Improving clinical immunoassays by removing bacterial alkaline phosphatase from purified water used in clinical analyzers

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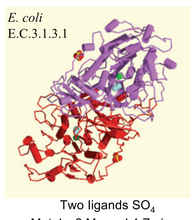
Research and Development - Lab Water – Merck Millipore - [1]St Quentin-Yvelines, France, [2]Billerica, MA, USA

Introduction



Two ligands SO_4
Metals: 2 Mg and 4 Zn ions

- Alkaline phosphatase (ALP) is involved in biochemical cascades, ELISA using ALP-conjugated secondary detection systems, and spectrophotometric assays in clinical cheamistry.
- The major source of interference with the assays originates from bacterial alkaline phosphatase released in the water.
- High purity water is used in clinical analyzers to prepare buffers, to rinse the tubing and needles, and to make dilutions. However, the water degrades quickly on standing in tubings and reservoirs during the downtimes of the instruments and high bacterial counts (15 to > 5 000 cfu/mL) are observed in most brands of clinical analyzers.
- The study conducted aimed:

(1) at establishing a correlation between bacterial counts and alkaline phosphatase levels in water
(2) at finding an efficient way to remove this enzyme from water to avoid interferences in the assays.

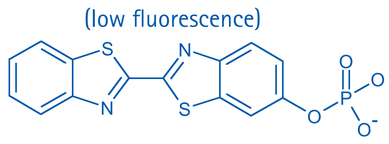
- The focus has been on the bacteria typically found in high purity water.

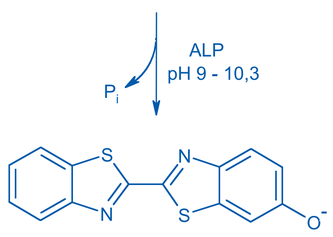
Bacteria levels and alkaline phosphatase concentrations

Challenge of RNases

Kit Roche – AttoPhos® system (JBL Scientific Inc, San Luis Obisco, CA, USA)
Reaction for 20 min at 37 °C; end-point measurement. Fluorescence emission measured in Relative Fluorescence Units (RFU) at 550 nm.
Calibration curve established for every assay in the range 0 to 10 micro units using the Sigma® ALP.
Quantification limit is 0.2 µU. Values reported below 0.2 µU are indicative. Detection limit is 0.02 µU/mL.
10 µL sample were used in all cases for AttoPhos® assay.
No ALP activity was ever detected in the culture media.

AttoPhos® Substrate
(low fluorescence)




BBT Anion
(high fluorescence)

Correlation bacterial strain - ALP activity

A few strains of bacteria can grow in purified water. Three strains were isolated in water sampled from clinical analyzers: *Sphingomonas paucimobilis, Ralstonia pickettii, and Caulobacter cresentus.* These bacteria were grown and the amount of ALP released was measured, using the Attophos® Roche kit.
One colony of each bacterial strain was added into a plastic sterile tube with 8 ml of liquid culture media (m-HPC medium, Merck Millipore). The incubation was done 24 hours at 30-35 °C.

Various amounts of ALP are detected depending on the strain of the bacteria

Bacteria type	Bacteria level (x10^6 cfu/ml)	ALP concentration (µ Unit/µL)
Sphingomonas paucimobilis	29.2	6.22
Caulobacter cresentus	9.7	9.95
Ralstonia pickettii	29.5	8.29

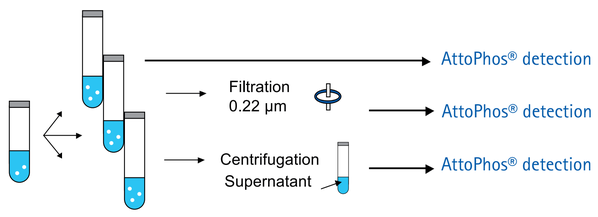
The bacterial count is done with the plate count method; bacteria culture is diluted into sterile ultrapure water (Milli-Q® Biocel water filtered with Stericup® 0.22 µm). Two dilution samples of 10 mL were filtered on membrane and incubated on agar media (Milliflex® system – Merck Millipore, manipulation under a laminar hood). Agar Media culture: R2A media plate, incubation condition : 72 h at 30-35 °C.

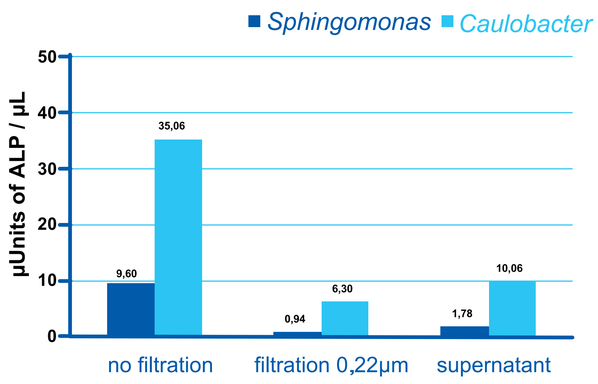
ALP is released by bacteria and is free in water

One colony of *Caulobacter* and *Sphingomonas* bacteria types was cultured in a plastic sterile tube with 6 mL of liquid culture media (m-HPC medium, Merck Millipore). The incubation condition was done for 7 days at 30-35 °C.

The 6 mL of bacteria culture were split into 3 tubes (2 mL each). The totality of the 2 mL mixture was filtrated with the filter 0.22 µm, the filtrate was analyzed. The totality of the 2 mL mixture was centrifuged at 14000 rpm/min – 20 minutes - and the supernatant was analyzed.

The data show that in the total ALP activity measured before filtration, part of the activity is conserved when bacteria are not present anymore (removed by filtration or centrifugation). This supports the presence of free ALP in the medium.

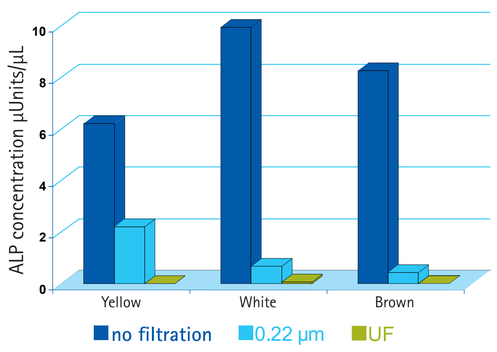




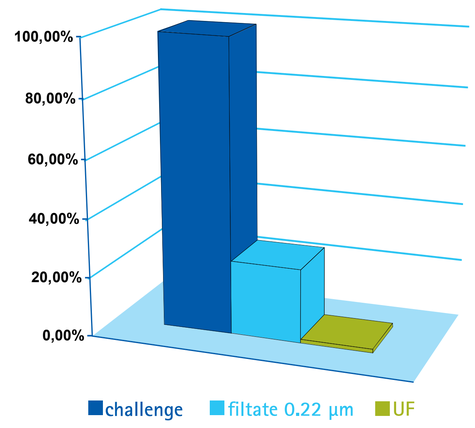
Efficiency of ultrafiltration for alkaline phosphatase removal

Challenge with bacteria cultures

Bacteria cultured for 24h at 30-35 °C. Volumes of 6 mL of bacteria culture split into 3 tubes. Process was applied to 3 bacteria strains. The totality of the 2 mL mixture was filtrated with the filter 0.22 µm, the filtrate was analyzed. The totality of the 2 mL mixture was filtrated with the Ultrafilter (UF), the filtrate was analyzed. The non filtrated solution was analyzed too.



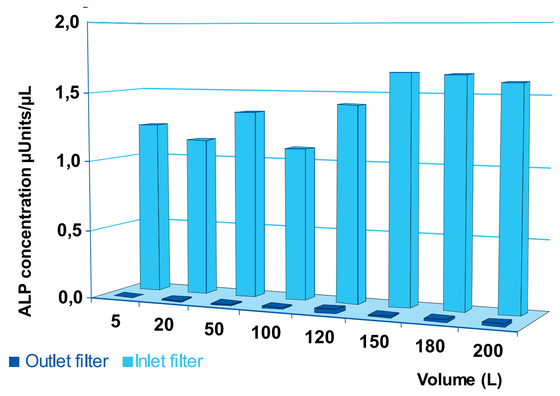
Challenge with pure ALP



- *Ultrafiltration demonstrates efficiency on the removal of pure ALP, as well as on the removal of ALP released by- and attached to- bacteria*

Challenge test with high concentration of ALP

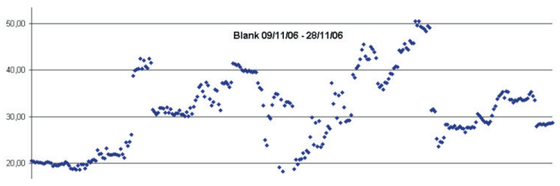
In order to have an idea of the capacity of the ultrafilter, a large volume of a concentrated solution of ALP was passed onto the cartridge. The challenge solution composition : 200 liters of Milli-Q® water + 300 Units of ALP (Sigma® P-4252, 1000 Units). The challenge solution was filtered on an ultrafiltration device (delivery pressure 1 bar, flow rate 1.4L/min). A sampling of the filtrate and the challenge solution were done at the same time for different volumes.



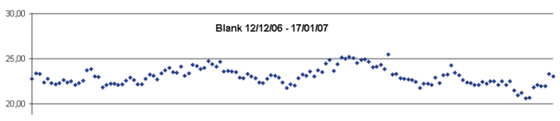
Real case study

The use of ultrafiltration has helped solving a long term issue on an immunoanalyzer. The CTNI assay is sensitive to the presence of ALP. The BioPak® has reduced the level of ALP and bacteria in the water, resulting in a much more stable blank.
Stable blank insures accuracy and precision in the final patient result.

Blank of the CTNI assay without Ultrafiltration



Blank of the CTNI assay with Ultrafiltration (BioPak®) used for water purification



Potential calculated efficiency of the ultrafiltration cartridge in regards to bacterial level

- A total of about 300 units of ALP were filtered.
- Concentration of ALP released by bacteria (from our experience): 1 x 10^7 cfu/mL bacteria release *ca* 10-15 unit/L of ALP.
- Type I – CLSI water < 10 cfu/mL. Assuming some contamination in the on-board reservoir up to 100 cfu/mL will lead to a release of 15 x 10^{-5} unit/L of ALP.
- Volume that would potentially be passed to reach 300 units of ALP: 300/15 x 10^{-5} unit/L = 2 x 10^6 liters