A RAPID AND SPECIFIC ASSAY TO DETECT BACTERIAL CONTAMINATION IN MILK

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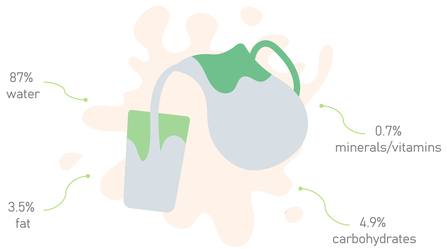
INTRODUCTION



The proliferation of germs, such as bacteria and fungi, constitutes one of the most common cause of milk spoilage. From the collection of the milk to its packaging, every processing step can contribute to milk contamination.

The use of refrigeration in most developed countries and heat treatments, such as pasteurisation and ultra-high temperature (UHT) processing, eliminate a majority of the biological contaminants to low bacterial levels. However, psychrotrophic bacteria have become a leading cause of milk spoilage due to their ability to grow at low temperatures and to produce heat-resistant proteolytic enzymes. Enzymatic degradation due to Gram-positive or Gram-negative bacteria critically impacts organoleptic properties and product shelf-life of processed milk.
With up to 30% losses in the dairy industry due to psychrotrophic bacterial contamination, there is an apparent need for rapid and sensitive testing to identify microbial contaminants in milk.

AIMS OF THE TECHNOLOGY

EnzoTact©PRO, a milk protease testing assay, aims for :

BACTERIAL PROTEASE DETECTION

Specific detection of bacterial proteases secreted by both Gram-positive (ex : *Bacillus* spp) and Gram-negative (ex : *Pseudomonas* spp) commonly isolated strains in post-pasteurised and UHT milk

QUALITY CONTROL

Robust indicator of dairy quality by assessment of milk protease content

PROTEASE ACTIVITY ASSAY

Rapid, affordable, easy-to-use and highly sensitive near-infrared based assay

METHODS

Detact Diagnostics® patented, novel and disruptive bacterial detection platform is based on the VIPER (Visualisation by Infrared Peptide Reaction) technology (**Figure 1**). The technology relies on the immediate release of a quenched fluorophore after specific cleavage of a bacteria-sensitive peptide substrate by a bacterial protease, which emits in Near-Infrared (NIR) light (> 780 nm). This extremely stable signal is then detected through substances and tissues by the Detact DeNIRO® NIR Fluorometer.

Acidic precipitation and centrifugation of milk samples

30-min incubation
Instant readout on the DeNIRO© fluorometer. Results in Relative Fluorescence Units (RFU), directly correlated with the milk protease concentration.

EnzoTact©PRO is a 2-step assay

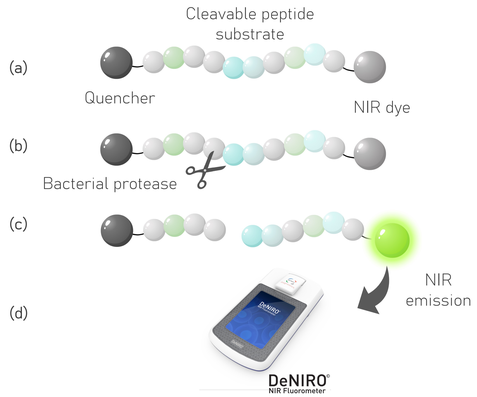


Figure 1 : Schematic overview of the VIPER technology
The technology is based on detecting the activity of proteases (also known as peptidases or proteinases), enzymes specialised in splitting peptide bonds in proteins.

(a) Detact Diagnostics® 's substrate is constituted of a quencher (a deactivator of fluorescence), a fluorophore (a fluorescent chemical re-emitting light upon excitation) and a specifically designed peptide.

(b) A bacterial endopeptidase recognises the substrate and binds to it.

(c) The protease hydrolyses the peptide bond at the cleavage site. The split peptide results in the detachment of the quencher and the fluorophore. Fluorescence is no longer inhibited.

(d) The NIR signal emitted by the free fluorophore is detected by the DeNIRO© NIR Fluorometer and transmitted in Relative Fluorescence Units, correlated with the protease concentration.

RESULTS

Specific proteolytic detection by EnzoTact© PRO substrate

EnzoTact©PRO substrate enables specific detection of proteases secreted by milk-contaminating bacterial strains, as *Bacillus* and *Pseudomonas* spp. Strains rarely isolated in milk, such as *Ochrobactrum anthropi* and *Bordetella bronchoseptica,* do not cleave EnzoTact© PRO substrate (**Figure 2**).

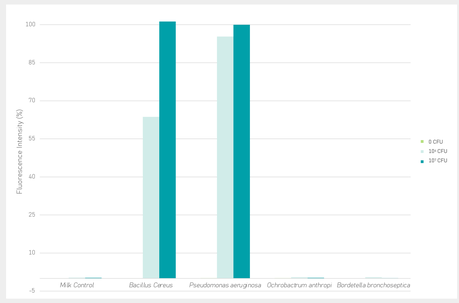


Figure 2 : Specific detection of proteases in milk by EnzoTact©PRO

Milk matrix sample preparation

Influence of milk fat and milk native proteins on enzymatic assay

As the VIPER technology was firstly developed in buffer, the impact of fat and native milk proteins on the substrate fluorescence and the enzymatic activity of a standard bacterial protease (subtilisin from *Bacillus licheniformis*) cleaving EnzoTact©PRO substrate was assessed.
Fat negatively influences the substrate fluorescence but does not significantly affect the enzyme reaction, while the presence of proteins have a huge negative impact on the enzyme reaction with the fluorescent substrate but does not impact the substrate fluorescence.

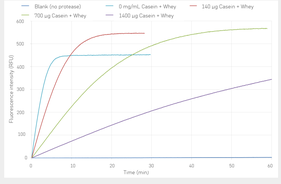


Figure 3 : Protease activity at different milk protein concentrations over time

As a competition between milk proteins and substrate can be suggested, protease activity on EnzoTact© PRO substrate was evaluated using different protein concentrations of casein : whey proteins (80 : 20, same ratio as in cow's milk). Casein proteins lead to a decrease in protease activity, impacting more significantly the fluorescence signal than whey proteins do (**Figure 3**).
These results indicate that removal of casein proteins prior to fluorescence reading appears to be a feasible route to setup a very sensitive enzyme activity assay for use in milk.

Optimisation of precipitation step

To precipitate casein from milk matrix, an acidification step before activity measurement was added and optimised. The influence on protease activity of decreasing sample pH to pH < 5 was studied.

Milk precipitation with an acidic buffer (pH ≤ 4) for a decrease in pH to < 5, incubated for 5 minutes followed by a centrifugation step of 5 minutes at 12 500 rpm resulted in the highest fluorescence response and very low protein concentrations in the supernatant.
Therefore, precipitation of milk casein micelles by a pH decrease before fluorescence intensity measurement is the key to a non-time-consuming sample preparation and a sensitive assay.

Conditions for sensitive proteolytic detection

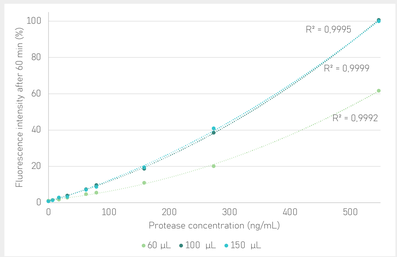


Figure 4 : Variation of sample volume and impact on fluorescence response in UHT milk

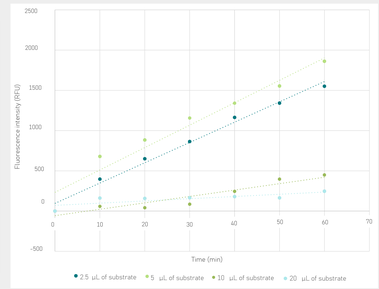


Figure 5 : Effect of substrate addition on the NIR fluorescence signal in UHT milk

In order to maximise the detection of the fluorescence signal and sensitivity of the enzymatic assay, the working conditions (buffer, substrate stability and concentration, sample volume) were optimised.
As expected, by raising the sample volume the fluorescence response was significantly increased. It can be noted that a volume limit was met, after reaching a certain large volume it did not result in a higher fluorescence emission (**Figure 4**).
On the other hand, increasing the amount of fluorophore-containing substrate unexpectedly resulted in a decrease of the fluorescence response (**Figure 5**). This can be explained by a common spectroscopy phenomenon called inner-filter effect or by self-quenching of the dye when highly concentrated.
Further experiments lead to implementing an optimal ratio of sample and buffer to ensure sensitive detection of the fluorescence signal, reaching a minimal limit of detection (LoD) of 9.6 ng/mL of protease in UHT milk.

CONCLUSION

Each year, 116 million tonnes of dairy products are discarded worldwide, with 1/6 of all milk produced globally wasted, half of it before even reaches stores. This represents millions of dollars of worthless costs for the dairy industry, without mentioning the major environmental consequences due to pointless CO_2 production.
Accurate evaluation of the quality of a batch of milk, increase in the average product shelf life and valuable insight on the most suitable end application could have a drastic effect in reducing the amount of milk waste throughout the dairy production chain.
Detact Diagnostics®, with support from NIZO, addresses those issues with the development of EnzoTact©PRO.

- PROTEASE ACTIVITY ASSAY IN MILK novel, rapid and specific for testing UHT milk
- SENSITIVE NIR DETECTION down to subnanomolar concentrations
- EASY-TO-USE quick sample preparation & no requirement for a trained microbiologist
- QUALITY CONTROL before commercialisation or for testing recalled dairy products
- APPLICATIONS farm site testing, industrial milk storage facilities & packaging factories

Detact Diagnostics® has now extended its range of milk-testing products with the development of **EnzoTact©RAW**, the first protease detection assay in raw milk.

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Contact us !

References : Lu and Wang, 2017 / Samaržija *et al.*, 2012