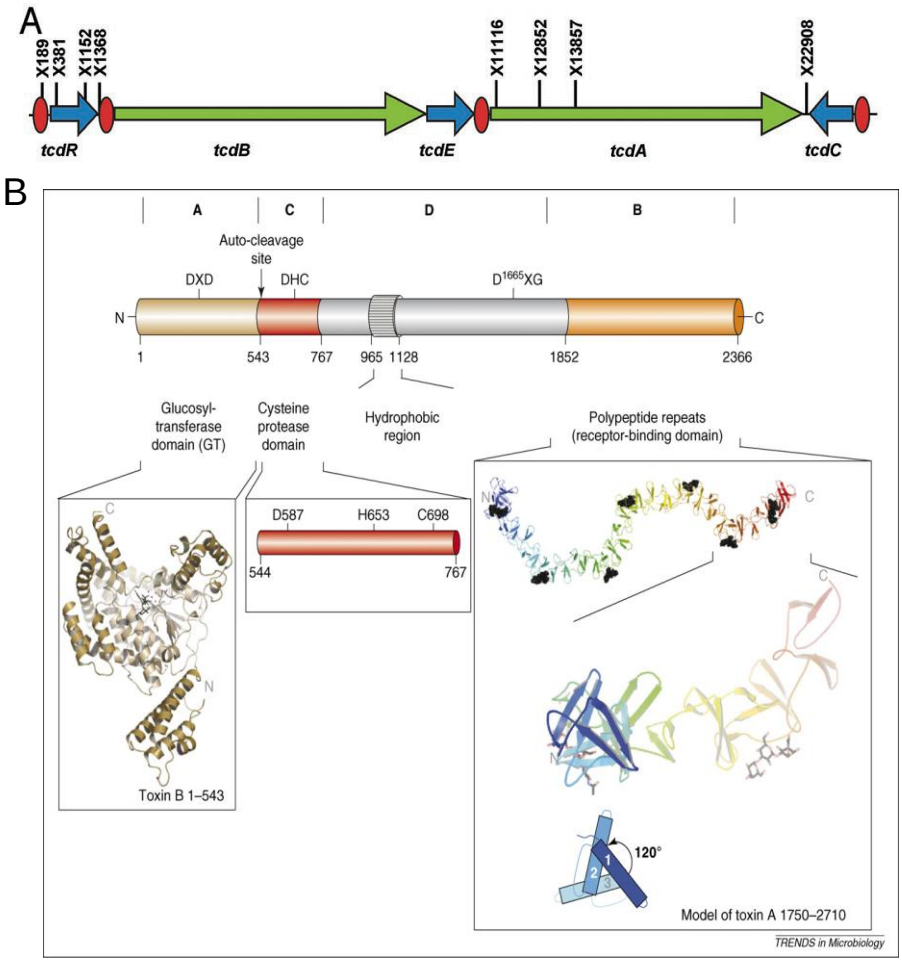


Fig. 5:
- A *Clostidium difficile* pathogenicity locus (PaLoc)
- B Domain structure of clostridial glucosylating toxins. The ABCD model (labeled in bold at top of figure) of clostridial glucosylating toxins is shown with Clostridium difficile toxin B as an example (fig. modified from Jank T., Aktories K. 2008).

Toxoids A&B

Treatment of the Toxins with formaldehyde results in the corresponding Toxoids A&B which are completely inactivated and possess at least partial retention of their immunogenicity (Torres J.F., et al. 1995). Maya S. and her colleagues (2008), demonstrate the stabilizing effect of formaldehyde crosslinking on the thermal stability. It has been shown that vaccination

employing both toxoids is effective in hamsters, healthy adults and patients with recurrent *Clostidium difficile* associated disease (Torres J.F., et al. 1995).

Genetic types of *Clostridium difficile*

Clostridium difficile strains show significant interspecies heterogeneity in a PaLoc region. The reference strain VPI 10463 represents the toxinotype 0 (zero). All strains that differ from this strain can be currently assigned to one of 24 variant toxinotypes, designated by roman numerals (I–XXIV). Their characteristics are summarized in fig. 6. Changes in the PaLoc seen in these variant toxinotypes are insertions, deletions and point mutations. Interestingly, the three types of mutations seem to prevail at specific parts of PaLoc (Maja R. 2008).

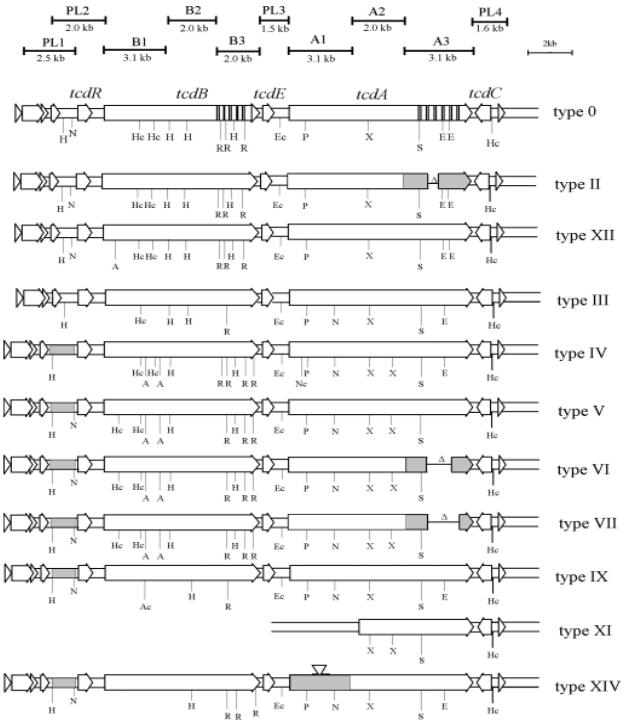


Fig. 6: Schematic presentation of the PaLoc region in toxinotype 0 and some representative variant toxinotypes (Maja R. 2008).

The consequences of major changes in the PaLoc are (1) the absence of reduction of one or both toxins and (2) production of toxins with altered properties (Maja R. 2008). In addition to the topic of toxins, the scientific community has been searching for strains of *Clostridium difficile* that have been prevalent more often than other strains. The scientific community believes that the presence of at least one of the toxins, either A or B, is necessary for disease, with enhanced virulence when both toxins are present (Martirosian, G., et al. 2005). This is important when considering that the Center for Disease Control (CDC) and researchers from Canada have named the strain believed to be the cause of the majority of the new outbreaks as hyper virulent toxin type III ribotype 027 strain (Pepin J., et al. 2005c). This strain has also been referred to as North American pulso-type I (NAP1)/ribotype 027 (Louie, T.J. 2005), as been reported also from USA and several countries in Europe (Zaiß N.H., et al. 2010). This strain has been implicated in a number of outbreak settings of CDAD (Cloud & Kelly. 2007). This strain has also been shown to produce about 15-20 times more toxin than a 'normal strain' of *Clostridium difficile* (Louie, T.J. 2005). This strain, or a close cousin to it, has also been identified in the UK (Pepin J., et al. 2005c). This strain is causing the new problems that are present now: increased prevalence of discharges, more co-morbidities, and higher mortality.

LABORATORY TESTING OF *Clostridium difficile* INFECTION (CDI)

Imaging techniques, such as radiography, Computed Tomography (CT) imaging, and endoscopy, have largely been superseded by laboratory testing for *Clostridium difficile*, because they are expensive, unpleasant to the patient, relatively insensitive, usually not specific, and unnecessary given the availability of a toxin assay. There are a variety of tests with advantages and disadvantages (table 1). Factors to consider when selecting a diagnostic test include turnaround time, sensitivity, specificity, cost, and availability.

Table 1: advantages and disadvantages of diagnostic testing methods for *Clostridium difficile* (Rebecca H., et al. 2006).

DIAGNOSTIC TEST	TURN-AROUND TIME	SENSITIVITY	ADVANTAGES	DISADVANTAGES
Endoscopy	2 hours	51%	Diagnostic of pseudomembranous colitis	Low sensitivity
Anaerobic culture	72 hours	89%–100%	Results useful for molecular typing	Does not distinguish toxin-producing strains
Tissue cytotoxic assay	48 hours	94%–100%	Detects A-B+ strains Gold standard	False-positives Results vary with experience of the technologist
Common antigen	15–45 minutes	58%–92%	Detects A-B+ strains Easy to use	Does not distinguish toxin-producing strains Cross-reacts with other anaerobes
Enzyme-linked immunosorbent assay (ELISA)—toxin A	2 hours	80%–95%	Easy to use	Does not detect A-B+ strains
ELISA—toxin A + B	2 hours		Detects A-B+ strains	Increased sensitivity for low-level toxin production
Immunochromatographic toxin A	< 1 hour	60%–85%	Simple to use Rapid	Does not detect A-B+ strains

BASED ON DATA FROM KELLY CP, POTHOULAKIS C, LAMONT JT. *CLOSTRIDIUM DIFFICILE* COLITIS. N ENGL J MED 1994; 330:257–262; GERDING DN, JOHNSON S, PETERSON LR, MULLIGAN ME, SILVA J, JR. *CLOSTRIDIUM DIFFICILE*-ASSOCIATED DIARRHEA AND COLITIS. INFECT CONTROL HOSP EPIDEMIOL 1995; 16:459–477; AND WILKINS TD, LYERLY DM. *CLOSTRIDIUM DIFFICILE* TESTING: AFTER 20 YEARS, STILL CHALLENGING. J CLIN MICROBIOL 2003; 41:531–534

Although no gold standard exists for diagnosis of CDI, the cell cytotoxicity assay is the best available test. It detects *Clostridium difficile* toxins at picogram levels, it was the first test described, and it is still the most sensitive available test for detection of toxin B (Glen H., et al. 2010).
Most laboratories use the EIA (toxin enzyme immunoassay) to detect toxin A or toxins A and B. Although the EIA has good specificity, it is only 70%–80% sensitive, requiring repeat testing, use of alternative tests, or initiation of empirical treatment for some patients. If an EIA is used, the assays for toxins A and B are preferred, because some cases of CDI involve strains that produce only toxin B. Detection limits for these methods range are around 1000 pg of toxin (Viscidi R., et al. 1984). One study reported a detection limit of 10,000 pg (Landry M.L., et al. 2001). These limits are much higher than the lower limit of detection for the cell cytotoxicity assay (10 pg) (Landry M.L. et al. 2001) . The cell cytotoxicity assay is the most sensitive and specific stool toxin assay for detection of *Clostridium difficile*, but it has a 24–48 hours turnaround time, is more expensive than the EIA, and is technically demanding. Stool culture is the most sensitive method but requires 48 hours and demonstration that the *Clostridium difficile* isolate is

toxigenic. The glutamate dehydrogenase GDH (common-antigen assay) lacks specificity but is sensitive and rapid and can be done as a screening test for a subsequent cell cytotoxicity assay. There are also molecular methods to detect the genetic components of toxin production (Glen H., et al. 2010).

At present, no single commercially available test offers good sensitivity and specificity in combination with a rapid turnaround time and low cost. For this reason usually laboratories use EIA in because it is inexpensive, fast, and technically easy to perform, followed by other tests (John R., et al. 2006).

Testing algorithms using a glutamate dehydrogenase (GDH) assay (which has presumptively higher sensitivity but lacks specificity) to screen for *Clostridium difficile* in stool samples, with reflex testing using a more specific assay, such as a toxin A/B EIA, have been proposed. GDH assays detect antigen present in both toxigenic and nontoxigenic strains of *Clostridium difficile* directly in stool samples. The time necessary to perform the GDH assay with EIA confirmation can be as long as 3 days. EIAs often lack sufficient sensitivity for confirmation of positive GDH assay results. The need to confirm GDH-positive specimens increases the turnaround time for positive results, delaying the notification of the physician ordering the test. PCR assays for various targets have been developed as a potential replacement for the less-sensitive (EIA) and less-specific (GDH) assays for *Clostridium difficile* detection. Since the cost of the PCR assays is, at the moment, prohibitive for most laboratories, the GDH or ELISA Toxin A and B assays are still most frequently used in the lab. (John. R. et al. 2006).

Some commercially available *Clostridium difficile* Toxin Detection Assays were compared from Kerrie E. and his colleagues (2009) and the results are show in the table 2.

Table 2: Comparison of commercial *Clostridium difficile* detection assays with results of cytotoxin testing (Kerrie E., et al. 2009).

Assay	No. of samples with the following assay result:				Cytotoxin test result	No. of samples tested	Sensitivity (%) (95% CI)[a]	Specificity (%) (95% CI)[a]
	Positive	Negative	Equivocal	Invalid				
Premier toxin A+B	99	9	0	0	Positive	108	91.7 (84.7–96.1)	97.1 (95.1–98.4)
	14	474	0	0	Negative	488		
GA *Clostridium difficile* antigen	83	25	0	0	Positive	108	76.8 (67.7–84.4)	90.9 (88.0–93.3)
	44	444	0	0	Negative	488		
Ridascreen toxin A/B	72	36	0	0	Positive	108	66.7 (56.9–75.4)	95.1 (92.6–96.7)
	23	464	1	0	Negative	488		
Techlab toxin A/B II	98	10	0	0	Positive	108	90.7 (83.6–95.5)	95.7 (93.4–97.3)
	21	467	0	0	Negative	488		
Remel ProSpecT	97	11	0	0	Positive	108	89.8 (82.5–94.8)	92.6 (89.8–94.7)
	36	452	0	0	Negative	488		
Vidas *C. difficile* toxin A/B	97	2	9	0	Positive	108	89.8 (82.5–94.8)	96.7 (94.6–98.0)
	5	472	11	0	Negative	488		
Remel Xpect	84	17	7	0	Positive	108	77.8 (68.8–85.2)	98.8 (97.2–99.5)
	5	482	1	0	Negative	488		
Techlab Tox A/B Quik Chek	91	17	0	0	Positive	108	84.3 (76.0–90.6)	98.6 (96.9–99.4)
	5	481	1	1	Negative	488		
Premier Immunocard A + B	84	15	0	9	Positive	108	77.8 (68.8–85.2)	92.8 (90.1–94.9)
	4	453	0	31	Negative	488		
Techlab C. diff Chek-60	82	9	0	0	Positive	91	90.1 (81.6–95.1)	92.9 (90.1–95.0)
	33	434	0	0	Negative	467		
BD GeneOhm *C. difficile*	83	7	0	0	Positive	90	92.2 (84.1–96.6)	94.0 (91.3–95.9)
	22	436	0	6	Negative	464		

[a] The sensitivity and specificity of the assays compared to the gold standard cytotoxin test results are shown. The sensitivity and specificity are given as percentages, and the 95% confidence interval (95% CI) values are shown in parentheses.

Recently, was developed by Meridian a high sensitive and specific assay for CDI called Illumigene® *Clostridium difficile*, it has a sensitivity of 95.2% and specificity of 95.3% when compare directly to cytotoxic bacterial culture (Package insert Illumigene®). This assay and the ELISA Premier toxins A&B were used as gold standard tests in this thesis for the Liaison® assay development.

Illumigene® *C. difficile*

The Illumigene® *Clostridium difficile* DNA amplification assay, performed on the Illumipro-10, is a qualitative in vitro diagnostic test for the direct detection of toxigenic *Clostridium difficile* in human stool specimens from patients suspected of having *Clostridium difficile*-associated disease (CDAD).

The Illumigene® *Clostridium difficile* assay utilizes loop-mediated isothermal DNA amplification (LAMP) technology to detect the pathogenicity locus (PaLoc) of toxigenic *Clostridium difficile*. The *Clostridium difficile* PaLoc codes for both the Toxin A gene (tcdA) and the Toxin B gene (tcdB), has conserved border regions, and is found at the same site on the *Clostridium difficile* genome for all toxigenic strains.

Illumipro-10 uses specifically designed primers to the PaLoc pathogenicity locus to provide for specific and continuous isothermal DNA amplification. A by-product of this amplification is the formation of magnesium

pyrophosphate, which forms a white precipitate leading to a turbid reaction solution. This presence of turbidity signifies a positive reaction while the absence of turbidity represents a negative reaction. The Illumigene® *Clostridium difficile* assay contains primers that specifically amplify a 204 BP region of the conserved 5' sequence of the tcdA gene within the PaLoc of toxigenic *Clostridium difficile* in diarrheal stool samples from patents suspected of having *Clostridium difficile*-associated disease (CDAD). The results of the assay are determined using the Meridian Illumipro-10 Incubator/Reader.

The Illumigene® *Clostridium difficile* assay detects the PaLoc by targeting a partial DNA fragment on the Toxin A gene. The tcdA target region was selected as an intact region remaining in all known A+B+ and A-B+ toxinotypes. In the fig. 7 is shown the Illumigene® assay procedure.

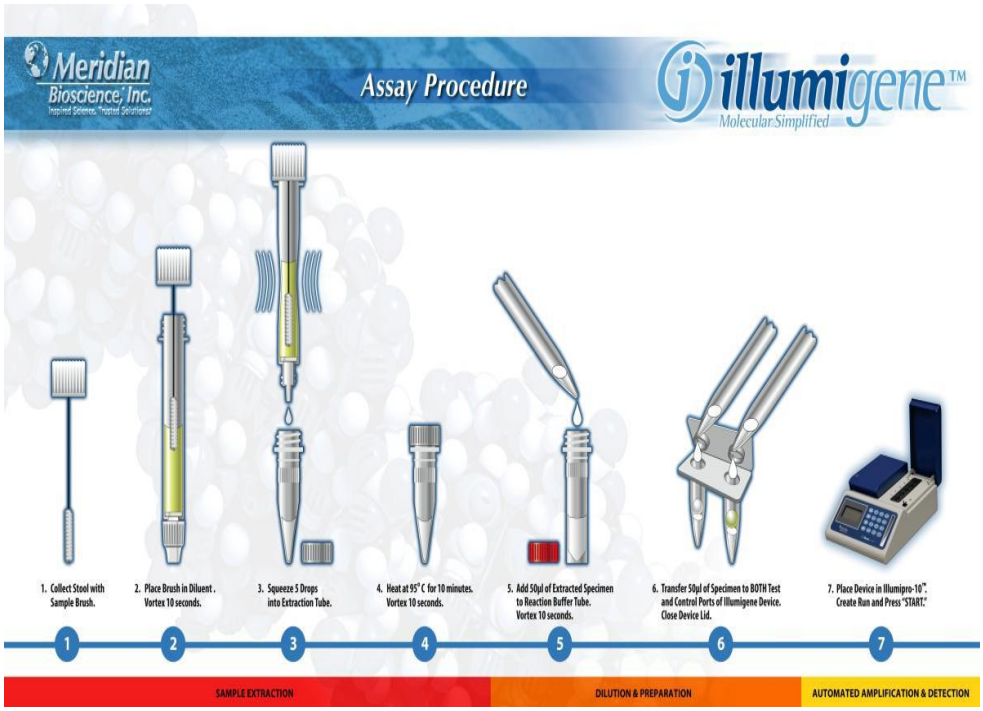


Fig. 7: Illumigene® *Clostridium difficile* assay procedure (Wince J. L., et al. POSTER BOARD NUMBER 204).

ELISA Premier Toxins A&B

Premier Toxins A&B is an enzyme immunoassay for the direct detection of *Clostridium difficile* toxin A and toxin B in stool samples. Breakaway microwells are coated with toxin specific monoclonal and polyclonal antibodies. Diluted patient specimens and HRP conjugated anti-toxin A and

B polyclonal antibodies are added to microwells. If either toxins is present in the diluted patient samples, HRP-conjugated toxin polyclonal antibodies (specific for both toxins) complexes are formed and detected by a substrate/cromagen solution.

Chlamydia trachomatis

HISTORY

Chlamydia trachomatis (*C. trachomatis*), the most prevalent sexually transmitted bacteria worldwide, is a known risk factor for pelvic inflammatory disease (PID), tubal factor infertility (TFI) and ectopic pregnancies, but the impact of a C. trachomatis infection on male fertility is unclear. It is also recently hypothesized that chronic infections with for example *C. trachomatis* might initiate/promote ovarian tumor development.
The effects of infection with *C. trachomatis* were first described in ancient Chinese writing in the Ebers papyrus (1500 B.C.) as trachoma of the eye (Stephens R.S. 1999). The name "trachoma" was first introduced in A.D. 60 and referred to the "roughness of the conjunctiva" that characterizes the ocular disease. The disease eventually became endemic but during the last century, the disease has disappeared from many parts of the world. The disappearance has been attributed to improvements in the standard of living and of hygienic practices. In hot, dry climates it still persists, and is a major cause of blindness in developing countries.
This finding boosted research on Chlamydia and by 1975 *C. trachomatis* was suggested to be the most common sexually transmitted bacterial pathogen worldwide (Schachter J., et al. 1975). *C. trachomatis* was by then already recognized as a very common cause of urethritis in men and cervical infection in women. In 1977 Mårdh and colleagues stated that *C. trachomatis* was a major cause of pelvic inflammatory disease (PID), (Mårdh, P.A. et al. 1977) and subsequent studies found that this organism could be associated with tubal factor infertility (Mårdh, P.A., Svensson, L. 1982) and ectopic pregnancy (Svensson, L., et al. 1985). It was in the early 1980´s that C. trachomatis was divided into two groups causing primarily either ocular disease or genital disease. Since then there has been a rapid progress in the knowledge of C. trachomatis and its effects and diseases in humans.
C. trachomatis is considered the worlds leading preventable cause of blindness, with about 6 million people blinded as a result of this disease (Thylefors B., et al. 1995). It is also the most common sexually transmitted bacteria in the world, with approximately 90 million new cases occurring each year (WHO. 2001).

EPIDEMIOLOGY

Up to as many as 85 to 90 percent of *C. trachomatis* infections in men and women are asymptomatic (Cottingham J., & Hunter D. 1992); (Crowley T., et al.1992) and can persist for several months and years. This results in a large reservoir of unrecognized, infected individual who are capable of transmitting the infection to sexual partners.

Urogenital *C. trachomatis* in female

The complications encountered by the female are many different such as urethritis, bartholinitis, cervicitis, endometritis, pelvic inflammatory disease (PID) sometimes with intraabdominal spread causing periappendicitis and perihepatitis, and in rare cases proctitis. Symptoms range from pain when urinating to lower abdominal pain, modest fever and adnexal and uterine tenderness on pelvic examination, but often there is only a midcycle bleeding or no symptoms at all. Late sequels are infertility, ectopic pregnancy, chronic pelvic pain and probably also uterine cervix squamous cell carcinoma (Demaio J., et al. 1991).

Urogenital *C. trachomatis* in males

When symptoms do occur, usually 1 to 3 weeks following the exposure, they are indistinguishable from those from gonorrhea. In men the spectrum of disease covers urethritis, prostatitis, orchitis and epididymitis (Dille B. J., et al.1993). Unilateral scrotal pain is primary symptoms, and common clinical sign of this infection include scrotal swelling, tenderness, and fever.

C. trachomatis infection in infants

C. trachomatis is the most common cause of neonatal conjunctivitis (Hammerschlag, M. R., et al. 1989). Prophylactic treatment of the eyes with silver nitrate does not prevent Chlamydia infection; 15 to 25% of treated infants who were exposed at birth develop conjunctivitis, and 3 to 16% develop pneumonia (Preece, P. M., et al. 1989). Symptoms of conjunctivitis usually develop within 2 weeks of delivery, and if the infection is untreated, *Chlamydia pneumonia* can develop at 4 to 17 weeks after delivery (Claesson, B. A., et al. 1989). These conditions are occasionally difficult to treat, and prolonged hospitalization may be necessary (Centers for Disease Control and Prevention. 1993).

C. trachomatis BACTERIUM AND METHOD OF ACTION

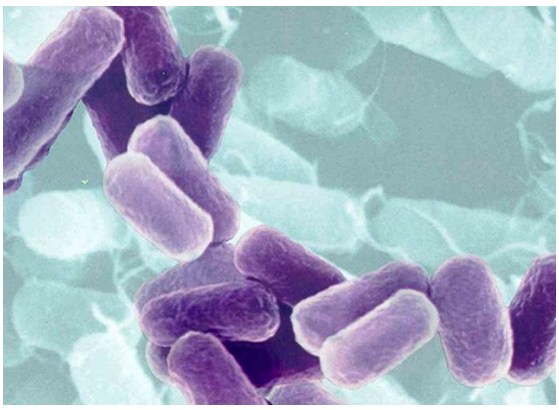


Fig. 8: *C. trachomatis* bacteria.

The *Chlamydiae* (fig. 8) are nonmotile, gram-negative bacteria pathogens that were once mistakenly thought to be viruses because of their obligate intracellular life cycle. *Chlamydiae* are metabolically deficient in their ability to synthesize ATP and thus require an exogenous source of this high-energy compound. (Bush, R.M. & Everett, K.D.E. 2001). They have been placed in their own order, *Chlamydiales*, with one family, *Chlamydiaceae*, and a single genus, *Chlamydia*. There has been some disagreement in the scientific community whether *Chlamydia* should be divided into two genera, *Chlamydia* and *Chlamydophila*, based on apparent differential clustering of the 16S rRNA gene, (Everett KD., et al. 1999) however this separation has not been commonly accepted (Stephens RS., et al. 2009). The genus Chlamydia consists of four major species, *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae* and *Chlamydia pecorum*. *C. trachomatis* has been divided into three biovariants (biovar): trachoma, lymphogranuloma venerum (LGV) and murine (mouse pneumonitis [MoPn] agent). The trachoma and LGV biovars are distinguished by different clinical features. LGV readily cause systemic infections and proliferate in lymph nodes, whereas growth of the trachoma biovar has been believed to be limited to columnar epithelial cells at mucosal surfaces. However, C*hlamydial* antigen and nucleic acid is also found in macrophages and smooth muscle cells deep within the lamina propria, (Stephens R.S. 1999) and electron microscopic investigation has revealed *C.trachomatis* elementary bodies within spermatozoa (Erbengi T. 1993). The trachoma biovar consists of prototypical serovariants (serovars), determined by the serological immune response, and designated by the letters A through K. The serovariants A through C give rise to trachoma of the eye, whereas serovariants D through K in adults give rise to genital manifestations and in newborns pneumonia and conjunctivitis.

Developmental cycle

The developmental cycle of *Chlamydia* consists of a small (0.3μm) extracellular, infectious elementary body (EB) and a larger (1μm) dividing intracellular reticulate body (RB) (Stephens RS. 1999) (fig. 9). The EB has an osmotically stable and poorly permeable cell envelope and a much reduced surface area compared to the RB. The EB are also metabolically inactive, but have the capability to recognize and enter the host cell, and to reorganize and grow 30- fold in volume to the division-capable RB form. The intracellular cycle all takes place in an inclusion, a membrane-limited vacuole. Within a few hours after inclusion, EBs differentiates into the larger, metabolically active RBs. As the chlamydiae multiply, the inclusion increases in size to accommodate the multiplying bacteria, which eventually turn into EBs that accumulate within the inclusion. The RBs continue to multiply until the cell lyses at 40 to 48 hours (*C. trachomatis*) post infection, and the infectious EBs are released.

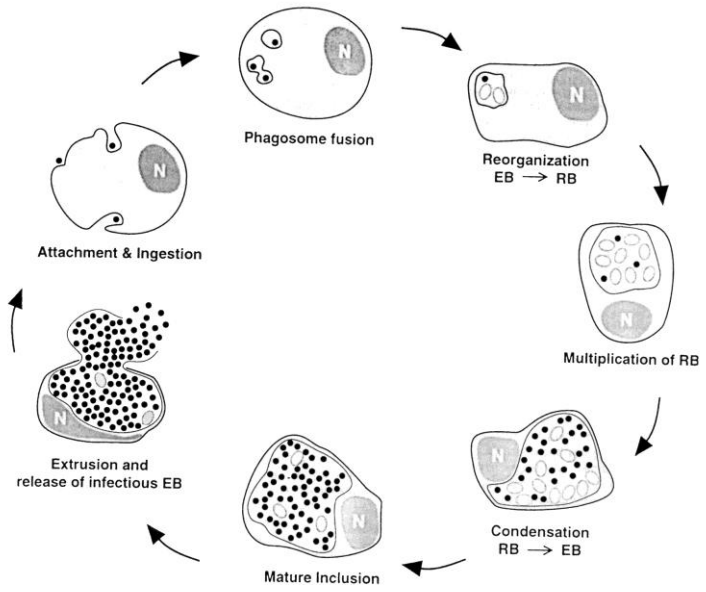


Fig. 9: Life cycle of *C.trachomatis* in tissue culture.

Persistence

Persistence of infection is the continuous presence of viable but non-infectious and non-cultivable bacteria (Deka S., et al. 2006). Today there is abundant evidence that chlamydiae are capable of causing enduring infections for months and years. Less is known in which developmental form chlamydiae survive long-term within the body, the susceptibility of such forms to antibiotic treatment, or the role of persistent chlamydial infection in disease. In vitro studies have shown that chlamydiae might have an abnormal development after exposure to antibiotics, developing aberrant RBs that enlarge, but resume normal developmental cycle after withdrawal of the antibiotic (Wolf K., et al. 2000). Exposure to a nutrient depleted environment has shown to give similar results, as well as cytokines, particularly gamma interferon, monocyte infection, continuous infection, (Hogan RJ., et al. 2004) co-infection with herpes simplex virus type 2 (HSV-2)13 or sustained antibiotic treatment (Mpiga P & Ravaoarinoro M. 2006). In vivo, chlamydial DNA and antigen have been detected in culture negative subjects in tubal, ovarian and endometrial tissues as well as in prostatic tissue and semen samples (Barlow RE., et al. 2001). Morphologically aberrant chlamydial forms resembling those observed in vitro have been visualized by electron microscopy (Nanagara R., et al. 1995). *Chlamydial* RNA has been detected in the absence of cultivability in experimental trachoma of primates (Holland SM., et al. 1992) as well as in synovial biopsy samples of patients with reactive arthritis or Reiter's syndrome, (Gerard HC., et al. 1998) and in the fallopian tubes of seven women with ectopic pregnancy who were DNA positive for *C. trachomatis* (Gerard HC, et al.1998). Since RNA is highly labile this indicates viable, metabolically active, but non-cultivable organisms. In animal infection models mice infected with either *C. trachomatis* (Cotter TW., et al. 1997) or *C. pneumoniae,*(Malinverni R., et al. 1995) infections that had become asymptomatic, reactivated after immunosuppression with cortisone or cyclophosphamide. Several studies addresses the question of persistent infections in the female genital tract, with a maximum follow-up period of five years (Golden MR., et al. 2000). The persistence rate varies between 29 and 87% depending on length of follow-up period, subjects included, detection method, test specimen etc. In a more recent study the persistence rate was 55% at one year, (Morre SA., et al. 2002) and in another 46% at 1 year, 18% at 2 years and 6% at 4 years (Molano M, et al. 2005). A *C. trachomatis* serovar-specific analysis was done and 53 out of 55 women were found to be infected by the same serovar at all occasions indicating persistence rather than re-infection. Oral contraceptive pill use and older age at first sexual intercourse was associated with increased clearance rate. In men, the duration of the followup period has lasted up to six months. 89% of

men eligible for follow-up were still *C. trachomatis* positive by PCR testing in a urine specimen after six months (Van Den Brule AJ., et al. 2002).

Chlamydia Heat Shock Proteins

Heat Shock Proteins (HSPs) are a group of highly conserved cellular proteins that acts as chaperones, with a key role in intracellular folding and refolding, assembly, and translocation of proteins. The expression of HSPs was initially found to be elevated in reaction to heat stress but is also expressed as well in reaction to proteolytic, mechanical or chemical stress (Pockley AG. 2003). There are four main groups of HSPs based on their molecular weights: HSP90, HSP70, HSP60 and the small HSPs.
During persistent infection the HSP60 production is up-regulated while the production of other proteins is down-regulated (Deka S., et al. 2006).
Chlamydial HSP60 is suggested to inhibit the apoptotic pathway of the host-cell supporting persistence of chlamydial infection (Dean D., & Powers VC. 2001). Human and chlamydial HSP60 share an approximately 50% amino acid homology (Neuer A., et al. 2000) and despite this homology, chlamydial HSP60s are highly immunogenic and are the HSPs most extensively studied in relation to infertility. The humoral immune response to cHSP60 is in several studies associated with tubal damage and subsequent infertility (Den Hartog JE., et al. 2005). An autoimmune cross-reaction between human and chlamydial HSP60, making the woman's immune system attack autologus HSP60, or a delayed hypersensitivity reaction, is suggested to be the mechanism for the inflammation and scarring of the tubes (Neuer A., et al. 2000). More recent research have demonstrated that *Chlamydia* infected cells produce pro-inflammatory chemokines, cytokines, growth factors and other cellular modulators, sufficient to account chronic and intense inflammation and the promotion of cellular proliferation, tissue remodeling and scarring in itself (Pockley AG. 2003). It is also suggested that chronic infection and inflammation, and inhibition of apoptosis by cHSP60, might have tumor promoting/initiating effects, increasing the risk for genital cancers such as cervical cancer (Paavonen J., et al. 2003). Furthermore, epithelial ovarian cancer is also suggested to be associated with *C. trachomatis* infections (Di Felice V., et al. 2005).

The major outer membrane protein (MOMP)

The MOMP is a surface-exposed, integral membrane protein of approximately 40 Kilodaltons (fig. 10). It has functional roles for the extracellular infectious elementary body (EB) and porin properties permitting uptake of essential nutrients such as ATP for the intracellular replicatory form, the reticulate body. Other studies (Baehr, W., et al. 1988) have demonstrated that the MOMP has discrete regions of antigenicity and immunoaccessibility. The human-pathogenic strains of *C. trachomatis* have been subdivided in to 15 serovars (Li, L2, L3, A through K, and Ba). By their antigenic properties, these serovars were grouped into three complexes: the B complex (B,Ba, D, E, Li, and L2), the intermediate complex (F, G, K, and L3), and the C complex (A, C, H, I, and J) (Conlan, W. J., et al. 1989). Intermediate serovars can also be subdivided into B-related (F and G) and C-related (K and L3) complex serovars. All 15 serovars of *C. trachomatis* bear a major outer membrane protein (MOMP), which constitutes 60%, by weight, of the chlamydial outer membrane proteins (Bolwell, C., et al. 1989). The MOMP genes encode a highly conserved protein structure that contains four evenly spaced domains, with sequences varying among the different serovars, called the four variable domains (VDs) (Krchnak, V., et al. 1987). The MOMP is also an immunoprotective antigen and is the target for neutralizing MAbs.

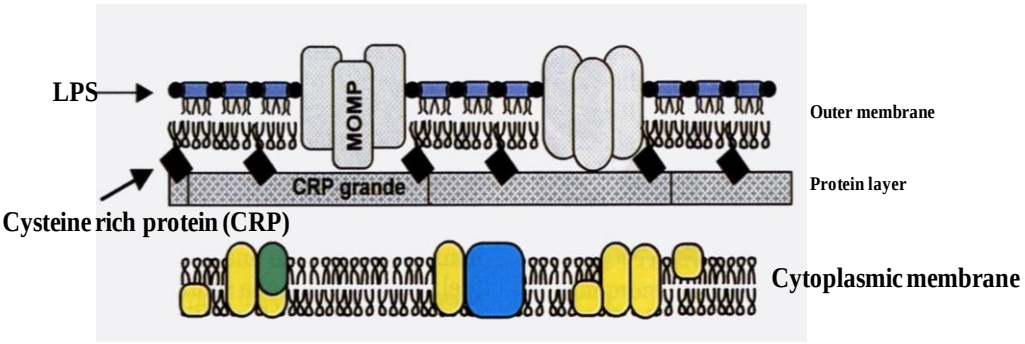


Fig. 10: *C.trachomatis* envelope organization.

LABORATORY TESTING OF *Chlamydia trachomatis* INFECTION

Test specimen

To get valid test information it is important to have the right test specimen, but in *C. trachomatis* diagnosis this is not always easily achieved. The infection might be localized in the endometrium, fallopian tubes, ovaries or the prostatic tissue (Toth M., et al. 2000) which are not easily accessible, and it requires invasive procedures to get tissue samples from these locations. The common routine diagnosis involves a first void urine sample or a cervical or urethral swab. Lately vaginal swabs have been introduced with good accuracy for detection of lower genital tract infections (Hobbs MM., et al. 2008). However, *C. trachomatis* antigen or DNA has been found in endometrium, fallopian tubes, ovaries, semen or prostatic tissue without being able to detect any bacteria in urine specimens or cervical secretions (Toth M., et al. 2000). *Chlamydia* serology, utilizing a blood sample, might in some instances be an alternative diagnostic method with an easily accessible test specimen giving information on the immune reaction to a chlamydial infection at any site of the body.

Culture

Culture was for many years the only method available for the diagnosis of *C. trachomatis*, but has in the light of newer methods, such as nucleic acid amplification tests (NAAT), it was abandoned for routine diagnosis in large-scale laboratories. The sensitivity is approximately 60-70% which makes it a poor diagnostic tool, but the specificity is 100% (Schachter J. 1999).

Nucleic acid amplification assays – NAATs

NAATs are widely used for *C. trachomatis* diagnosis today and have proven to be more sensitive and specific than previous diagnostic tests (culture, IF and EIA) because they don't need viable chlamydiae (more tolerant to transports) and due to the amplification process. A further advantage is that they can be used in non-invasive specimens such as a first-void urine or vaginal swabs with nearly identical sensitivity and specificity to those in cervical or urethral samples (Cook RL., 2005). Principally NAATs amplify are: either a) the target nucleic acid, DNA (polymerase chain reaction, PCR; strand displacement assay, SDA) or ribosomal RNA (rRNA) (transcription mediatedamplification, TMA); or b) the probe after it has annealed to the target nucleic acid (ligase chain reaction, LCR). The major targets for amplification based tests against *C. trachomatis* are generally multiple-copy gene products, such as the cryptic chlamydial plasmid (PCR, LCR, SDA) which is present in EBs with 7 to 10 copies, or rRNA (TMA) which may

have several thousands of copies per bacterial cell. The high number of target copies in TMA might theoretically be advantageous with respect to sensitivity. Specificities and sensitivities among the different NAATs are reported to be similar ranging from 95% to 100% for specificity and 80% to 93% for sensitivity (Cook RL., 2005). Different specimens can in some instances affect sensitivity due to inhibitors to the amplification process.

Serology

Serologic testing for *C. trachomatis* has been used to detect antichlamydial antibodies (Den Hartog J.E., et al. 2008). The microimmunofluorescence assay (IF) uses chlamydial EBs treated in a process to remove the group specific lipopolysaccharide (LPS) of the outer membrane. It is considered to be the 'gold standard' for the serological diagnosis of C. trachomatis infections (Dowell S.F., et al. 2001). However, IF is labor-intensive and reading of the assay is operator-dependant and subjective. To overcome these drawbacks, *C. trachomatis*-specific enzyme-linked immunosorbent assay (ELISA) tests have been developed. These ELISA generally use recombinant peptides of the major outer membrane protein (MOMP) or the 60-kDa chlamydial heat shock protein (cHSP60). A number of studies have demonstrated strong correlations between the presence of anti-MOMP antibodies (Jones C.S., et al. 2003) or cHSP60 (Gazzard C.M., et al. 2006) and the severity of genital *C. trachomatis* disease, PID, infertility and tubal pathology.

ELISA Savyon® Diagnostics

Savyon, Israel has developed an assay in which *C.trachomatis* species specific epitopes, derived from different serotypes, are coated to ELISA plates (Sero-CT-IgG). The test excludes cross-species reactive epitopes and is accurate and specific in the determination of *C.trachomatis* IgG antibodies, it has a sensitivity of 95% and and specificity of 100 %**.**
This method is used in this thesis as a reference for the development of the new CLIA assay.

THE LIAISON® SYSTEM

The Liaison® system is an instrument designed to perform immunometric analyses of biological fluid samples (such as serum or plasma) in a completely automated way (fig. 11). Up to 15 different tests can be performed at once on up to 144 samples in a sequential or random access mode. The output of the analysis is generated through the formation of an immune complex, followed by a chemiluminescent reaction that produces an emission of light.

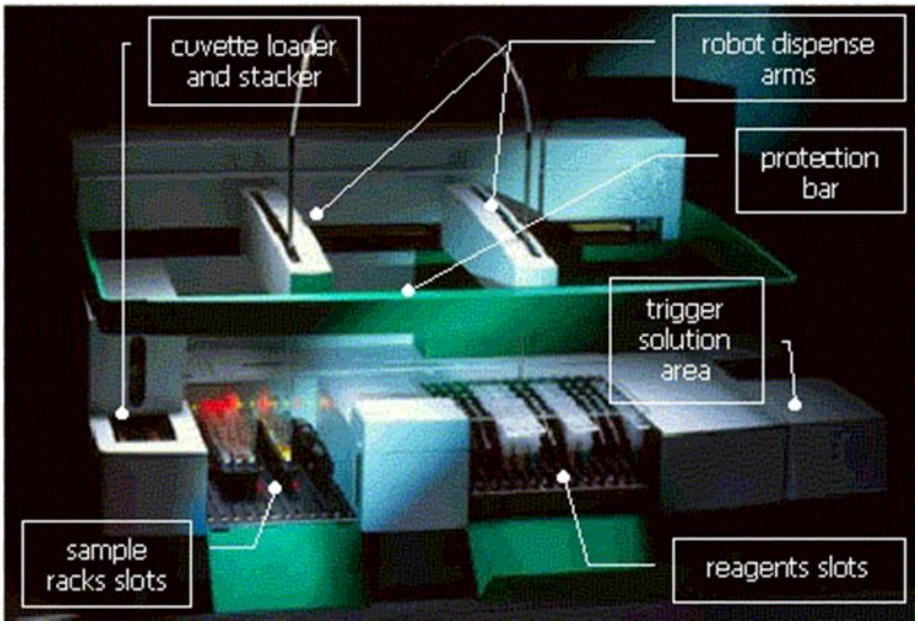


Fig. 11: The Liaison® system.

The instrument is composed of two modules, designed to be both allocated on a single workbench; the first module is a personal computer with touch screen, that hosts the user interface software and all the system data (assay protocols, reagent cartridges database, output of analyses, calibration history, network controls etc.). The second module is the actual analyzer, that performs the analysis from sample loading all the way to the final output for the user. Key components of the analyzer include:

- Cuvette loader and stacker: two conveyor belts allow continuous loading of the reaction modules, that are stored on a multilevel rack (7 levels).
- Sample rack slots: in the left-hand part of the instrument, a storage area can hold up to 12 sample racks, each carrying up to 12 samples. A barcode reader allows error-free catalogation of samples.

- Reagent slots: in the right-hand part of the instrument, another storage area can hold up to 15 different reagent cartridge simultaneously. This area is kept at a constant temperature of 15°C for optimal conservation of the reagents, while a stirring device keeps the microbeads always in homogenous suspension. Barcode reader for cartridge identification.
- Robot dispense arms: two robotic arms each carrying a dispensing needle. One arm is usually dedicated to dispensing samples, another to dispensing reagents. Each one has a separate washing well to clean the needle after each pipetting.
- Incubator: this area hosts the reaction cuvettes during incubation times, at a constant temperature of 37°C.
- Washing station: through the application of a magnetic field, this part allows retention of the paramagnetic microbeads and removal of the reaction liquids. Any number of washing steps with the desired washing buffer can be set.
- Read area: contains the injection devices of trigger reagents and the photomultiplier tube

The Liaison® system is based on two key features: the use of paramagnetic microbeads as the solid phase and the generation of signal by means of chemiluminescence.
The adoption of microbeads as the solid phase instead of the classic immunoassay supports, as the ELISA microwells gives a clear edge in terms of available reaction surface, which in turn increases the kinetic rates of the antigen-antibody complex formation.

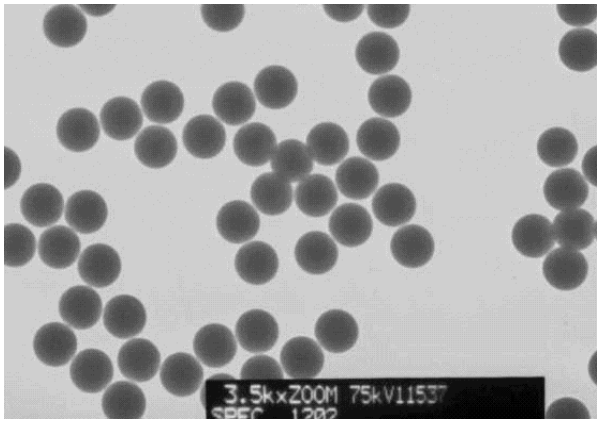


Fig. 12: The paramagnetic microbeads used in the Liaison® system.

Moreover, diffusion of both the analyte and the solid phase in the reaction volume is allowed, while in ELISA system only the analyte can diffuse,

decreasing the possibility of the immune complex formation. The microbeads adopted in the Liaison® system (fig. 12) are colloidal particles composed of a ferric oxide core covered by a polystyrene layer formed by spontaneous coalescence of polystyrene linear chains. This structure is in turn coated with another layer composed of polyurethane activated with tosyl- groups. The tosyl- group (4-toluenesulfonyl chloride) can undergo nucleophilic attack (fig. 13), allowing the beads to covalently bind proteins through their available aminic groups (ε-amino groups of lysines, N-terminal end).

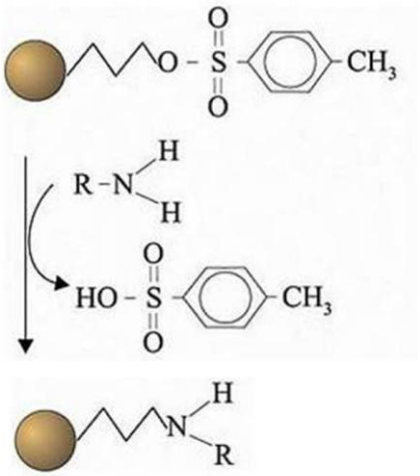


Fig. 13: Chemistry of the covalent binding of amines to the tosyl-activated beads.

The paramagnetic properties of these microbeads allow easy manipulation through the application of a magnetic field. The particles respond to a magnet but are not magnetic themselves and retain no residual magnetism after removal of the magnet.
The tracer molecule is an antigen or antibody conjugated to a signal generating compound. Chemiluminescent tracers are formed by conjugating the antibody (or antigen) to a molecule that can generate a photon emission upon addition of certain reagents. The entity of this photon emission is measured with a luminometer, usually equipped with a photomultiplier tube.
The chemiluminescent molecule used in the Liaison® system is the luminol derivate ABEI (N-(4-Amino-Butyl)-N-Ethyl-Isoluminol), which is converted to its activated ester to allow conjugation with the antibody or antigen (fig. 14).

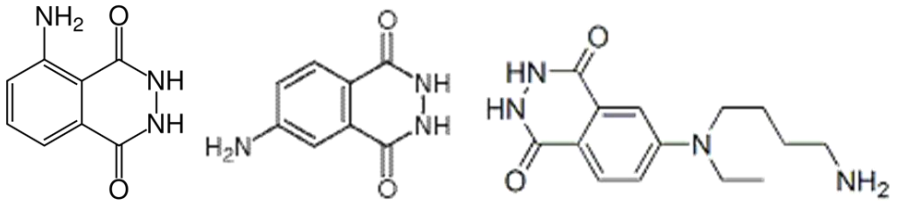


Fig. 14: Luminol, isoluminol, and ABEI.

In presence of H_20_2 and a microperoxidase (deuteroferriheme), ABEI achieves an excited state in consequence of a chemical reaction. This excited level decays to the ground level generating energy in form of light (fig. 15).

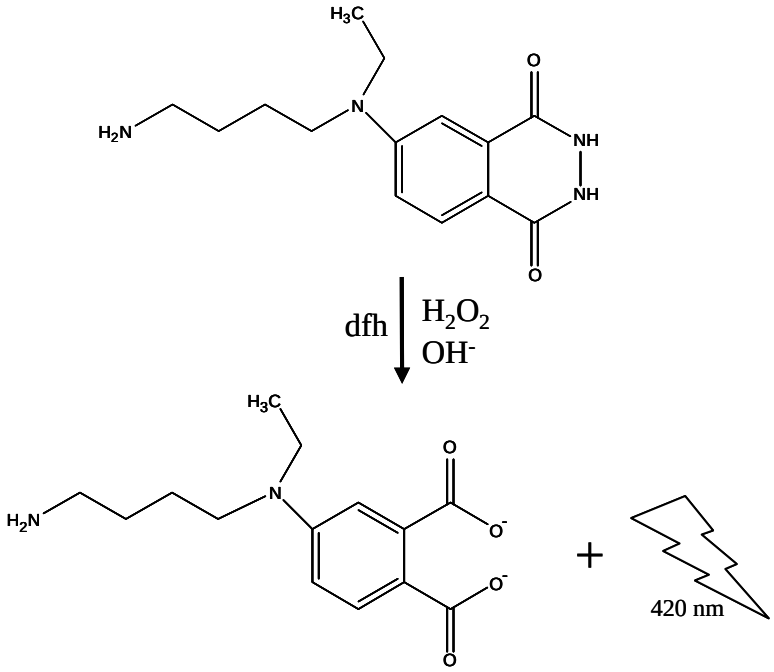


Fig. 15: Chemiluminescence reaction of ABEI.

The emission of light is recorded by the photomutiplier tube for an interval of just 3 seconds ("flash" chemiluminescence) and the signal is integrated over this interval (fig. 16). The final result is expressed in RLUs (Relative Light Units).
Using chemiluminesce is a great improvement over enzymatic signal generation of classic ELISA format assays. Sensitivity is highly increased and a greater dynamic range can be achieved.

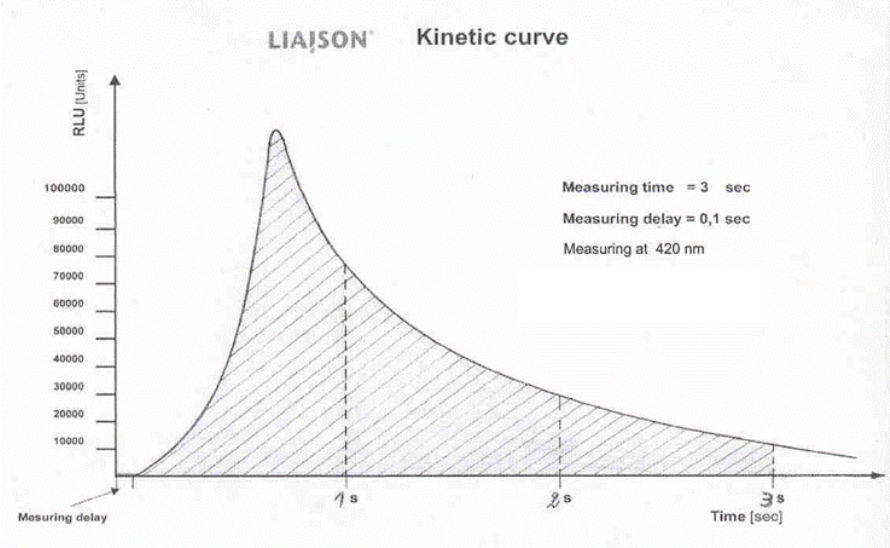


Fig. 16: "Flash" chemiluminescence signal integration of the Liaison® system.

Lower molecular weight and steric hindrance of ABEI compared to horseradish peroxidase allow conjugation of more signal generating molecules per tracer molecule. Moreover, generation and recording of signal is completed in a very short time (3 seconds), with a sensible throughput increase.

ProteOn ™

For the rapid screening of monoclonal antibodies anti Toxin A and Toxin B, created by DiaSorin for the development of the *C. difficile* assay, was used the **ProteOn ™ XPR 36 protein interaction array system.** This instrument has a multiplex surface plasmon resonance (SPR) optical biosensor that provides real-time data on the affinity, specificity, and interaction kinetics of protein interactions. SPR occurs when light interacts with a metal film placed at the interface between two media with different refractive indices, such as glass and water. SPR biosensors respond in real time to changes in the refractive index resulting from the binding and subsequent separation of two proteins. One protein (ligand) is chemically bound to a gold layer on the sensor surface. As a protein in solution (analyte) flowing over the surface binds to the immobilized protein, the refractive index near the sensor surface increases, leading to a shift in the SPR angle. When the protein solution is replaced with solution devoid of protein, the protein complex on the sensor surface dissociates, and the SPR angle shifts back. The shift in the SPR angle

is measured in response units (RU) and recorded as a function of time in the form of a sensorgram (fig. 17). The sensogram diplays the time course of binding of analite to ligand surface.

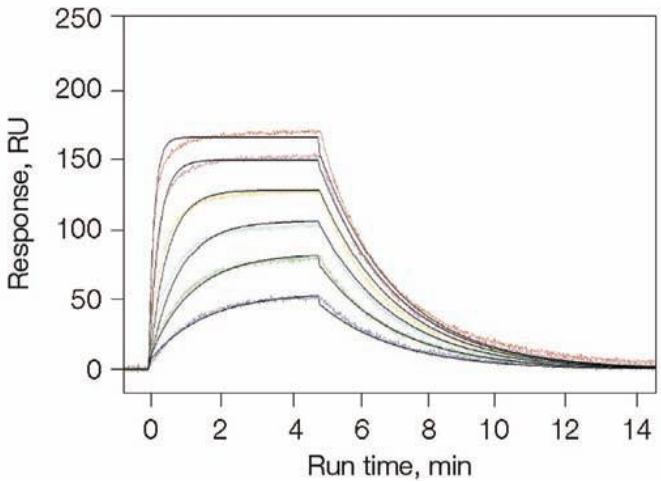


Fig. 17: Representative sensorgram from SPR analysis of protein–protein interaction kinetics. Each of the six curves represents one of the six horizontal channels on the ProteOn XPR system, each of which contains a different concentration of the analyte (Mohammed Y., 2007).

This system generates a 6 x 6 interaction array for the simultaneous analysis of up to six ligands with up to six analytes. The ProteOn XPR36 system increases the throughput, flexibility, and versatility of experiment design, enabling the completion of more experiments in less time:

- Analyze up to 36 different protein interactions in a single run, on a single chip.
- Perform a complete kinetic analysis in a single run.
- Measure a variety of experimental conditions simultaneously in parallel.
- Screen multiple panels of analytes.

AIM OF THE WORK

As mentioned in the introduction, the *Clostridium difficile* and *Chlamydia trachomatis* infections are largely diffused worldwide. For that reason is important the development of a new automated high-throughput assays, working on the automated LIAISON® system, that can diagnose the bacteria infection and reduce the number of sick and dead people for these diseases. These two chemiluminescence semi-quantitative immunoassays must have a good sensitivity and specificity in order to be competitive on the market.

Clostridium difficile

In this work a chemiluminescent immunoassay was developed for the semi-quantitative detection of Toxins A&B in human stool specimens. This will be the first fully automated test with high-throughput for the detection of Toxin A&B, in human stool specimens, available on the market and will be used as a rapid screening of CDI. The LIAISON® *C.difficile* Toxin A&B kit is direct two steps "sandwich" immunoassay. During the first step the Toxins A&B contained in stool supernatant bind the conjugate (MoMAbs anti Toxin A and MoMAbs anti Toxin B labeled with ABEI). In the second step the PMPs (coated with MoMAbs anti Toxin A and MoMAbs anti Toxin B) are added forming a sandwich with the previous complex. After the addition of trigger solutions the chemiluminescent reaction is measured as relative light units (fig. 18). The antibodies specific for the recognition of Toxins A & B were obtained by DiaSorin through the immunization of mice with Toxoid A and B.

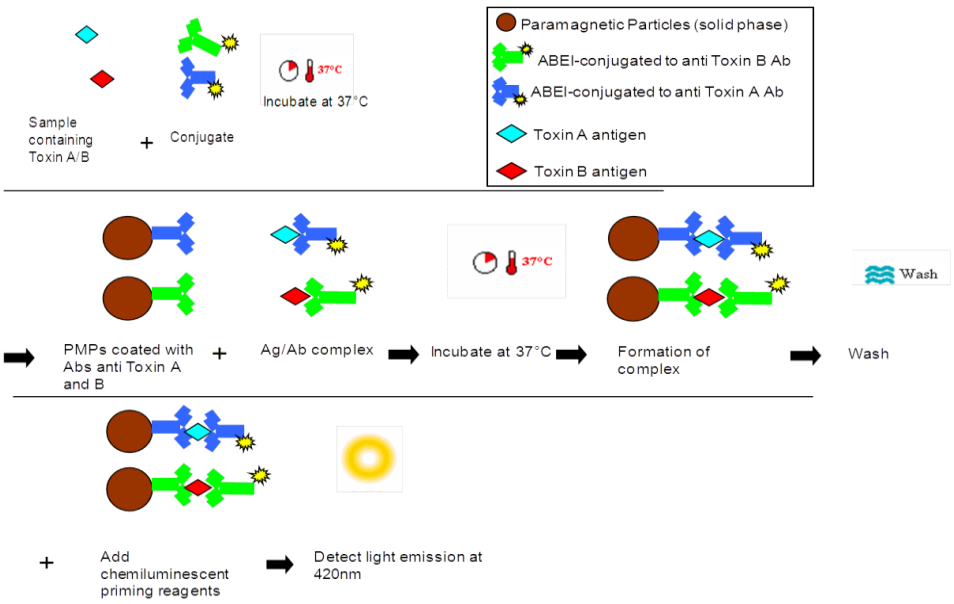


Fig. 18: LIAISON® *C.difficile* Toxin A&B assay protocol.

Stool-based assays represent an expansion of the LIAISON® automated diagnostic assay panel of infectious disease testing products offered for DiaSorin customers. As these assays are a novel direction of diagnostic testing for DiaSorin which we have limited technical experience, it is prudent to assess the ability of stool components to cause interference in general. Fecal material is a hodgepodge of digestive, biochemical, and bodily waste products that must be routinely eliminated. The average human produces 100-250 grams of fecal material per day of which 75% is water and only 25% is solid matter. This solid matter is mainly composed of indigestible food, bacteria, fats and cholesterol, and proteins. Numerous species of bacteria are known to colonize the large intestine including Salmonella, Staphylococcus, and Streptococcus. The bacterial strains and substances normally present in the feces were tested as potential interfering compounds.
The following design inputs (table 3) were developed in order to satisfy user needs and intended use based upon a review of marketing assessment. The Table 3 shows only the design inputs main points.

Table3: *C.difficile* design inputs.

Parameter	Acceptable	Target
Analyte	Toxin A and B	
Assay Form	Sandwich	
Expression of Results	Semi-quantitative	
Sample Type	Stool	
Limit of Detection	Better than the PTAB Meridian assay	
Strain reactivity requirements	A+/B+ and A-/B+	
Sensitivity	Greater than or equal to 85% Agreement to Illumigene®	Greater than or equal to 90% Agreement to Illumigene®
Specificity	Greater than or equal to 95% Agreement to Illumigene®	100% Agreement to Illumigene®
Cross-Reactivity and Interferences	No change in sample classification (Pos or Neg)	
Time to Result	<60 minutes	<35 minutes
Throughput	>45 tests/hr	>90 test/hr

Chlamydia trachomatis

This immunoassay was developed with the aim of achieving a semi-quantitative determination of human IgG anti *C.trachomatis* in serum specimens. For the *C. trachomatis* immunoassay development a mixture of 4 peptides derived from specific regions within the MOMP protein were used. These peptides are capable of interacting with antibodies against MOMP from any one of the serovars of *C.trachomatis* (described in patent WO9900414) and are currently used in an ELISA test by Savyon (Israel). The 4 peptides were produced by DiaSorin Spa by the peptide synthesizer (AAPPTEC APE 396) use.
The first approach used for the development of this essay was the use of the 4 peptides for on the solid phase (ELISA Savyon like):
STEP 1- The serum sample with the anti *C. trachomatis* human IgG bind the 4 peptides (on the solid phase).
STEP 2- After the washing stage, the conjugate (anti human IgG ABEI labeled) is added to detect the first antibody bound to the solid phase.
The coating was also made with two modified peptides (with the cysteines groups alkylation) in order to increasing recognition by the *C. trachomatis* human IgG.
The second strategy that has allowed the development of this essay was through the 4 biotinylated peptides used and streptavidine PMPs (as described in materials and methods and results chapters).

MATERIALS AND METHODS

PRODUCTION OF MoMAbs SPECIFICS FOR THE *C. difficile* TOXIN A & TOXIN B DETECTION

The necessary steps for the production of Mouse Monoclonal antibodies (MoMAbs) specific for a particular antigen are shown in the fig.19:

(1) Immunization of a mouse
(2) Isolation of B cells from the spleen
(3) Cultivation of myeloma cells
(4) Fusion of myeloma and B cells
(5) Screening of hybridomas
(6) Separation of hybridomas cell lines
(7) Selection of antigen specific secreting cell lines

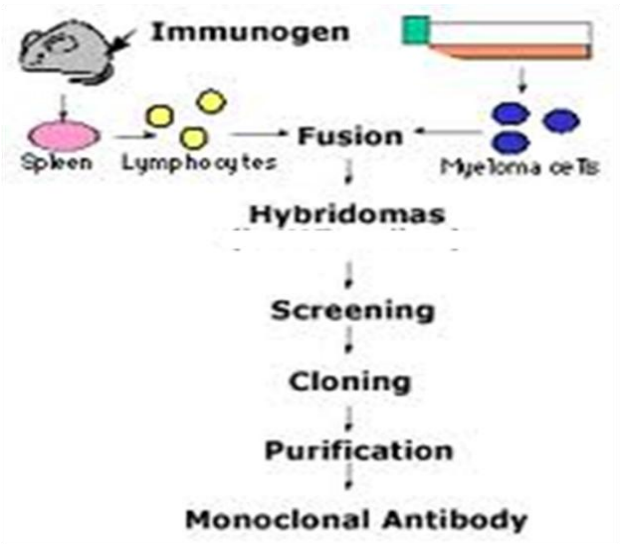


Fig.19: Steps required for the MoMAbs production specific for an antigen.

Toxin Immunization

8 female Balb/c mice (10 weeks/old) were immunized with 50ug of Toxoid A or Toxoid B (are purchased from List Biological) once a month for a total of 2 injections, using FREUND'S adjuvant (Sigma Aldrich). The protocol used is reported in Thi Man & Glenn E. Morris (1996).

Day 1: 1st injection 50µg antigen + COMPLETE FREUND'S ADJUVANT (200µL).
Day 30: 2nd injection 50µg antigen + INCOMPLETE FREUND'S ADJUVANT.
Day 40: BLEEDING to check immune response.
From day 60: BOOST FUSION:

- 5 DAYS BEFORE FUSION: 1st BOOST: 50µg antigen NO ADJUVANT (200µL).
- 4 DAYS BEFORE FUSION: 2nd BOOST: 50µg antigen NO ADJUVANT (200µL).
- 3 DAYS BEFORE FUSION: 3rd BOOST: 50µg antigen NO ADJUVANT (200µL).

Fusion

Fusions between mouse splenic B cells and myeloma were done as reported in Prat et al., (1991) on the basis of the methodology described by Kohler and Milstein (1975). Immune spleen B lymphocytes were fused with P3.X63.Ag8.653 myeloma cells. Hybridomas were selected with HAT medium. Supernatants were assayed in ELISA on Toxin A and Toxin B, using HRP-conjugated goat anti-mouse IgG Fc specific (AbD-SEROTEC, 1:10000) as secondary antibody. After washing, chromogen/substrate solution was added and OD was read at 450nm. The hybridomas, whose supernatants were positive in these assays, were cloned twice by limiting dilutions.

Screening

ELISA

The plate was coated with Toxin A or Toxin B at 1µg/ml for 2h, followed by saturation with BSA. First incubation was carried out by adding 100 µl anti-Toxin A or Toxin B mAb supernatant for 1 hour at r.t.. After washing three times with the washing buffer, 100 µl of HRPconjugated anti-mouse IgG antibodies were incubated for 1 hour at room temperature (goat anti mouse IgG, 1:10000, AbD Serotec).

Isotyping

Isotyping assessment was performed using PIERCE RAPID ELISA MOUSE ANTIBODY ISOTYPING KIT (antigen-independent assay) see fig. 20.

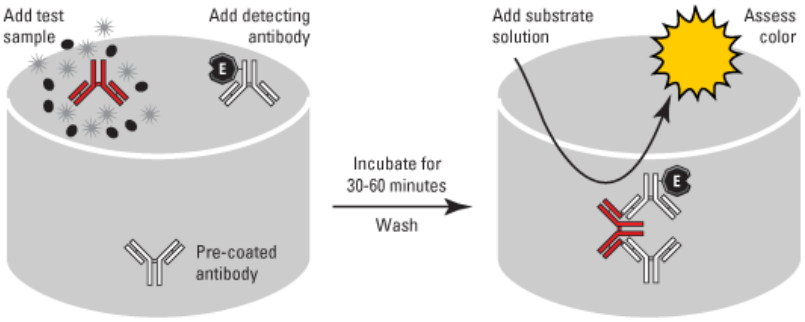


Fig.20: Plate precoated with isotype-specific antibody. Detection with HRP-conjugated anti-mouse.

Antibody affinity assessment on ProteOn ™

Purified capture antibodies (anti –mouse IgG) were immobilized in six parallel channels (fig.21-1) of the ProteOn ™ Sensor Chip. The chip used has NeutrAvidin bound to the carbossilic groups of modified alginate molecule (NLC) for the capturing of the biotinylated anti-mouse antibody. Unpurified supernatant containing antibodies from six different hybridoma clones for Toxin A or Toxin B antigen are injected orthogonally (fig.21-2). Next, the purified antigen Toxin A or Toxin B is injected in the same orientation as the original capture antibodies, at a different concentration in each of the six channels (fig.21-3). ProteOn™ XPR36 analysis yields 36 sensorgrams (arranged in sets of six representing the six different hybridoma analyzed) that detail the kinetics of all 36 interactions. Data are fitted using 1:1 Langmuir binding model, which considers a 1:1 interaction between antigen and antibody. The complex is then removed from biotinylated capture antibody and it is possible to perform another set of 6x6 interaction. Supernatant. Supernatants containing mouse monoclonal antibodies were analyzed undiluted, using cell culture medium (RPMI, 10% FBS, UltraQ, Na-Pyruvate, antibiotics) as reference capture antibody (biotinylated anti-mouse) was dilute to a final concentration of 25ng/ml into PBS Tween 0.05% pH 7.4. Native lyophilized *C. difficile* Toxin A&B were resuspended

in dd H_2O to a final concentration of 1mg/mL and then diluted into PBS Tween 0.05% pH 7.4 to the working concentrations of: 1µM, 100 µM, 50 µM, 10nM and 1 µM.

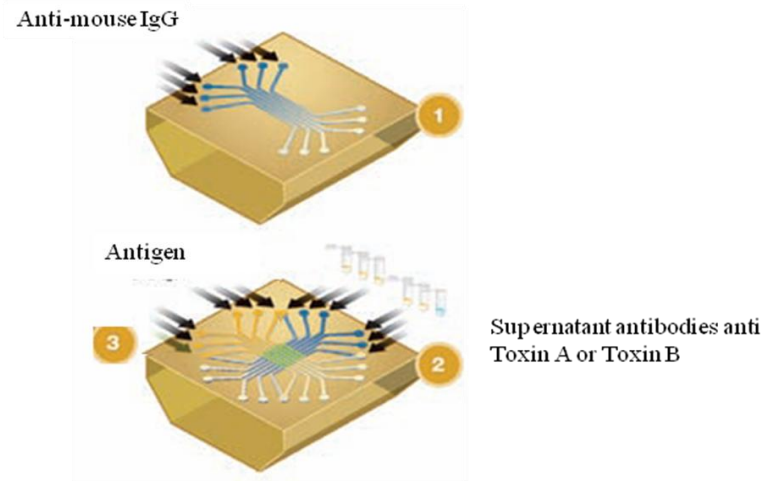


Fig. 21: Procedure for affinity assessment of hybridoma supernatant.

PARAMAGNETIC PARTICLES (PMPs)

Antigen or antibody coating procedure on PMPs

The PMPs used for the coating of anti Toxin A and Toxin B MoMAbs and the *C. trachomatis* peptides were the Dynabeads® M-280 Tosylactivated. The coating were made following the procedure recommended by DYNAL:

1. Resuspend microparticles in resuspension buffer (Borate 0.1 M, pH 9.5)
2. Add the MoMAbs or *C. trachomatis* peptides
3. 18 h @ 37 C
4. Wash twice with Tris 0.2 M, BSA 0.1% (w/v), pH 8.5
5. 18 h @ 37 C
6. Wash with PBS 10 mM, BSA 0.1% (w/v), pH 7.4
7. Re-suspend in PBS 10 mM, BSA 0.1% (w/v), pH 7.4

The MoMAbs were coated at 200µg/ml and for the peptides were done different coating concentrations (24, 100, 400 µg/ml).

The PMPs used with the *C.trachomatis* biotinylated peptides are the M280 Streptavidin-coupled Dynabeads®.

CONJUGATE AND CONJUGATE DILUENT

C. difficile

The two monoclonal antibodies made by DiaSorin have been labeled with isoluminol-derived compound (ABEI). The current excess of each conjugate antibody is 5X in excess of 100 ng/ml (high Toxin sample concentration) of Toxin in the assay reaction.

The conjugate diluent consists of 0.1 M Phosphate buffer at pH 7.0 with proteins and 0.1% Tween 20, 0.1% Proclin 300, 0.05% Gentamicin sulfate, at pH 7.0.

C. trachomatis

The *C. trachomatis* conjugate is an anti humam IgG labeled with the ABEI. It is diluted in a 0.1 M Phosphate buffer at pH 7.0 with preservatives at 100ng/ml.

SAMPLE DILUENT

C. difficile

The *C. difficile* Toxins A&B Sample Diluent is used to homogenize the stools in order to extract Toxin A and Toxin B out of the patient stool so that it is solubilized in the sample diluent. The Sample Diluent consists in 50 mM Tris buffer, with proteins and preservatives.

Stool Extraction Protocol

The following protocol is used to extract Toxins A & B from Stools.

1. Add 750 µL of LIAISON® *C. difficile* Toxins A&B Sample Diluent to 2 mL processing tube.

2. Mix stool as thoroughly as possible prior to withdrawing sample.

    a. **Liquid or Semi-Solid Stools**: Using disposable transfer pipet, Measure and Transfer 750 µL of stool into tube containing the same volume of LIAISON® *C. difficile* Toxins A&B Sample Diluent. Rinse transfer pipet with sample diluent stool mixture if necessary, to rinse as much sample as possible out of transfer pipet.
    b. **Solid Stools:** Using wooden applicator stick(s) or similar device, Measure and Transfer a 4-5 mm diameter stool sample into the tube containing LIAISON® *C. difficile* Toxins A&B Sample Diluent.

3. Cap and Vortex each tube vigorously for 20-30 seconds to loosen and disperse stool as much as possible to release toxins into sample diluent stool mixture.

4. Centrifuge tube in microcentrifuge set at maximum speed for 10 minutes at 18 - 22°C.

a. **No Debris in Supernatant:** pipette a minimum of 500 µL of stool supernatant from top of tube without disturbing pellet, add to an appropriate LIAISON® tube and load onto LIAISON® instrument for testing with the LIAISON® *C. difficile* Toxins A&B kit.
b. **Debris present in Supernatant:** Occasionally, fat-like or clot-like material may fail to pellet during centrifugation. In this case, Transfer the stool supernatant containing the debris into a 13x100 mm filtration tube, and push the filtration plunger to the bottom of the tube containing the stool supernatant, while pushing the cleared supernatant up into the interior tube of the Filtration Plunger. Pour or pipette a minimum of 500 µL cleared stool supernatant from the interior tube of the filtration plunger into an appropriate LIAISON® tube and load onto LIAISON® instrument for testing using the LIAISON® *C. difficile* Toxins A&B kit.

C. trachomatis

The sample diluent in this case is useful for the serum dilution in reaction. It consists in 0.1 M Phosphate buffer at pH 7.0, with proteins and preservatives.

MASTER STANDARDS CURVE

C. difficile

The LIAISON® *C. difficile* Toxins A&B Master Standards Calibrators (MSC) are prepared with purified Toxin or Toxoid A&B in a 1:1 proportion. Both Toxoids are purchased from List Biological. The MSC are made in a coltrol matrix buffer consists of 10 mM Phosphate buffer, proteins and preservatives. The Calibrators and Kit Controls are lyophilized and reconstituted with a 2 mL DI water fill.

ASSAY PROTOCOL

C. difficile

The LIAISON® *C. difficile* Toxins A&B assay is a two-site sandwich assay. The assay uses 200 µL of extracted stool supernatant incubated for 22 minutes with 100 µL of Conjugate. Finally 20 µL of PMPs are incubated with the sample conjugate mixture as a Delayed addition for 8 minutes, the PMPs are washed and the signal is generated.

C. trachomatis with biotinylated peptides

The LIAISON® *C. trachomatis* Toxins A&B assay is a two step-assay. 15 µL of sample are incubated for 10 minutes with 200µL of biotinylated peptides (and 20µL streptavidinated PMPs. After the washing step the PMPs are incubated for 10minutes with 200µL of conjugate.
The biotinylated peptides are diluted in 0.1 M Phosphate buffer pH 7.0 with preservatives at 25ng/mL; ratio 1:1:1:1.

C.difficile CROSS REACTIVITY and STRAIN REACTIVITY TEST

The microorganisms were cultured at Meridian Bioscience Inc. and diluted to a McFarland #4 standard reference for turbidity. The bacteria and yeast were diluted from their McFarland #4 cultures by 1:10 in the Stool samples, and the viruses were diluted 1:100 in the stool samples, in order to achieve the final concentrations listed in the results tables.

INTERFERING SUBSTANCES

Some of the stool interference compounds were purchased from Sigma Aldrich.

SAMPLES

The *C. difficile* stool samples positive and negative were purchased from USA hospitals. The *C. trachomatis* serums positive and negative were purchased from various hospitals in Europe. The methods used by these hospitals to classify the samples as positive or negative were for *C. difficile* by cell cytotoxicity assay + ELISA, and for *C. trachomatis* by Micro Immune Fluorescence (MIF).

Synthesis of *C.trachomatis* peptides

The peptides were synthesized, using a solid phase peptide synthesis (SPPS) protocol. The general principle of SPPS relies on repeated cycles of coupling-wash-deprotection-wash. The free N-terminal amine of a solid-phase attached peptide is coupled (fig. 22) to a single N-protected amino acid unit, eventually protected at its side-chain by an orthogonal protecting group. This unit is then deprotected, revealing a new N-terminal amine to which a further amino acid may be attached. The superiority of the solid phase technique partially lies in the ability to perform wash cycles after each reaction, removing excess reagent with all of the growing peptide of interest remaining covalently attached to the insoluble resin.
The solid phase synthesis can be performed by using two alternative protecting groups to temporarily mask the α-amino group of the amino acid and allow further chain elongation: tert-butyloxycarbonyl, Boc, and fluorenyl-9-methoxycarbonyl, Fmoc. Generally, the Fmoc strategy is more suitable and typically employed, as the Fmoc group is a base-labile protecting group which can be easily removed using secondary amines like piperidine and the cleavage of the peptide from the resin occurs under mild acidic conditions.
The peptides presented in this work were prepared by automated SPPS using Fmoc chemistry on an APEX396 synthesizer (AAPPTEC). The starting resin was a polystyrene Rink amide resin with a functionality of 0.34 mmol/g. Each coupling step was achieved by *in situ* activation of the aminoacidic carboxylic function using HBTU/HOBt as activator agents and DIPEA (N, N-diisopropylethylamine) as base, in DMF. 5-fold molar excess of amino acid/HBTU/HOBt and 10-fold excess of DIPEA, over the resin functional groups, were used, with a coupling time of 45min. After each coupling, Fmoc cleavage was accomplished by treating the peptidyl-resin with 20% piperidine in DMF for 10min. At the end of the synthesis, the resin was washed with diethylether and dried under vacuum. The final cleavage of the peptides from the resin and simultaneous side-chain deprotection was achieved by treatment for 1h30' with a cleavage mixture consisting in 88% TFA, 5% phenol, 5% water, 2% triisopropylsilane. The peptides were then precipitated from ice-cold tert-butyl-methyl ether, centrifuged and subjected to three ether-washing/centrifugation cycles to remove the scavengers. The crude peptides dried from ether were then lyophilized and characterized by UPLC-MS. Afetrwards they were purified by reveres phase HPLC.

UPLC-MS analysis
UPLC-MS analyses were performed using a Waters Acquity® system on a reverse phase Acquity UPLC® BEH C18 1.7um 2.1x100 mm column heated at 45°C. Eluents: water 0.1% TFA (A), acetonitrile 0.1% TFA (B). Flow: 0.4 mL/min. Gradient used: 1min isocratic condition at 20% B, then to 60% B in 4min, afterwards to 80% B in 0.2min followed by 0.5min isocratic run and return to the starting conditions. Absorbance read at 214 nm.

HPLC purification
The peptides were purified through reverse phase HPLC using a Waters preparative system on a XBridge™ Prep C18 5µm 30 x150 mm column (Waters). Eluent A: water, 0.1% TFA; eluent B: acetonitrile, 0.1% TFA. Gradient: 20% eluent B for 5 min, 20 to 50% eluent B in 20 min, to 80% eluent B in 5 min, flow rate 30 mL/min, absorbance read at 214 nm.
The collected fractions were analyzed by UPLC and those containing the target species at high purities were pooled together and lyophilized. The analytical characterization of the purified pools by UPLC/MS was finally carried out.

Peptides alkylation

Alkylation of the cysteine thiol groups is achieved by incubating the peptides with iodoacetamide (5-fold molar excess) at pH 7.5 for 1h. Alkylated peptides were then purified through reverse phase HPLC and the purified pool characterized by UPLC/MS.

Biotinylated peptides

The biotinylated peptides were purchased from IRBM Science Park.

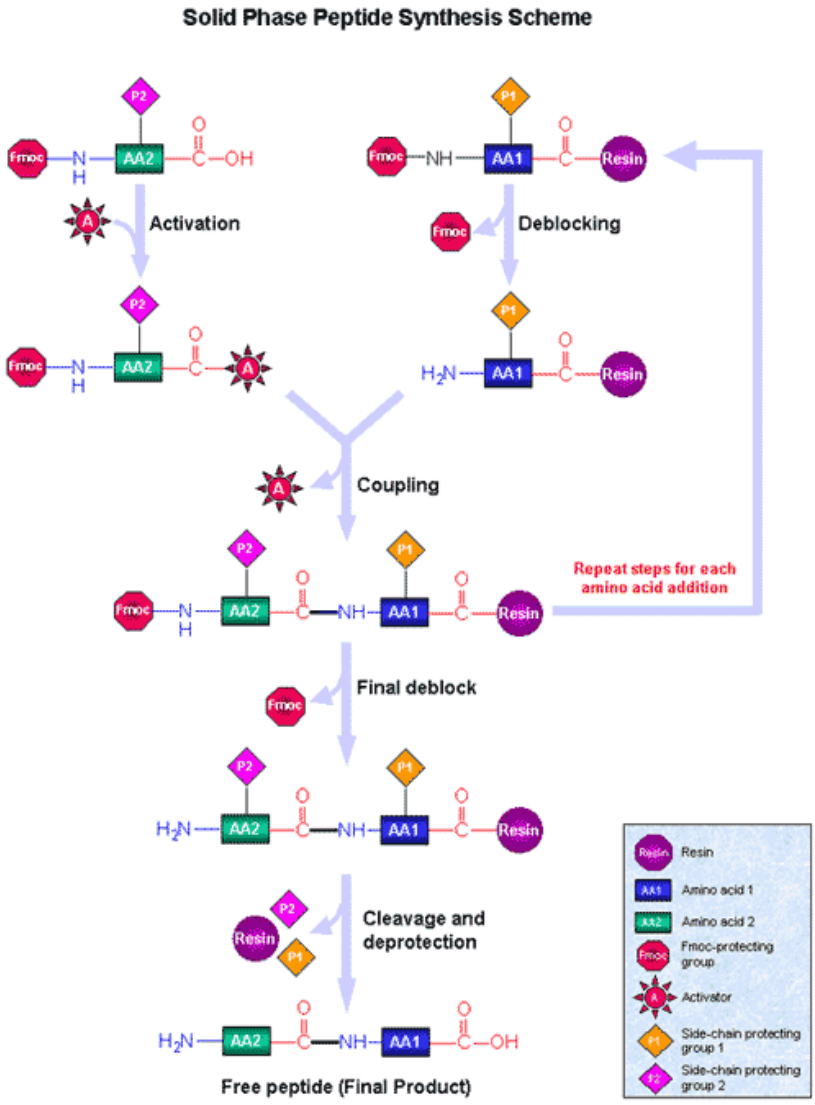


Fig. 22: Solid phase peptide synthesis scheme.

RESULTS

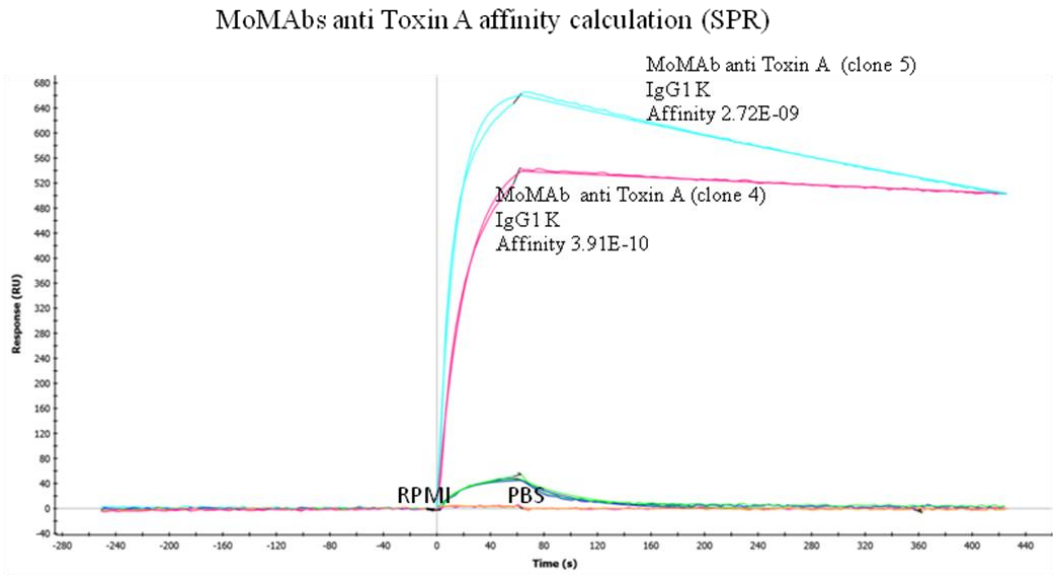
Clostridium difficile **RESULTS**

Toxin A&B Monoclonal Abs

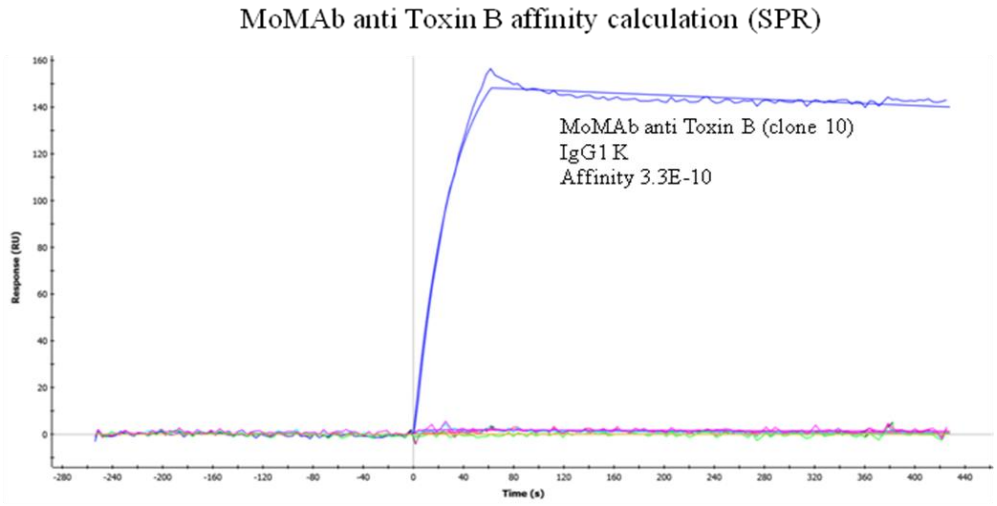
For the development of the LIAISON® *C. difficile* Toxins A&B assay, 2 antibodies able to recognize the toxin A and toxin B with high specificity and affinity were required. Following the procedure described in materials and methods section, 2 monoclonal antibodies were made by DiaSorin. These antibodies have been developed by mice immunization with the *C. difficile* Toxoid A and Toxoid B. Among the screened antibodies, those who have showed a greater affinity for the Toxin A or B are reported in the table 4 and graphs 1, 2.

Table 4: MoMAbs isotype and immunoreactivity analysis by the ELISA assay and affinity determination by ProteOn.

Cloned ID	ISOTYPE (ELISA)	REACTIVITY (ELISA Abs signal)	AFFINITY (Proteon)
4 (anti Toxin A)	IgG 1	3	3.91E-10
5 (anti Toxin A)	IgG 1	3	2.72E-09
10 (anti Toxin B)	IgG 1	3	3.3E-10



Graph1: 2 Mouse Monoclonal Antibodies Toxin A affinity calculation by SPR.

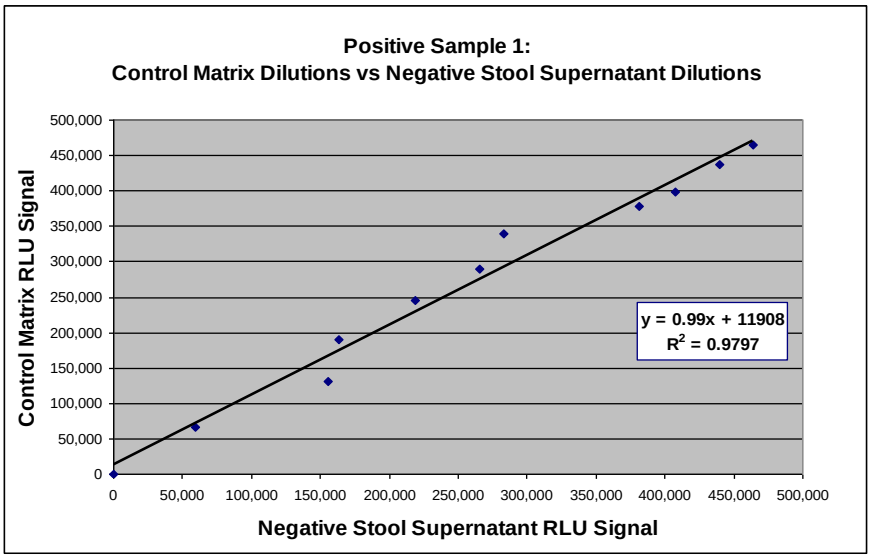


Graph 2: Mouse Monoclonal Antibody Toxin B affinity calculation by SPR.

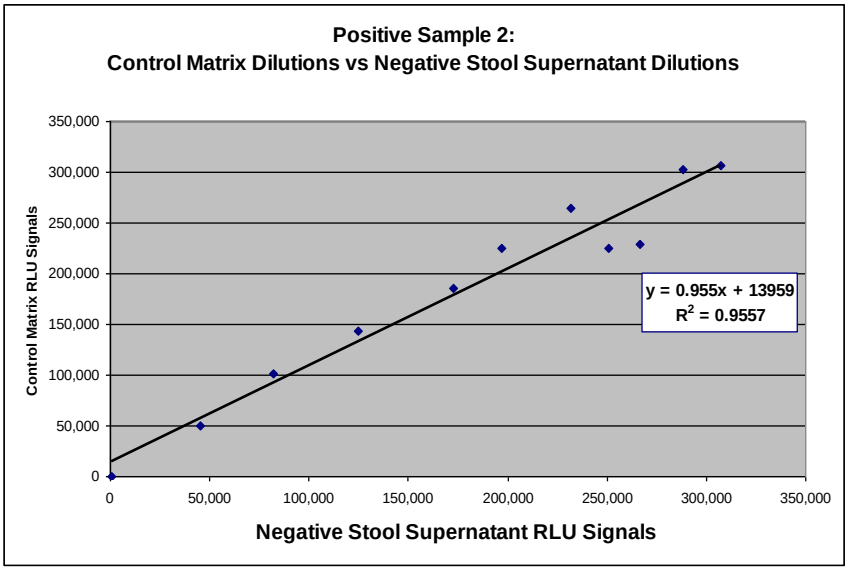
Comments: the antibodies obtained from clones 4 and 10, that showed high affinity for the toxin A and B respectively, were selected for the LIAISON® *C. difficile* Toxins A&B assay development.

MATRIX EQUIVALENCE TESTING

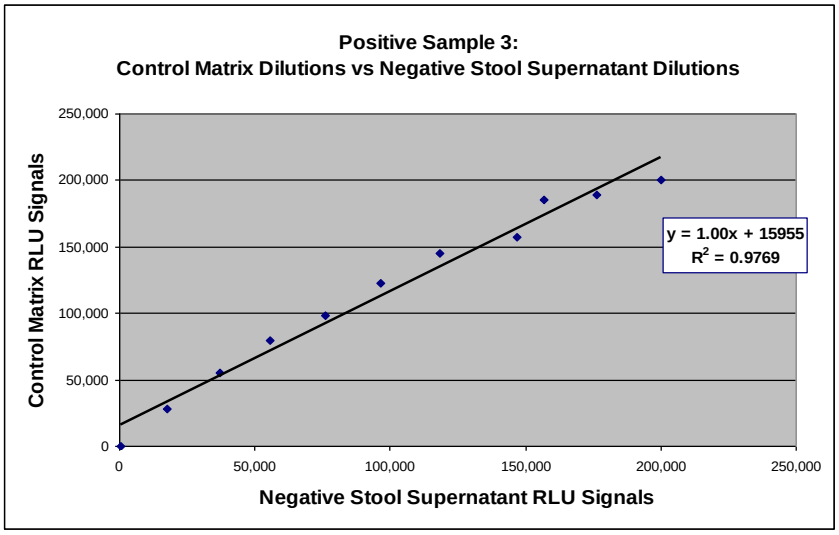
It is important to prove that the buffer based matrix (Matrix buffer) for *C. difficile* Toxins A&B dilutes a Positive Stool Supernatant equivalently to a Negative Stool Supernatant. This experiment was required to show that Kit Controls and other panels/controls do not need to be manufactured using Human Stool.
The requirement for this portion is that the graphs of dilutions of 4 Positive Stool Supernatants diluted by Control Matrix graphed against dilutions by Negative Stool Supernatants yields a Slope Value from the regression in the range of 0.90 – 1.10 (graphs 3, 4, 5, 6).



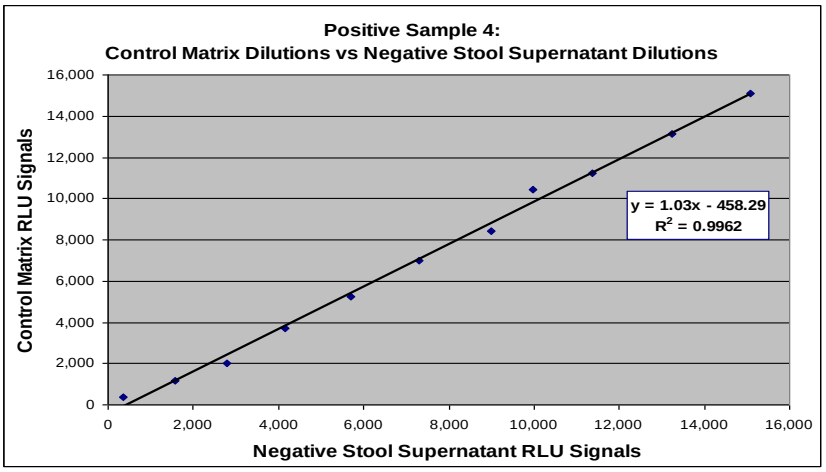
Graph 3: Control Matrix dilutions vs Stool Supernatant dilutions on positive sample 1.



Graph 4: Control Matrix dilutions vs Stool Supernatant dilutions on positive sample 2.



Graph 5: Control Matrix dilutions vs Stool Supernatant dilutions on positive sample 3.



Graph 6: Control Matrix dilutions vs Stool Supernatant dilutions on positive sample 4.

Comments: the Four Positive dilution samples have Slope Values between 0.90 – 1.10 for dilution by Control Matrix vs Negative Stool Supernatant. The Kit Control Matrix is a PASS for this testing and Kit Controls, Internal Controls, Precision Panels etc., do not need to be manufactured using human stool samples.

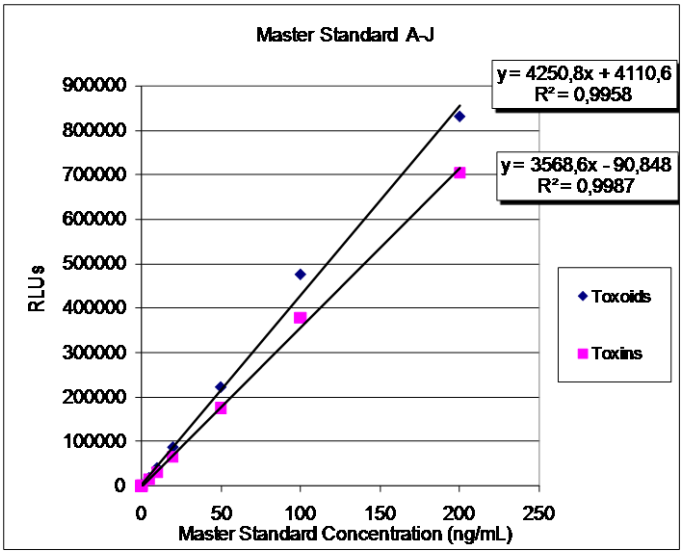
MASTER STANDARD CURVE PREPARATION AND CUT OFF DETERMINATION

The *Clostridium difficile* Toxins A&B Master Standards points (calibration curve) were all made with the same buffer matrix formulation. The Calibrators and Kit Controls were lyophilized and reconstituted with a 2 mL DI water fill. The sample size for the C. difficile Toxins A&B assay is 200 µL and requires that Calibrators and Kit Controls were supplied with sufficient volume to the customer. Two Master Standards curve were prepared, one with purified Toxoid A and Toxoid B and one with Toxin A and Toxin B in a 1:1 proportion (table 5).

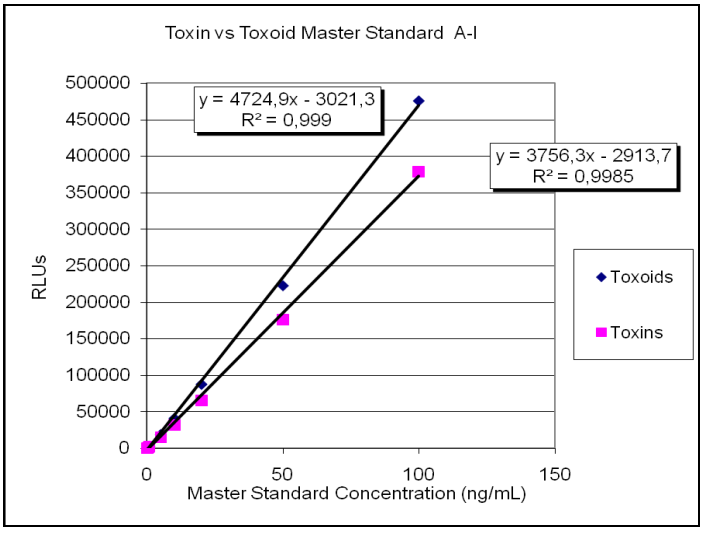
Table 5:Master Standard curve made with Toxins A&B or Toxoids A&B.

Master Standard	**Toxin or Toxoid A (ng/mL)**	**Toxin or Toxoid B (ng/mL)**	**Total concentration (ng/mL)**
A	0	0	0
B	0.1	0.1	0.2
C	0.25	0.25	0.5
D	0.4	0.4	0.8
E	2.5	2.5	5
F	5	5	10
G	10	10	20
H	25	25	50
I	50	50	100
J	100	100	200

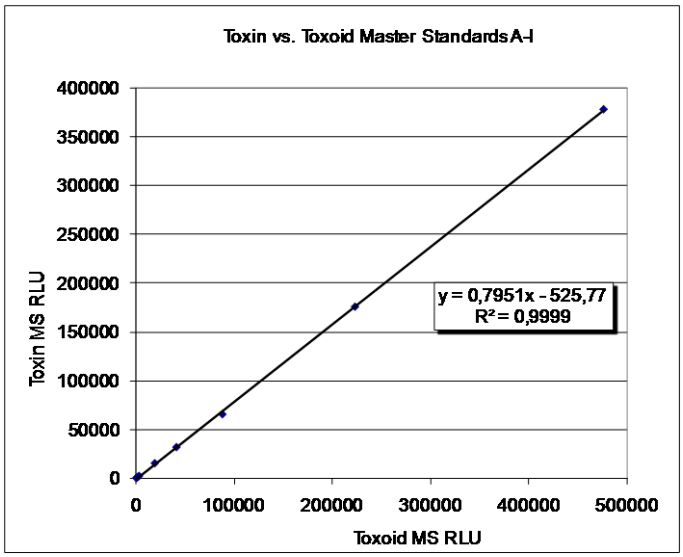
The linear regression was made on Toxin and Toxoid Master Standard (graph 9). Highest J point Standard in both Toxin/Toxoid curves has a signal of ~1,000,000 RLU's – this is causing nonlinearity at the top of the assay (graph 7), so we used only the Master Standards A – I (graph 8). Due to the toxins instability the Toxoid Master Standard was chosen for the C.difficile calibration in the subsequent experiments.
At each point of the curve was then assigned an Index Value correlated with the RLUs value (table 6).
To decide the cutoff index value and RLU, 80 stool samples *C.diff*. negative for Toxigenic Culture and Illumigene 10 instrument, were tested with 2 LIAISON® instruments. The results are not show, but **the RLU cutoff chosen was at 1015 whit the corresponding index value = 1.**



Graph 7: Toxins A&B and Toxoid A&B Master Curve A-J points.



Graph 8: Toxins A&B and Toxoid A&B Master Curve A-I points.



Graph 9: Toxins A&B vs Toxoid A&B Master Curve A-I points.

Table 6:Toxoid Master Standard RLUs and Index Values.

Toxoid Master Standard	**Average MS Index**	**Average MS RLU**
A	0.5	460
B	1.1	1141
C	2.1	2155
D	3.0	3059
E	18	18348
F	38	38673
G	81	81709
H	215	218361

DIAGNOSTIC ACCURACY

The LIAISON® *Clostridium difficile* Toxins A&B assay was compared to the Meridian Bioscience Illumigene® *Clostridium difficile* DNA amplification assay performed on the illumipro-10 instrument and the PTAB ELISA (from Meridian). The stool samples (117 negative and 44 positive) were obtained from Hennepin County Medical Center in Minneapolis, MN. This test was done on 117 negative and 44 positive stool samples and the results are show in the table 7.
The Design Inputs indicate the following requirements for Sensitivity and Specificity of the LIAISON® *Clostridium difficile* Toxins A&B assay.

Parameter	Acceptable	Target
Sensitivity on symptomatic patients	Greater than or equal to 85% Agreement to Illumigene®	Greater than or equal to 90% Agreement to Illumigene®
Specificity on symptomatic patients	Greater than or equal to 95% Agreement to Illumigene®	100% Agreement to Illumigene®

Table 7: LIAISON® *Clostridium difficile* Toxins A&B diagnostic accuracy.

LIAISON	Illumigene +	Illumigene -	Total
LIAISON +	**43**	**1**	**44**
LIAISON –	**1**	**116**	**117**
Total	**44**	**117**	**161**

Negative Agreement, Specificity: (116/117)*100 = 99.1%
Positive Agreement, Sensitivity: (43/44)*100 = 97.7%
Overall Agreement: (43+116)/161*100 = 98.8%

Comments: the *Clostridium difficile* LIAISON® kit meets the Target design Input Acceptance Criteria for Sensitivity and Specificity performance requirement.

LIMIT OF DETECTION

The **detection limit**, **lower limit of detection**, or **LOD** (limit of detection), is the lowest quantity of a substance that can be distinguished from the absence of that substance (a *blank value*) within a stated confidence limit (generally 1%). The detection limit is estimated from the mean of the blank, the standard deviation of the blank and some confidence factor. In this procedure 60 replicates of the blank or zero, and 12 replicates of each of the following 4 levels of spiked purified Toxins A and B; 50 pg/mL, 100 pg/mL, 200 pg/mL and 250 pg/mL were tested in buffer based matrix. The same number of replicates were used for the "Stool Extracted" LoD values, but the 4 levels of purified Toxins A and B spiked into negative stool were at; 200 pg/mL, 400 pg/mL, 800 pg/mL and 1000 pg/mL. In the Buffer Based LoD method the control matrix buffer was measured as a zero, and the same matrix was spiked with purified Toxin A and Toxin B. These solutions were then tested directly on the LIAISON®.
In the Extracted Stool LoD method an autoclaved, negative stool pool was aliquoted and a portion of the stool pool was spiked with purified Toxin A and a portion was spiked with Toxin B antigen. The remainder of the unspiked negative stool, and the two spiked stool pools were all extracted using the sample diluent, imitating the processing and extraction of stools. The supernatants resulting from each sample type were pooled, mixed well and tested on the LIAISON® (Table 8). The supernatant stool pools were also tested on the Meridian Premier Toxins A and B (PTAB) EIA kit, to measure if the PTAB kit detected the spiked stool samples, using that kits extraction method (table 9). The Buffer Based method is only a measure of the Assay performance, while the Extracted Stool method includes the performance of both the Extraction and Assay combined.

The Design Inputs indicate the following requirements for Limit of Detection of the LIAISON *Clostridium difficile* Toxins A&B assay. The Design Inputs are relevant only to the Extracted Stool LoD. The Buffer Based LoD results are for information only, since during development this method was often used to determine the functionality of the Assay, in order to optimize for sensitivity.

Parameter	Acceptable	Target
Limit of detection	Better than the PTAB Meridian assay at 95% confidence level.	

Table 8: LIAISON® LoD (Limit of Detection) and LoB (Limit of Blank).

LoD/LoB Type	Toxin Type	LoD/LoB Concentration (pg/mL)
Extracted Stool LoD	***Toxin A***	**158**
	Toxin B	**79**
Extracted Stool LoB	***Toxin A***	66
	Toxin B	28
Buffer Based LoD	***Toxin A***	**102**
	Toxin B	**71**
Buffer Based LoB	***Toxin A***	46
	Toxin B	30

Table 9**:** PTAB (6 replicates) and LIAISON® Toxins A and B (12 replicates) detection from extracted stool spikes. If the Abs is ≥0.100 the sample is positive.

Negative Stool Level	LIAISON Mean RLU Value	PTAB Mean Abs Signal Detection Dual Wavelength 450-630 = ≥0.100
Zero	588	0.0005
Toxin A 200 pg/mL	765	0.0005
400 pg/mL	938	0.0017
800 pg/mL	1394	0.0013
1000 pg/mL	1586	0.0018
Toxin B 200 pg/mL	1037	0.0010
400 pg/mL	1566	0.0017
800 pg/mL	2315	0.0022
1000 pg/mL	2968	0.0032

Comments: the *Clostridium difficile* Toxins A&B Assay is a PASS for Limit of Detection since it is "better than" the Premier Toxins A&B kit by detecting incremental changes by 200 pg/mL from Zero Negative Stool, while the PTAB kit does not.

CROSS REACTIVITY

Cross Reactivity to Bacteria, fungi and viruses in stool was tested. The comparison of Bacteria, fungi and virus spiked samples to corresponding dilution controls for each spike, should exhibit no change in classification when compared to the dilution control classification. The microorganisms (39) were tested in 6 groups, over 2 different days of testing, by spiking both High Negative and Low Positive Stools with the microorganisms according to the final concentrations listed in the table 10, then extracting the stools and testing the supernatants on the LIAISON®. Each group contained the High Negative and Low Positive Stools without spiked material as controls, and also High Negative and Low Positive Stools which were spiked with PBS of the same volume used to spike from the microorganism culture into the stool, and then extracted using same method as those stools spiked with the microorganism.
The Design Inputs indicate the following requirements for Cross Reactivity of the LIAISON® *Clostridium difficile* Toxins A&B assay.

Parameter	Target
Cross Reactivity	**No change in sample classification (Pos or Neg)**

Cross Reactivity Results:
Bacterial or Yeast CFU = colony forming unit
Viral TCID50/mL = median tissue culture infective dose; that amount of a pathogenic agent that will produce pathological change in 50% of cell cultures inoculated.

Table 10: Cross reactivity results.

Microorganism	Final Conc High Negative & Low Positive Spikes	Index High Negative Extraction Control	Index High Negative PBS Extraction Control	Spiked Microbe High Negative	Index Low Positive Extraction Control	Index Low Positive PBS Extraction Control	Spiked Microbe Low Positive
Aeromonas hydrophila	**1.2 x 10^8 CFU/mL**	0.74	0.67	**0.65**	1.35	1.24	**1.32**
Campylobacter Coli	**1.2 x 10^8 CFU/mL**	0.74	0.67	**0.64**	1.35	1.24	**1.24**
Campylobacter fetus	**1.2 x 10^8 CFU/mL**	0.74	0.67	**0.67**	1.35	1.24	**1.30**
Campylobacter jejuni	**1.2 x 10^8 CFU/mL**	0.74	0.65	**0.67**	1.45	1.33	**1.27**
Candida albicans	**1.2 x 10^8 CFU/mL**	0.74	0.65	**0.72**	1.45	1.33	**1.30**
Citrobacter freundii	**1.2 x 10^8 CFU/mL**	0.74	0.65	**0.66**	1.45	1.33	**1.26**
Clostridium perfringens	**1.2 x 10^8 CFU/mL**	0.74	0.65	**0.69**	1.45	1.33	**1.33**
Clostridium sordellii	**1.2 x 10^8 CFU/mL**	0.74	0.65	**0.62**	1.45	1.33	**1.28**
Enterobacter cloacae	**1.2 x 10^8 CFU/mL**	0.77	0.69	**0.73**	1.52	1.32	**1.38**
Enterococcus faecalis	**1.2 x 10^8 CFU/mL**	0.77	0.69	**0.68**	1.52	1.32	**1.29**
Escherichia coli	**1.2 x 10^8 CFU/mL**	0.74	0.65	**0.66**	1.45	1.33	**1.33**
Escherichia hermannii	**1.2 x 10^8 CFU/mL**	0.74	0.65	**0.64**	1.45	1.33	**1.27**
Escherichia fergusonii	**1.2 x 10^8 CFU/mL**	0.74	0.65	**0.65**	1.45	1.33	**1.19**
Helicobacter pylori	**1.2 x 10^8 CFU/mL**	0.77	0.69	**0.71**	1.52	1.32	**1.32**
Klebsiella pneumonia	**1.2 x 10^8 CFU/mL**	0.77	0.69	**0.64**	1.52	1.32	**1.28**
Lactobacillus delbrueckii	**1.2 x 10^8 CFU/mL**	0.77	0.69	**0.72**	1.52	1.32	**1.29**
Listeria monocytogenes	**1.2 x 10^8 CFU/mL**	0.77	0.69	**0.67**	1.52	1.32	**1.28**
Peptostreptococcus anaerobius	**1.2 x 10^8 CFU/mL**	0.77	0.69	**0.67**	1.52	1.32	**1.40**
Plesiomonas shigelloides	**1.2 x 10^8 CFU/mL**	0.77	0.69	**0.70**	1.52	1.32	**1.25**
Proteus vulgaris	**1.2 x 10^8 CFU/mL**	0.77	0.69	**0.79**	1.52	1.32	**1.33**

Pseudomonas aeruginosa	**1.2 x 10^8 CFU/mL**	0.80	0.72	**0.72**	1.53	1.27	**1.32**
Pseudomonus fluorescens	**1.2 x 10^8 CFU/mL**	0.77	0.69	**0.68**	1.52	1.32	**1.30**
***Salmonella* Group B**	**1.2 x 10^8 CFU/mL**	0.80	0.72	**0.68**	1.53	1.27	**1.30**
***Salmonella* Group C**	**1.2 x 10^8 CFU/mL**	0.80	0.72	**0.71**	1.53	1.27	**1.36**
***Salmonella* Group D**	**1.2 x 10^8 CFU/mL**	0.80	0.72	**0.69**	1.53	1.27	**1.27**
***Salmonella* Group E**	**1.2 x 10^8 CFU/mL**	0.80	0.72	**0.70**	1.53	1.27	**1.35**
Serratia liquefaciens	**1.2 x 10^8 CFU/mL**	0.80	0.72	**0.69**	1.53	1.27	**1.32**
Shigella boydii	**1.2 x 10^8 CFU/mL**	0.80	0.72	**0.72**	1.53	1.27	**1.36**
Shigella flexneri	**1.2 x 10^8 CFU/mL**	0.80	0.72	**0.70**	1.53	1.27	**1.27**
Shigella sonnei	**1.2 x 10^8 CFU/mL**	0.80	0.72	**0.74**	1.53	1.27	**1.38**
Staphylococcus aureus	**1.2 x 10^8 CFU/mL**	0.80	0.72	**0.68**	1.53	1.27	**1.26**
Staphylococcus epidermidis	**1.2 x 10^8 CFU/mL**	0.78	0.68	**0.68**	1.43	1.31	**1.29**
Vibrio parahaemolyticus	**1.2 x 10^8 CFU/mL**	0.78	0.68	**0.72**	1.43	1.31	**1.31**
Yersinia enterocolitica	**1.2 x 10^8 CFU/mL**	0.78	0.68	**0.67**	1.43	1.31	**1.25**
Adenovirus Type 40	**1 x $10^{5.29}$ $TCID_{50}$/mL**	0.78	0.73	**0.74**	1.43	1.51	**1.40**
Adenovirus Type 41	**1 x $10^{5.93}$ $TCID_{50}$/mL**	0.78	0.73	**0.70**	1.43	1.51	**1.28**
Coxsackievirus	**1 x $10^{5.06}$ $TCID_{50}$/mL**	0.78	0.73	**0.72**	1.43	1.51	**1.58**
Echovirus	**1 x 105.93 TCID50/mL**	0.78	0.73	**0.70**	1.43	1.51	**1.30**
Rotavirus	**1 x 105.29 TCID50/mL**	0.78	0.73	**0.73**	1.43	1.51	**1.40**

Comments: all microorganisms exhibited no cross reactivity in the LIAISON *Clostridium difficile* Toxins A&B assay.

C.difficile STRAIN REACTIVITY

The *Clostridium difficile* LIAISON® Toxins A&B test was also evaluated using several reference strains of *Clostridium difficile*. The Strain Reactivity was made with 9 different Strains of toxigenic *Clostridium difficile*. The Strains were tested by spiking a negative liquid stool with the microorganisms according to the final concentrations listed in the Table below. After 48 hours at 37°C the extracted spiked stool supernatant were testing on the LIAISON®. The Negative Stool without spiked material was extracted as a control, and the Negative Stool spiked with PBS of the same volume used to spike from the *Clostridium difficile* cultures into the stool, and then extracted using same method as those stools spiked with the microorganisms (table 11).
The Design Inputs indicate the following requirements for *Clostridium difficile* Strain Reactivity of the LIAISON® *Clostridium difficile* Toxins A&B assay.

Parameter	**Acceptable**	**Target**
Strain Reactivity Requirements	**A+/B+** **A-/B+** **(Same Reactivity as Meridian)**	

Table 11: *C.difficile* strain reactivity.

Microorganism	Colony Forming Unit/mL spiked	Toxinotype	Phenotype	Index Neg. stool Extraction Control	Index Neg. stool with PBS Extraction Control	Index Spiked Microbe in Neg. stool
C. difficile **Strain 8864**	**1.2 x 10^8 CFU/mL**	**Type X** 1 of 4 known A-/B+ types	**A-/B+**	0.32	0.55	**2.38**
C. difficile **Strain 43598**	**6.0 x 10^8 CFU/mL**	**Type VIII** 1 of 4 known A-/B+ types	**Largest group of A-/B+** More than 100 isolates worldwide	0.39	0.29	**1.97**
C. difficile **Strain CF1**	**1.2 x 10^9 CFU/mL**	**Type VIII** 1 of 4 known A-/B+ types	**Largest group of A-/B+** More than 100 isolates worldwide	0.40	0.35	**6.58**
C. difficile **Strain 2007858**	**1.2 x 10^8 CFU/mL**	**Type IX / XXIII**	**A+/B+**	0.32	0.55	**4.52**
C. difficile **Strain BI8**	**1.2 x 10^8 CFU/mL**	**Type III**	**A+/B+**	0.32	0.55	**86.3**
C. difficile **Strain 10463**	**1.2 x 10^8 CFU/mL**	**Type 0** 1st toxigenic strain sequenced = Reference Strain	**A+/B+**	0.32	0.55	**54.8**
C. difficile **Strain 2007435**	**1.2 x 10^8 CFU/mL**	**Type XII**	**A+/B+**	0.32	0.55	**32.2**
C. difficile **Strain 2007431**	**1.2 x 10^8 CFU/mL**	**Type III**	**A+/B+**	0.32	0.55	**20.0**
C. difficile **Strain 2006240**	**1.2 x 10^8 CFU/mL**	**Type V**	**A+/B+**	0.32	0.55	**6.68**

Comments: all *C. difficile* Toxins A&B Strains react in the LIAISON C. difficile Toxins A&B assay to cause a Positive result. The extent of reactivity varies based on the expression levels for each culture and bacterial strain.

INTERFERING SUBSTANCES

Some of the compounds present in stool specimens were tested on the *C.difficile* LIAISON® Toxins A&B, to assess their ability to cause interference in general. The following substances (at the specified concentrations) were diluted in stool sample diluents table 12.

Table 12: Stool interfering substances.

COMPOUND	CONCENTRATION	Sample classification
Barium sulfate	5 mg/mL	Negative
Stearic acid/Palmitic acid (fecal fat)	2.65mg/mL/1.3 mg/mL	Negative
Hemoglobin	3.2 mg/mL	Negative
Imodium AD® (Loperamide HCl)	6.67x10-3 mg/mL	Negative
Kaopectate® (Bismuth subsalicylate)	0.87 mg/mL	Negative
Metronidazole	12.5 mg/mL	Negative
Mucin	3.33 mg/mL	Negative
Mylanta® (Ammonium hydroxide w/ magnesium hydroxide)	4.2 mg/mL	Negative
Pepto Bismol ® (Bismuth subsalicylate)	0.87 mg/mL	Negative
Polyethylene glycol 3350	79.05 mg/mL	Negative
Prilosec® (Omeprazole)	0.5 mg/mL	Negative
Simethicone	0.625 mg/mL	Negative
Tagamet® (Cimetidine)	0.5 mg/mL	Negative
Tums® (Calcium carbonate)	0.5 mg/mL	Negative
Vancomycin hydrochloride	2.5 mg/mL	Negative
White blood cells 5%	5%	Negative
Whole blood	25%	Negative

Comments: some of the listed substances initially generated a aspecific signal, but this problem has been solved through the blocking agents addition to the sample diluent and conjugate diluent. The final version of the essay as shown in the table 12 does not show any kind of interference.

Chlamydia trachomatis RESULTS

Analytical characterization of the purified peptides

The four peptides (Ct A, Ct B, Ct C, Ct D) described in the patent W09900414, were synthesized by peptide synthesizer (as described in Materials and Methods). Below are reported the MS profiles of the four purified peptides. The Ct A expected MW is 1733, the expected signals are 1734.0 (MH^+), 867.5 ($M+2H^+$) (fig. 23).

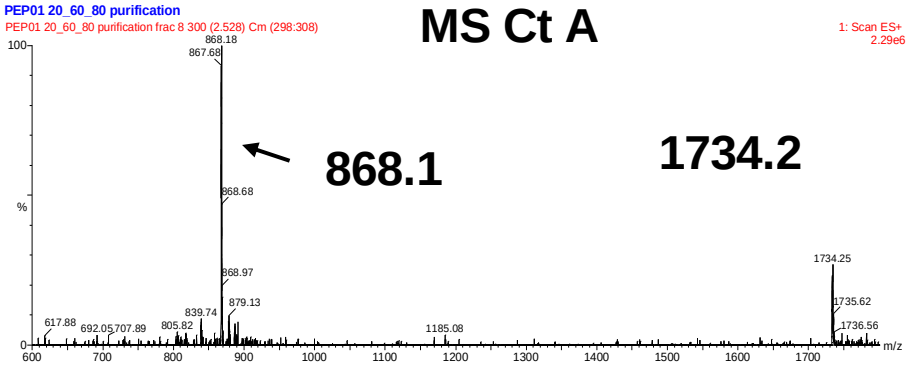


Fig. 23: Ct A purified MS profile.

The Ct B expected MW is 1760, the expected signals are 1761 (MH^+), 881 ($M+2H^+$) (fig. 24).

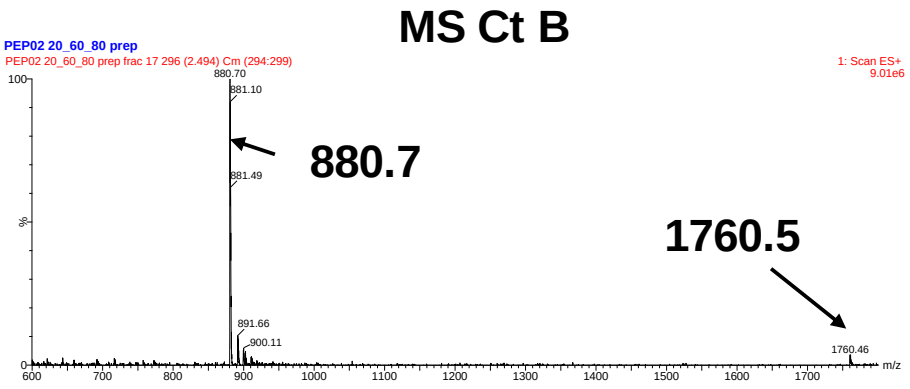


Fig. 24: Ct B purified MS profile.

The Ct C expected MW is 1921.2, the expected signals are 961.6 ($M+2H^+$), 641.4 ($M+3H^+$) (fig. 25).

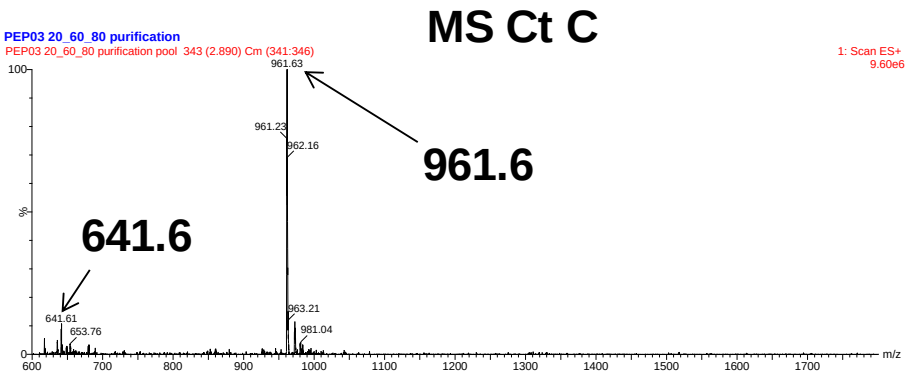


Fig. 25: Ct C purified MS profile.

The Ct D expected MW is 2354.8, the expected signals are 1178.4 ($M+2H^+$), 785.9 ($M+2H^+$) (fig. 26).

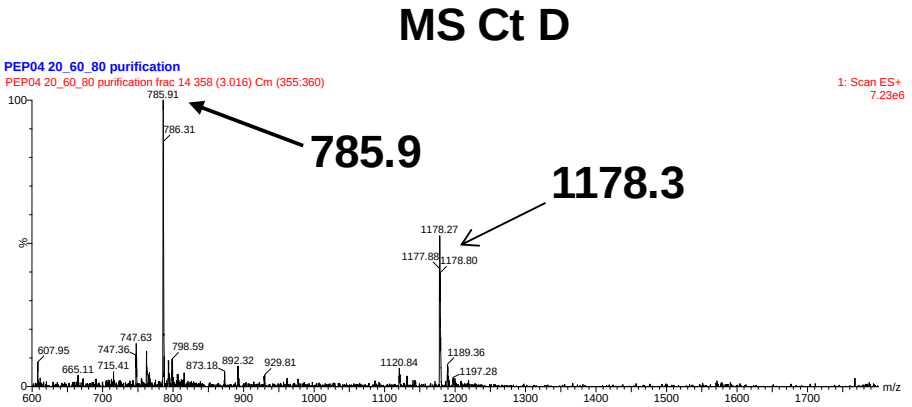


Fig. 26: Ct D purified MS profile.

Comments: the MS profiles obtained from the four *C. trachomatis* peptides correspond to the expected.

PHASE 1: DIRECT IMMOBILISATION OF PEPTIDES ON PARAMAGNETIC PARTICLES (PMPs)

The first approach used for the development of the *C. trachomatis* assay was the direct coating of the 4 peptides on the PMPs (solid phase). Different coating concentrations were done.

Coating concentration

The four *C. trachomatis* peptides were immobilised on Dynal M280 paramagnetic particles reacting with the tosyl groups. Each peptide was coated separately and then combined in a 1:1:1:1 mixure. Two different coating concentrations were made, 24 and 100 µg/mL (graphs 10, 11).

Conjugate

Since the goal of this immunoassay is to diagnose the anti *C. trachomatis* human IgG, the Abs anti human IgG were used as conjugate at 100ng/ml.

Samples

Thirteen serum samples consisting of two negative and eleven positive for *C. trachomatis*, were classified according to the results of the ELISA Savyon reference assay (table 13). The samples in the *C. trachomatis* LIAISON® assay prototype are diluted in reaction by phosphate buffer.

Table 13:ELISA results on the thirteen serum samples, if the ELISA COI is: <1,0 the sample is *C. trachomatis* negative; 1-1,1 is borderline; >1,1 is *C. trachomatis* positive.

Sample ID	ELISA Cutoff Index (COI)
Neg 1	0,295
Neg 2	0,353
Pos 1	2,047
Pos 2	2,315
Pos 3	2,388
Pos 4	2,403
Pos 5	2,735
Pos 6	3,513
Pos 7	4,091
Pos 8	4,235
Pos 9	5,261
Pos 10	5,543
Pos 11	6,200

Assay procedure

In these preliminary tests the analytical procedure was a two step assay with a total incubation time of 40 minutes, as in the following format:

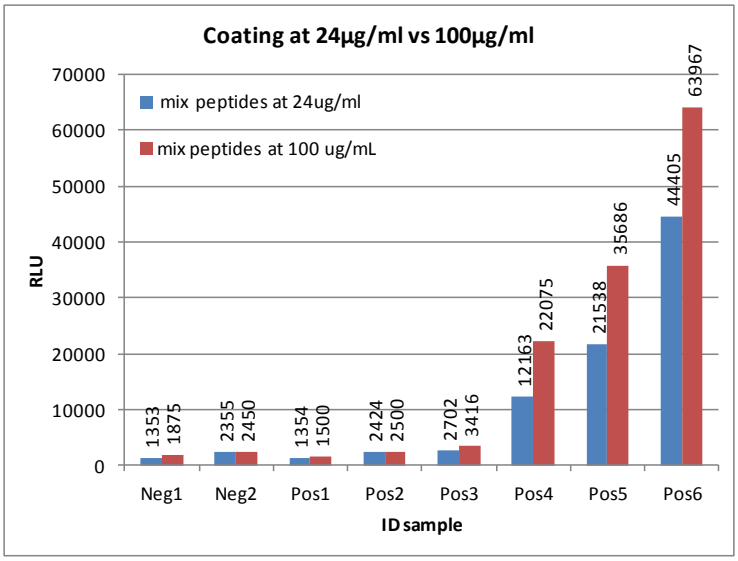
- 1st step : 10 µL sample + 200 µL phosphate buffer + 20 µL PMPs ➔ 20 minutes

  Wash

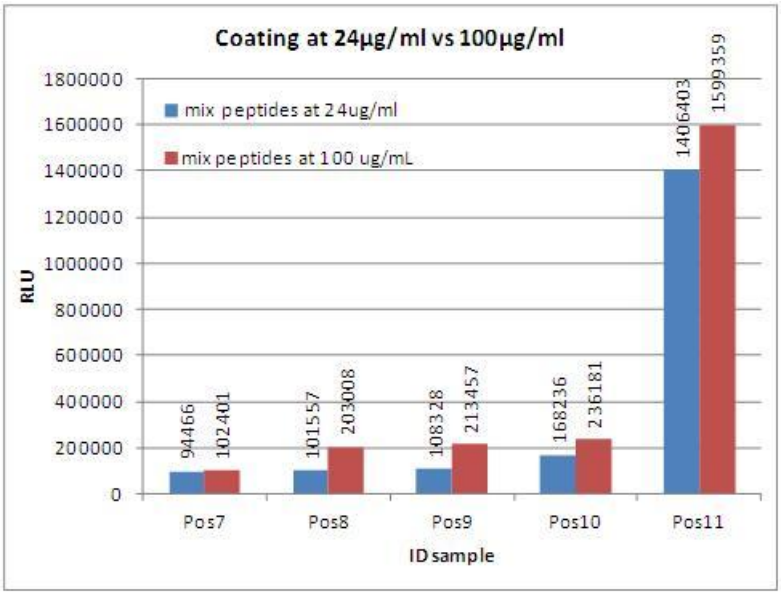
- 2nd step : 200 µL conjugate (IgG anti hIgG at 100 ng / mL) ➔ 20 minutes

  Wash & read

In the first step the anti *C. trachomatis* human IgGs in the samples bind the peptides coated on the PMPs. After the washing step the conjugate anti human IgG is added, the unbound reagents are removed and through the addition of trigger solutions the chemiluminescent reaction is measured as relative light units.



Graph 10: Results obtained with the 2 negative and first 6 positive samples with the coating of the 4 peptides at 24 and 100 µg/mL.

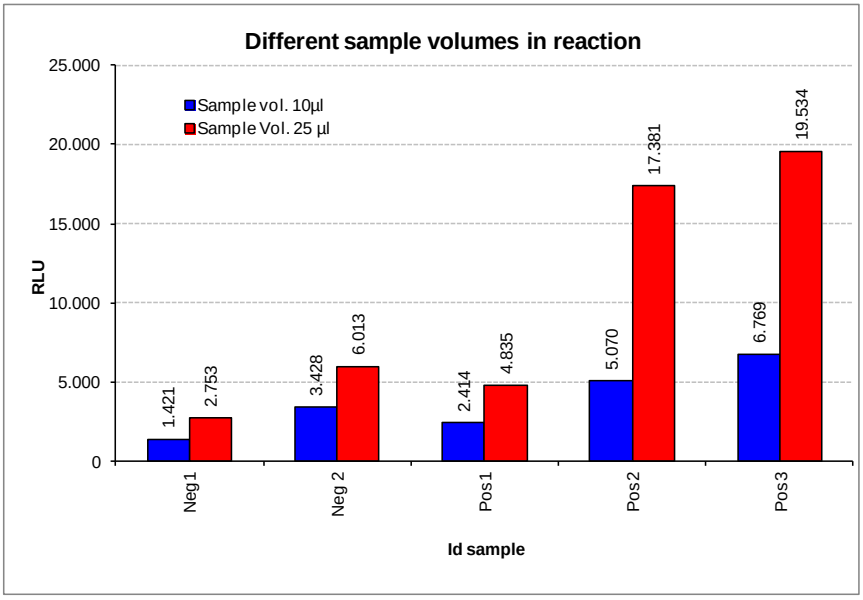


Graph 11: Results obtained on the positive samples number 7, 8, 9, 10, 11 with the coating of the 4 peptides at 24 and 100 µg/ml.

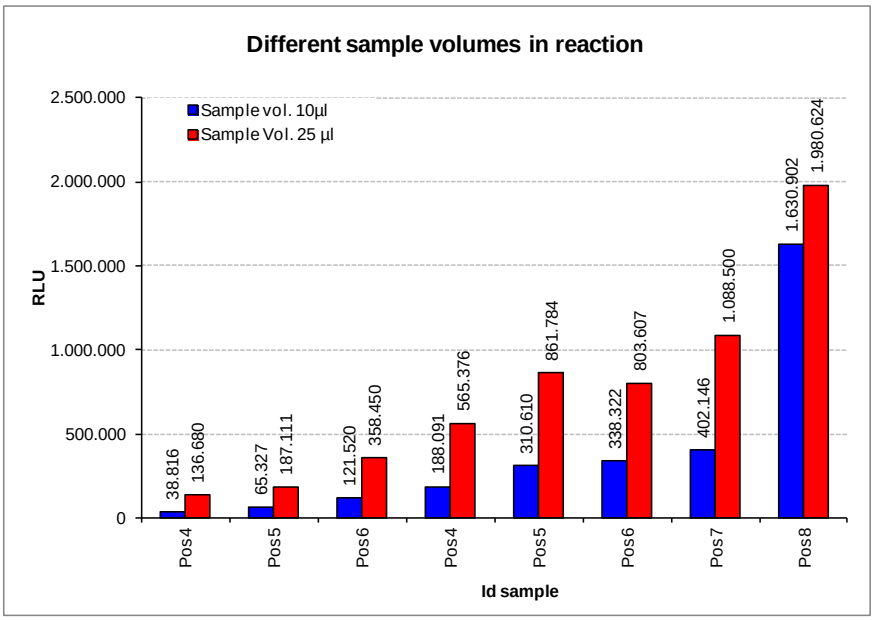
Comments: the results obtained with the panel of samples were unsatisfactory, three positive samples showed low signal, similar to the two negative samples with both coating concentrations. Also the 400 µg/ml were used but without any improvement (data not shown).

Increase of the sample volume in reaction and coating concentration

In order to increase the signal of the three false negatives (Pos1, and Pos 2 and Pos 3) on the *C. trachomatis* LIAISON® kit prototype, the sample volume was then brought at 25 µL/test and the coating of peptides was made at 200 µg/mL (graphs 12, 13).



Graph 12: Different sample volumes with negative 1, 2 and positive 1, 2, 3.

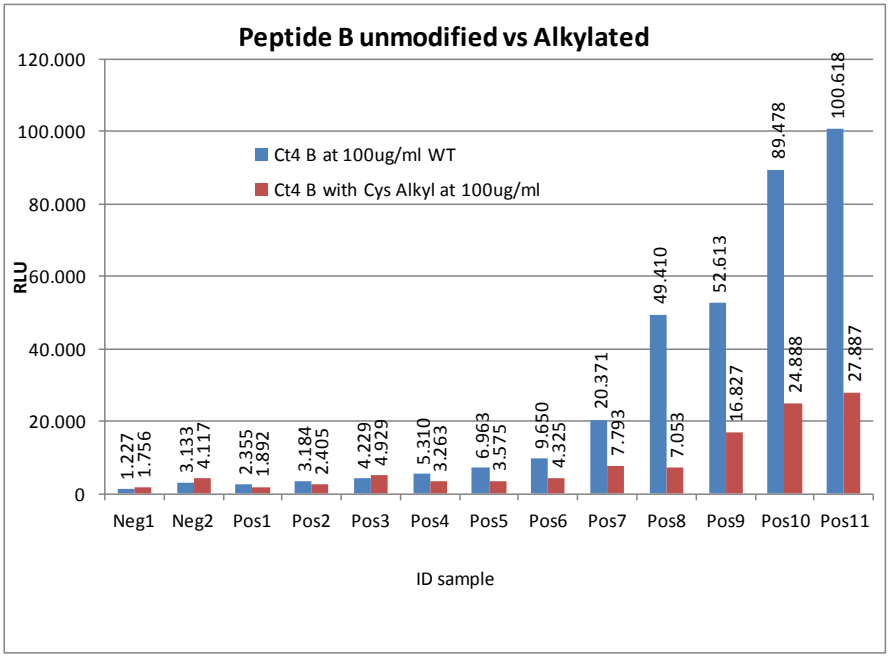


Graph 13: Different sample volumes with positive 4, 5, 6, 7, 8.

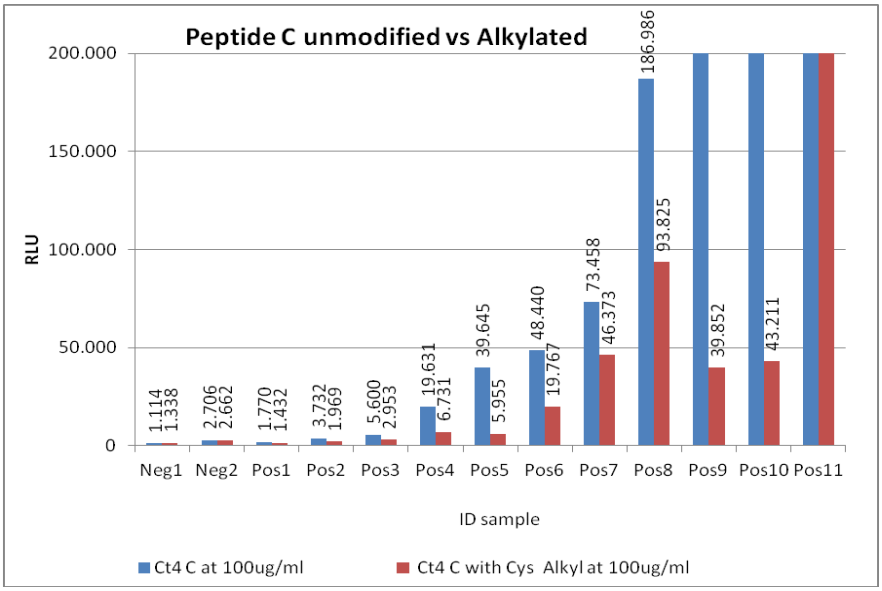
Comments: this change allowed the "recovery" of two of these “false negative” (Pos2, Pos3) samples while the third (Pos1) still showed poor reactivity (graph 12).

Peptides alkylation

The paramagnetic particles currently used in our studies (Dynal) are activated with tosyl groups. These typically react with amino groups (from N-terminus and lysine side-chains) and thiol groups (cysteine side-chains) giving rise to stable covalent bonds.
Generally, peptides coated on Tosyl-activated beads do not confer good performance of the resulting immunoassay compared to proteins. This is mainly due to their smaller size and, in particular, to the following reasons: (i) steric hindrance effect due to the close proximity of the immonoreactive region to the beads surface, (ii) coating may involve amino acids’ side-chains belonging to the antigenic determinant, (iii) immobilization may occur through two or more residues giving rise to loop structures with decreased immunoreactivity. Moreover, cysteine residues in the peptide sequences can oxidize forming intra- or inter- molecular disulfide bridges bringing about a drop in the immunoreactivity.
The four peptide sequences evaluated in our study contain at least one Lys residue plus the N-terminal amino group available for the covalent immobilization on the tosyl beads. Two peptides, Ct B and Ct C, contain also a cysteine residue and represent those with the most critical sequences. The peptide Ct B has the following sequence: VDITTLNPTIAGCGSVAK, where the internal cysteine thiol group can react with the tosyl group on the PMPs, thus potentially interfering with the immunorecognition. The peptide Ct C, sequence CVFDVTTLNPTIAGAGDVK, has an N-terminal cysteine residue. Since the N-terminal region of this peptide seems to be crucial for the immunoreactivity, as reported in the patent WO9900414, the coating occurring through the cysteine may be detrimental. Therefore, in both the cases, the covalent immobilization through the cysteine residues would likely cause a loss of immunoreactivity of these peptides and result in false negative samples. To prevent their involvement during the coating on the PMPs, the thiol groups of these cysteines were alkylated, and the modified peptides were tested with a coating at 100ug/ml and 10µl of sample. The modified peptides were compared to the unmodified ones (graphs 14, 15) and to the mix of peptides at 100µg/ml (table 14).



Graph 14: Peptide B unmodified compared to the Alkylated analog.



Graph 15: Peptide C unmodified compared with the Alkylated.

Table 14: RLU of peptides mix coated at 100µg/ml compared with the alchylated peptides B and C.

ID sample	Ref. Mix of pep. at 100ug/ml	Pep. B with Cys Alkyl. at 100ug/ml	Pep. C with Cys Alkyl. at 100ug/ml
Neg1	1261	1.756	1.338
Neg2	2398	4.117	2.662
Pos1	1790	1.892	1.432
Pos2	2600	2.405	1.969
Pos3	3550	4.929	2.953
Pos4	20330	3.263	6.731
Pos5	48521	3.575	5.955
Pos6	58964	4.325	19.767
Pos7	94104	7.793	46.373
Pos8	208303	7.053	93.825
Pos9	218794	16.827	39.852
Pos10	222208	24.888	43.211
Pos11	1526775	27.887	1.252.646

Comments: the cysteines alkylation of the peptides B and C decreases the immunoreactivity of the peptides. These changes result in a significant signal reduction of all samples positive for *C. trachomatis*.

PHASE 2: APPLICATION OF BIOTINYLATED PEPTIDES

After the disappointing results obtained with the direct immobilisation on the solid phase, the assay structure was then modified with the use of the four *C. trachomatis* biotinylated peptides. The biotin group allows the capture of peptides on streptavidin PMPs with proper orientation. The biotin was added in the C-terminal position of each peptide through the ε-amino group of a C-terminal extra-lysine and a polyethylenoxide-based linker (Ttds) plus a glutamic acid residue, as water solubilizing spacer, between the C-terminal end of its parent sequence and the C-terminal biotinylated lysine (fig. 27).

Peptide **K– Ttds-E - Lys(Biotin)**

Fig. 27: *C. trachomatis* biotinylated peptides structure.

The assay protocol was modified while maintaining a "2 step" reaction, different sample volumes, peptide, PMPs and conjugate concentrations were tested (data not shown), but the best condition is the following:

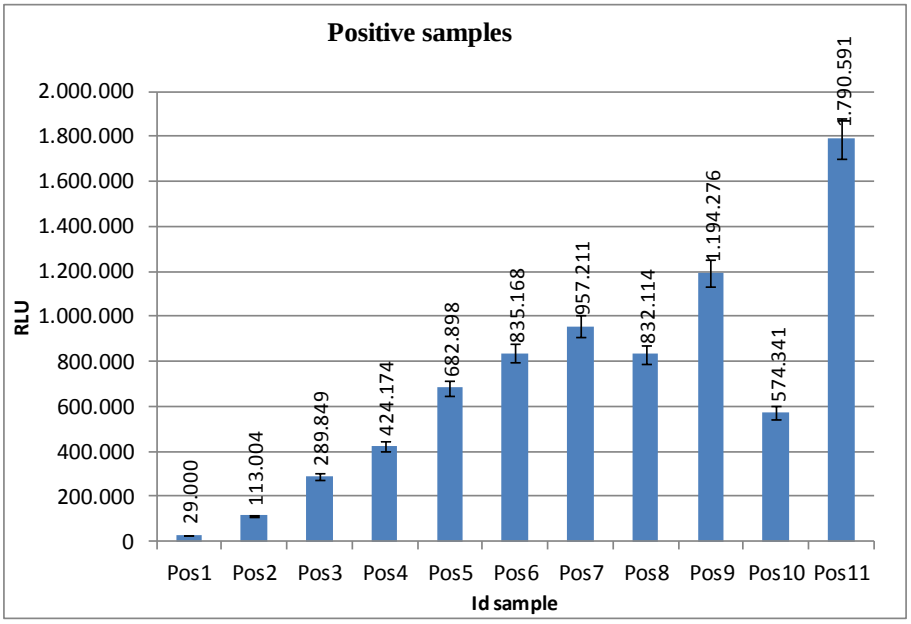
- 1st step : 15 µL sample + 200 µL Mix of biotinylated peptides (at 25ng/ml; ratio 1:1:1:1) + 20 µL streptavidinated PMPs at 0.25% ➔10 minutes

Wash

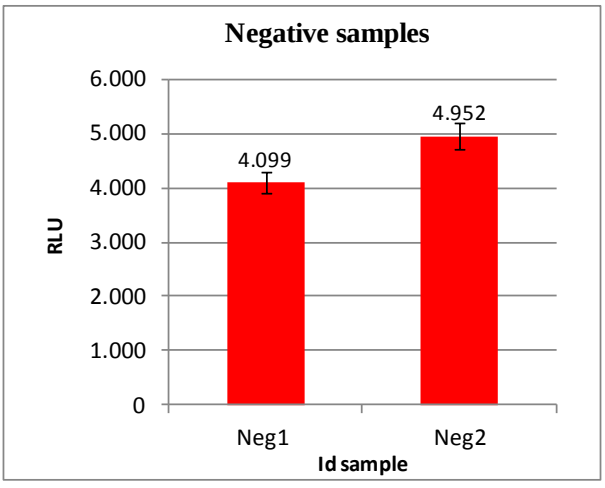
- 2nd step : 200 µL conjugate (IgG anti human IgG at 100 ng / mL) ➔10 minutes

Wash & read

In the first step the anti *C. trachomatis* human IgGs in the samples bind the four biotinylated peptides, these are simultaneously bound to the streptavidin coated onto paramagnetic particles. After the washing step the conjugate anti human IgG is added, the unbound reagents are removed and through the addition of trigger solutions the chemiluminescent reaction is measured as relative light units. The results obtained on the reference samples are shown in the graphs 16, 17.



Graph 16: 11 *C. trachomatis* positive samples tested by the biotinylated peptides.



Graph 17: Negative samples for *C. trachomatis* tested by the biotinylated peptides.

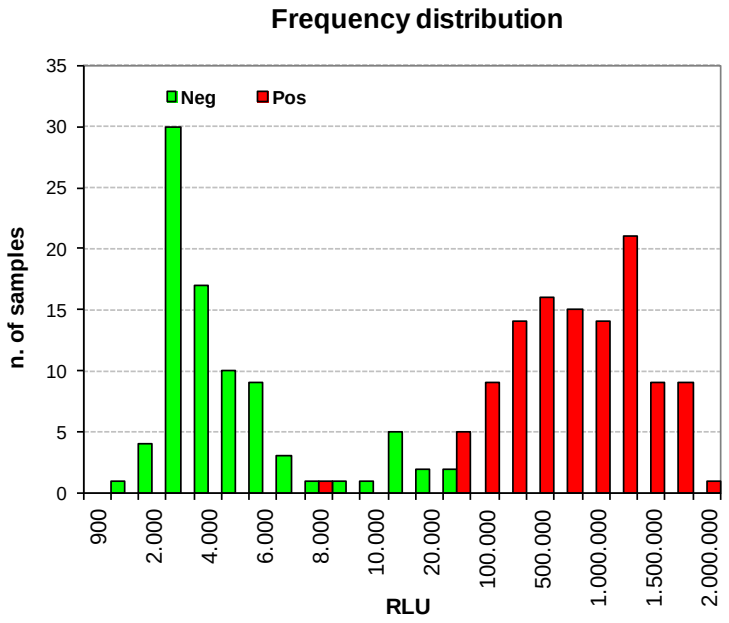
Comments: The results obtained with the biotinylated peptides show a large RLU separation between the positive and negative samples for *C. trachomatis*.

DIAGNOSTIC ACCURACY

The LIAISON® *C. trachomatis* assay was compared to the Savyon ELISA assay. The serum samples (86 positive and 114 negative) were obtained from different hospitals (see see Materials and Methods), the results are summarized in the graph 17 and table 15.
Concerning the expected LIAISON® kit performance, the Micro Immune Fluorescence (MIF) is considered as the reference test, with 99% agreement on positive and 95% on negative. Also the Savyon kit performance is considered. The Design Inputs indicate the following requirements for Sensitivity and Specificity of the *C. trachomatis* LIAISON® assay.

Parameter	Target
Sensitivity	99%
Specificity	95%

This samples analysis also allowed the determination of a **cutoff = 20000 RLU**. This cutoff was determined in order to have the best overall agreement.



Graph 17: Frequency distribution of 86 positive, 114 negative samples analyzed with the LIAISON® *C. trachomatis* assay.

Table 15: LIAISON® *C. trachomatis* assay accuracy.

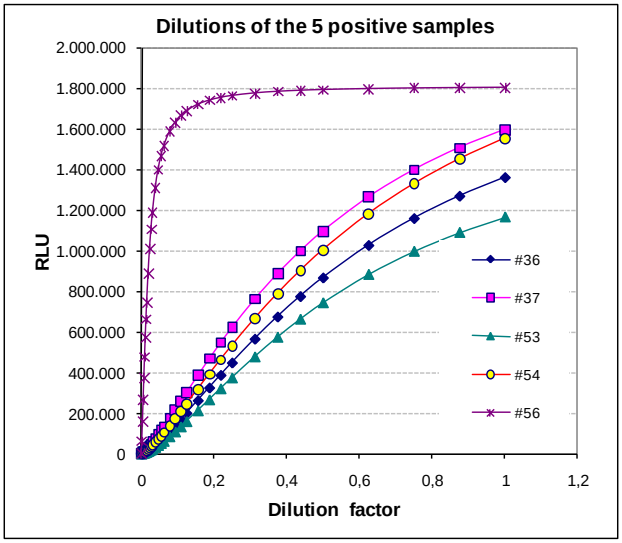
LIAISON testing	ELISA Savyon +	ELISA Savyon -	Total
LIAISON +	113	2	115
LIAISON –	1	84	85
Total	114	86	200

Negative Agreement, Specificity: (84/86)*100 = 97.7%
Positive Agreement, Sensitivity: (113/114)*100= 99.1%
Overall Agreement: (113+84)/200*100 = 98.5%

Comments: the *C. trachomatis* LIAISON® kit meets the Target Design Input Acceptance Criteria for Sensitivity and Specificity performance requirement.

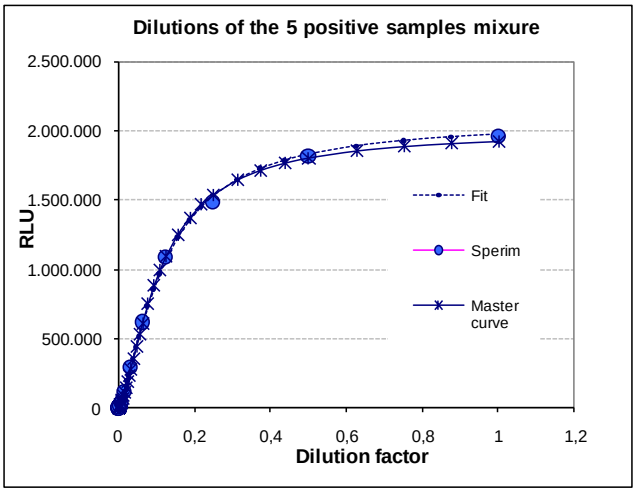
MASTER STANDARD CURVE (calibration curve) PREPARATION

For the *C. trachomatis* master standard preparation, 5 high positive samples (#36, #37, #53, #54, #56) were assayed, in its entirety by dilutions to 1: 128 in *C. trachomatis* negative serum, on the LIAISON® *C. trachomatis* assay prototype (graph 18). This first test was used to see the behavior of the samples in order to avoid dilution and abnormal samples in the standard calibration curve.



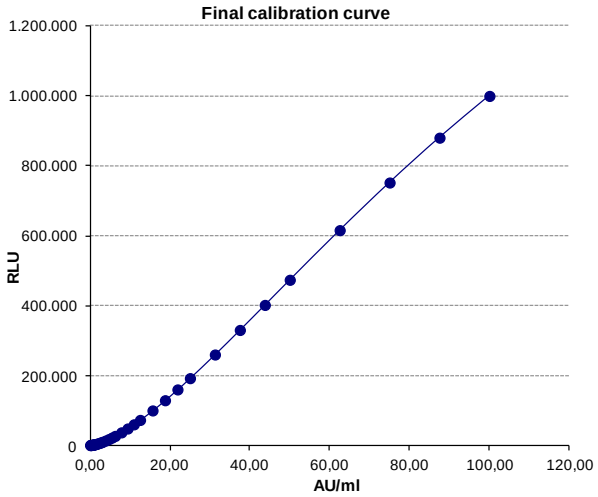
Graph 18: 5 high positive samples for *C. trachomatis* diluted in a negative samples.

The graph shows that the samples are high positive for *C. trachomatis*. All samples have a linear trend on the dilutions made except for the #56 because it has a too high RLU signal. Subsequently a second test was made where the samples were mixed 1:1:1:1 to see the mixture diluted behavior (graph 19).



Graph 19: Dilutions made on the 5 positive samples (#36, #37, #53, #54, #56) mixure.

Verified the consistency of the curve obtained with the dilutions of the 5 positive samples mixed (graph), a second lot of master curve was made. The five positive samples were joined in a big stock solution, that was then diluted with nine serial dilution 1:2 (graph 20 and table 16). The dilution that gave a signal of around 1000000 RLU was assigned a value corresponding to 100 Arbitrary Units/ml (AU/ml). Values greater than this number are not necessary in this assay for the calibration curve.



Graph 20: Final master curve obtained with the 5 positive samples.

Table 16:*C. trachomatis* Master Curve.

ID Calibrator	**Dilution of the Positive Pool in Negative Serum**	**AU / ml**	**RLU**
Cal [0]	Negative serum	0,00	2334
Cal 1	1/2048	0,39	3150
Cal 2	1/1024	0,78	3972
Cal 3	1/512	1,56	6009
Cal 4	1/256	3,13	12133
Cal 5	1/128	6,25	28930
Cal 6	1/64	12,50	80012
Cal 7	1/32	25,00	209086
Cal 8	1/16	50,00	485922
Cal 9	1/8	100,00	922895

Comments: This is the *C. trachomatis* Master Standard Curve and will be used in all future calibrations of the kit for the detection of *C. trachomatis* infection.

DISCUSSION

With this thesis work DiaSorin is going to include the LIAISON® *Clostridium difficile* Toxins A&B and *Chlamydia trachomatis* immunoassays to increase the list of specialty tests on its fully automated platform and to offer a new panel of tests to customers already working with the LIAISON® instrument.

Clostridium difficile

Clostridium difficile is the major cause of healthcare-associated infections. Toxins-producing *Clostridium difficile* strains are important pathogens among patients who are treated with antibiotics or chemotherapeutic agents as described in the introduction. Rapid and accurate diagnosis of CDI is very important to stop the spread of this infection. The LIAISON® *Clostridium difficile* kit could be used as a screening assay in combination with other more specific assay (PCR) or with cytotoxic bacterial culture. The PCR test (in particular the new test of Cepheid gene Xpert) is very sensitive and fast (1 hours) although the cost (20-40€) makes it suitable as a II level test. The sensitivity and specificity of the LIAISON® *Clostridium difficile* Toxins A&B assay was compared with the sensitivity and specificity of the Illumigene (Meridian). The reason for this has to be identified in the testing methodology recommended in the Guidelines for CDI (like IDSA and ECCMID for US and Europe) to reach a higher level of sensitivity and specificity. The 2-step testing algorithm is based on the detection of GDH as first screening assay, followed by a more specific assay performed on positive samples (Toxin A&B detection). For all these reasons, in this work a semi-quantitative immunoassays determination of *C.difficile* Toxin A&B with a direct two steps "sandwich" chemiluminescent immunoassay has been developed. The monoclonal antibodies developed by DiaSorin showed a high Toxins A & B immunoreactivity allowing the LIAISON® assay kit development with high sensitivity and specificity. The LIAISON® *C. difficile* kit specificity is 99.1% and the sensitivity is 97.7%, both these parameters are greater, therefore better, than the design input acceptance criteria. Another parameter evaluated was the LIAISON® kit Limit of Detection, this was compared with the PTAB Meridian assay. The kit developed in this thesis has shown a better Limit of Detection than the comparison kit by detecting incremental changes by 200pg/ml from zero (negative stool) while the PTAB kit does not.
As the stool assay is a new direction of diagnostic testing for DiaSorin, the ability of stool components to cause interference in general was evaluated. Both the 39 organisms (spiked in negative and low positive stools) and 17

compounds normally present in the stool does not exhibited cross reactivity or interference problems, this is important to avoid false positives.
C. difficile strains show significant interspecies heterogeneity in a PaLoc region, 9 different strains (A-/B+ and A+/B+) of toxigenic *C. difficile* were tested by spiking a negative stool with the bacteria. All these microorganisms used react in the LIAISON® *Clostridium difficile* Toxins A&B assay reflecting a positive result.
For the construction of a calibration curve and kit controls has been necessary to create a buffer to simulate the composition of a negative stool supernatant. This matrix buffer is shown equivalent performance to negative stool supernatant in the positive samples dilutions. This result is very important because the kit controls and calibrators do not need to be made using human stool samples, for this assay and the future stool assay that will be developed by DiaSorin.
In conclusion, the results obtained in this work demonstrate the high performance of the LIAISON® *Clostridium difficile* Toxins A&B assay, it met all criteria necessary for the marketing of the kit, including the short time to results (30 minutes), and the high throughput (> 90 tests / h) that were required to the Design Inputs.

Chlamydia trachomatis

Chlamydia trachomatis is the commonest sexually transmitted bacterial infection in the developed countries. The human-pathogenic strains of *C. trachomatis* have been subdivided into 15 serovars. The 4 peptides mixture (Ct A, Ct B, Ct C and Ct D) used in this thesis derived from the major outer membrane protein (MOMP), which together are capable of specifically reacting with antibodies specific to any one of the 15 serovars. These peptides, described in patent WO9900414, were synthesized using the peptide synthesizer at DiaSorin. The molecular weight found by the analysis of each peptide with UPLC-MS corresponds with that was expected, this proves that the synthesis has been done successfully (fig. from 22 to 25). During the first assay attempt (phase 1), the peptides were coated on solid phase. Different coatings concentrations (24 µg/ml, 100 µg/ml, 200 µg/ml and 400µg/ml) and samples volumes in reaction were used but in all these cases, however, some of the samples positive for *C. trachomatis* were not seen as positive by the LIAISON system. A careful analysis of the peptides sequences has revealed the presence of cysteines groups in the Ct B and Ct C. These could cause a loss of immunoreactivity of these peptides for the reasons explained in the results. The alkylation of these groups was made to

solve these problems, but also in this case some of the samples panel were not seen as positive with the *Chlamydia trachomatis* LIAISON prototype. The strategy that allowed the correct functioning of the *Chlamydia trachomatis* LIAISON® kit, has involved the use of biotinylated peptides. These were made to react simultaneously with the solid phase (streptavidin PMPs) and human anti-*Chlamydia trachomatis* antibodies in the samples (sera). With the following step the IgG anti human IgG are added as a conjugate. Through this system, the peptides are oriented correctly and so they can be easily recognized by the antibodies in the serum. The specificity calculated with this assay is 97.7% and the sensitivity is 99.1%, both these parameters are greater, than those required in the *Chlamydia trachomatis* design inputs so the goal was successfully achieved.

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ABSTRACT

The aim of my work was to develop high-throughput chemiluminescent immunoassays, working on the automated LIAISON® system. Two semi-quantitative immunoassays were created to diagnose bacterial infections caused by: 1- *Clostridium difficile* (detection of Toxin A&B in human stool specimens); 2- *Chlamydia trachomatis* (detection of anti *C.trachomatis* human IgG in human serum specimens).

Clostridium difficile

Clostridium difficile is a gram-positive, spore-forming anaerobic bacillus. It is a major cause of antibiotic-associated diarrhea and colitis. Antibiotic-associated colitis is an infection of the colon commonly found in individuals who have been using antibiotics. It is the most common acquired infection in hospitalized or extended care patients. *Clostridium difficile* manifests itself by the production of Toxins A&B. The LIAISON® *Clostridium difficile* Toxin A&B kit could be used as a screening assay in combination with other more specific assay. For the development of this assay 2 MoMAbs anti Toxin A and Toxin B were produced. These were used as solid phase (PMPs) and conjugate, in a 2 steps "sandwich" chemiluminescent immunoassay, in order to detect the 2 *C.difficile* Toxins. Some parameters of this new assay were analyzed in this work with the following result: high specificity (99.1%), sensitivity (97.7%) and Limit of Detection (200pg/ml). These values show the high performance of the kit, the Limit of Detection in particular is better than the reference kit of Meridian (PTAB). Nine different strains of toxigenic *C. difficile* (A-/B+ and A+/B+) were tested by spiking a negative stool with the bacteria. All these microorganisms react in the LIAISON® *Clostridium difficile* Toxins A&B assay, reflecting a positive result. The ability of stool components to cause interference in general was evaluated. Both the 39 organisms (spiked in negative and low positive stools) and 17 compounds normally present in the stool does not exhibited cross reactivity or interference problems. Finally the short time to results (30 minutes), and the high throughput (> 90 tests / h), together with all parameters see above met the necessary criteria for the marketing of this LIAISON® *Clostridium difficile* Toxin A&B kit.

Chlamydia trachomatis

C.trachomatis is the most prevalent sexually transmitted bacteria worldwide. It is an obligate intracellular pathogen that can cause numerous disease states in both men and women. Both sexes can display urethritis, proctitis, trachoma, and infertility. The bacterium can cause prostatitis and epididymitis in men. In women, cervicitis, pelvic inflammatory disease (PID), ectopic pregnancy, and acute or chronic pelvic pain are frequent complications. *C. trachomatis* is also an important neonatal pathogen, where it can lead to infections of the eye (trachoma) and pulmonary complications. The human-pathogenic strains of *C. trachomatis* have been subdivided into 15 serovars. The 4 peptides mixture (Ct A, Ct B, Ct C and Ct D), used in this thesis, derived from the *C.trachomatis* major outer membrane protein (MOMP) and are currently used by the *C. trachomatis* ELISA Savyion Kit. These peptides together are capable to specifically reacting with antibodies specific to any one of the 15 serovars. Several attempts have been tried in the aim to develop this assay: 4 coating concentrations of peptides on solid phase were evaluated with 2 different sample volumes, then 2 modified peptides (with cysteines alkylated) were used in order to improve the recognition by the antibodies (present in the *C.trachomatis* positive samples). The strategy that allowed the correct functioning of the *C. trachomatis* LIAISON® kit, has involved the use of biotinylated peptides. These were made to react simultaneously with the solid phase (streptavidin PMPs) and human anti *C. trachomatis* antibodies in the samples (sera). With the following step the IgG anti human IgG are added as a conjugate. The specificity calculated with this assay is 97.7% and the sensitivity is 99.1%; both these parameters largely satisfy the criteria that were indicated necessary for the marketing of this kit, so the goals were successfully achieved.