Validation of ZymoSnap for Alkaline Phosphatase (ALP) Detection in Dairy Products

Introduction

Why Alkaline Phosphatase?

Alkaline Phosphatase (ALP) is a naturally present enzyme in all raw milks. It catalyzes the dephosphorylation of many types of proteins and nucleotides. Since the heat required to inactivate the enzyme exceeds that of most microbial spoilage organisms, including *Mycobacterium*, it serves as a powerful indicator of successful pasteurization (along with rapid cooling). In fact, inactivation of this enzyme to an acceptable level is used to measure effective pasteurization. This value varies among different pasteurized milk types: < 350 mU/L for cow's milk (ISO Standard 11816-1), < 300 mU/L for goat's milk, and <500 mU/L for sheep's milk. ALP measurements above these levels would, therefore, be considered indicative of a significant issue in the pasteurization process. Because ALP is more heat stable than most pathogenic organisms, ALP can also serve as an indicator of overall product safety, although it cannot guarantee the tested product is completely free of undesirable microorganisms.

How to test for ALP?

Two methods for testing for ALP are indicated below: ZymoSnap and Fluorophos® (reference method). This study compares the performance of the two.

ZymoSnap

ZymoSnap is a rapid, bioluminogenic method that determines the level of ALP enzyme via conversion of Relative Light Units (RLUs) to milliunits per liter (mU/L) of ALP in pasteurized milk samples. A value below 100 mU/L will give a pass result, results above 350 mU/L will give a fail result. These limits align with the regulations for post-pasteurization ALP levels.

The assay uses a single, self-contained device in one simple procedure. Sample is added to the ZymoSnap ALP tube, the device is activated to release detection reagent and is incubated for 5 minutes. The resulting ALP enzymatic activity is measured in a luminometer, specifically, the EnSURE® Touch. To establish Pass/Fail criteria, a positive and negative control must be prepared using the ZymoSnap ALP Positive Control Kit and tested prior to testing unknown samples. ZymoSnap is AOAC approved and is awaiting NCIMS approval.

Fluorophos

The Fluorophos Test System is an alternative method that is often used for measuring ALP. This system uses a fluorometer, the FLM200, to measure ALP levels in dairy samples. The instrument requires a dedicated space of approximately 17 x 13 x 7 inches, along with an additional heating block. The system requires a 15-minute start-up time to equilibrate the operating temperature. It must also be calibrated daily with positive and negative controls, and three calibrators, with the selection of sample type before samples are tested. ALP substrate must be prepared before use. Fluorophos is AOAC, NCIMS and ISO approved, so it is often referred to as the ALP reference method.

Method & Materials

Equipment, Supplies, and Reagents

- ZymoSnap ALP (Part No. ZS-ALP-100)
- ZymoSnap ALP Positive Control Kit (Part No. ZS-ALP-PC)
- Incubator/Heating block set at 37 ± 1 °C
- EnSURE Touch Luminometer (Part No. ETOUCH)
- Pipettor and Pipette Tips
- Pipette (10 mL) and test tubes (0.5" x 4.0")
- Fluorophos Test System – Fluorometer
- Fluorophos Calibrator Set
- Fluorophos Controls
- Fluorophos Test Reagent Set
- Vortex

Sample Preparation and Incubation

ZymoSnap

Once a ZymoSnap ALP device has equilibrated to room temperature, lift the Snap-Valve bulb out of the device and add 75 µL of sample to the tube. Replace the bulb, shake and flick the device to ensure all the sample drains to the bottom of the tube. Activate the device by bending the Snap-Valve bulb forward and backward to release the content of the bulb and squeeze the bulb to release the reagents into the tube.

Incubate the device for 5 min ± 10 s at 37 ± 1 °C. Near the end of the incubation time, turn on the EnSURE Touch instrument and prepare it for reading the results. After incubation, shake the ZymoSnap device for 5 seconds to mix thoroughly. Immediately place the device into the EnSURE Touch and record the results in mU/L, representing the ALP activity.

Fluorophos

Before testing a sample, the system must be calibrated for the specific product type being tested (see below). In addition, reagents must be prepared by mixing the ALP substrate with the ALP Substrate Buffer. Three calibrators (A, B and C) are used to prepare the instrument for sample testing. Next, reagents are all reconstituted and the substrate is added to a glass cuvette and placed in the heating block. A sample is pipetted into the cuvette, vortexed, and then placed into the fluorometer. After 60 seconds, the fluorometer begins reading the sample and at the end of three minutes, the fluorometer will display the fluorescence in mU/L, representing the ALP activity.

Positive and Negative Control Testing/Calibration

ZymoSnap

Prepare a negative control by heat-inactivating a sample similar to what is being tested. To heat-inactivate, heat a large volume (10 mL) of the sample in a test tube (0.5" x 4.0") in an incubator or water bath set at 72 °C for 10 minutes. Cool the sample rapidly on ice (to prevent reactivating the enzyme) – this is the Negative Control. Use 1 mL of this heat-inactivated sample to reconstitute the Positive Control vials to create the positive control sample. Note: this must be performed for each sample type.

For Control Testing, it is recommended to run three replicates from each Positive and Negative Control for each sample type. Testing is performed using the same procedure as for the samples (detailed above).

Fluorophos

Daily, instrument controls must be prepared by heating the control in a cuvette for 15 minutes in the heat block. The control is then placed into the instrument and the value recorded once the display stabilizes. Next, the same is done with the reconstituted substrate (15 min in a heat block, then reading the cuvette in the instrument). Values for both need to be within a specified range and recorded for later reference. Once this is complete, samples can be tested as above.

Results

Comparative Analysis

A comparison of ZymoSnap versus Fluorophos was conducted with ten dairy brands and three fat solid densities (0.1%, 2% and 4%). Samples were spiked with various levels of bovine alkaline phosphatase (ALP) to compare assay performance. ALP spike levels used were zero, 100 mU/L, 350 mU/L and 1,000 mU/L. Five replicates per assay were performed with each sample. The average result per sample is shown in Table 1 below.

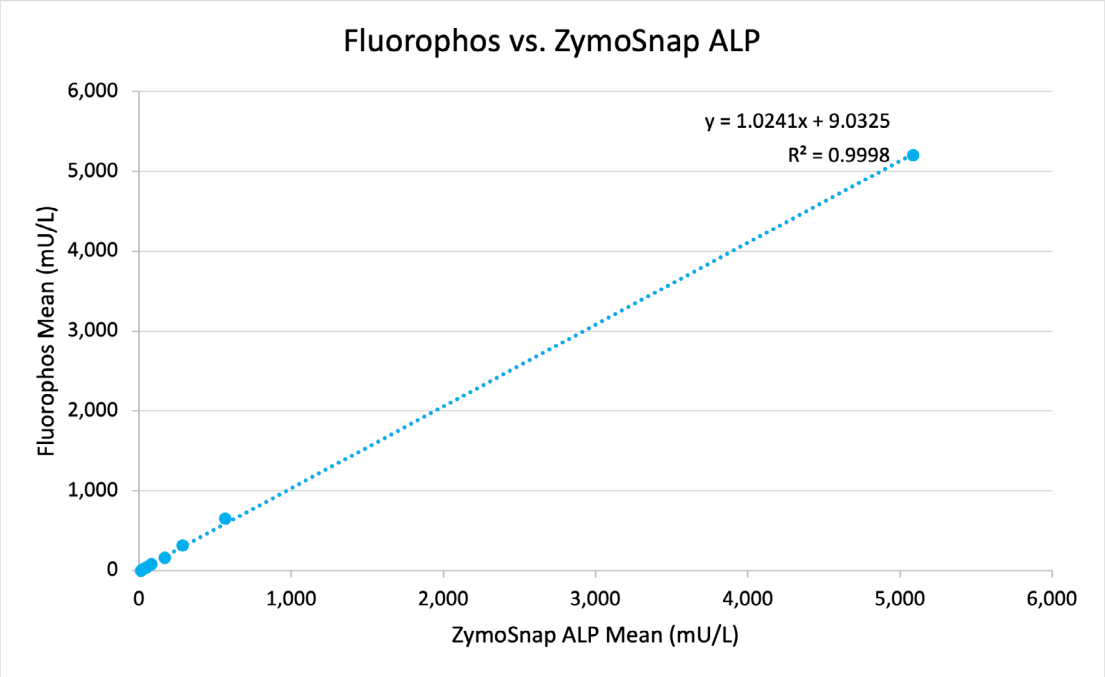
Table 1: Comparison of ALP Levels in Cow's Milk

Sample type	1,000 mU/L		350 mU/L		100 mU/L		0 mU/L	
	Fluorophos	ZymoSnap	Fluorophos	ZymoSnap	Fluorophos	ZymoSnap	Fluorophos	ZymoSnap
4.0% cow's milk	2,462	2,997	377	395	82	66	<10	11
2.0% cow's milk	2,253	3,473	228	295	73	96	<10	10
0.1% cow's milk	2,136	6,470	167	561	41	182	<10	9

Accuracy and Linearity Analyses

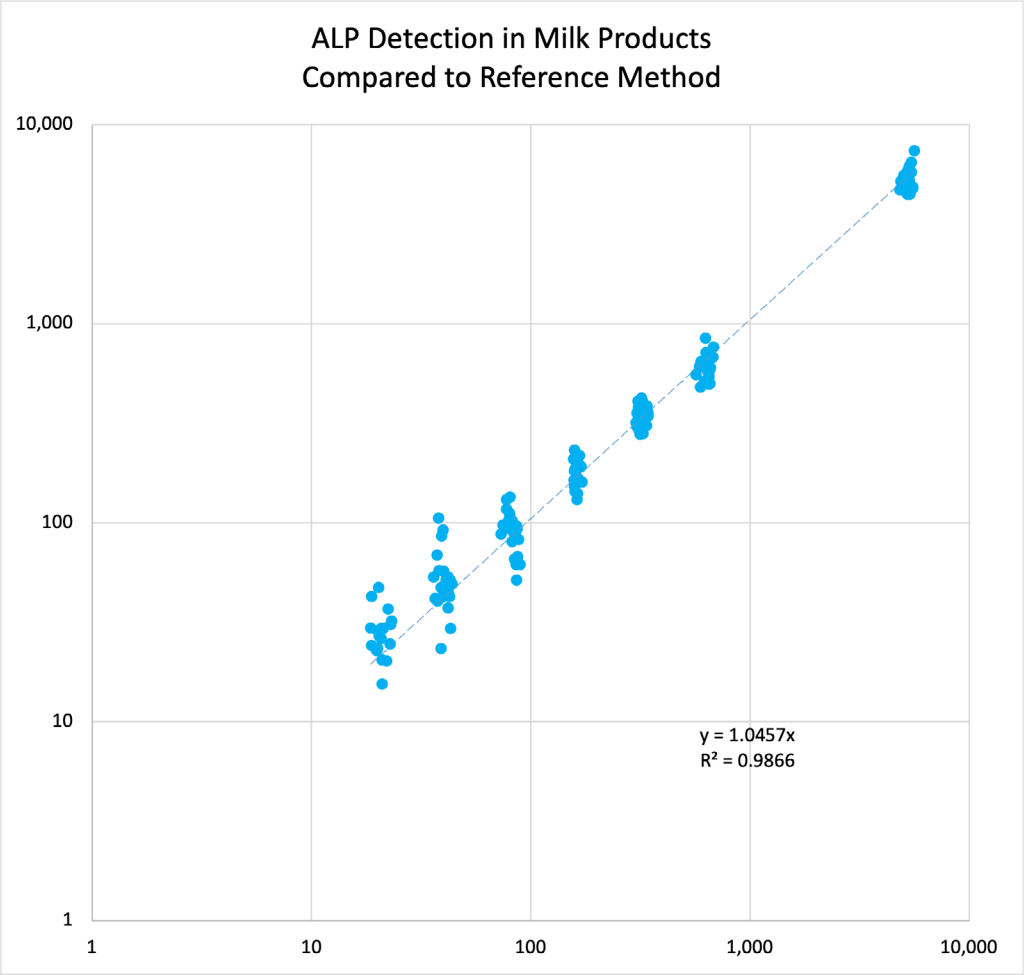
To verify assay linearity versus the reference method (Fluorophos), samples were tested where ALP was spiked at contaminations levels of 5,000, 625, 320, 160, 80, 40, 20, and 0 mU/L in triplicate. Correlation data was graphed (Figure 1) and the slope of the line determined along with the coefficient of determination. The R^2 was found to be 0.9998, demonstrating an extremely accurate correlation between both methods.

Figure 1: Correlation of ALP Determination



Similar results were obtained when these ALP spike levels were tested in a variety of dairy products, including cow's milk (at varying fat levels from 0.1% to 4%), cream (20%, 40%), chocolate milk (0.1%) and strawberry milk. Results are displayed in Figure 2, again demonstrating a very tight correlation between Fluorophos and ZymoSnap.

Figure 2: Correlation of ALP Levels in Milk Using Fluorophos and ZymoSnap



Other Animal Milks

ZymoSnap has been tested on other animal milks in addition to cow's milk. Similar performance was obtained when testing sheep and goat milk, further demonstrating that ZymoSnap was fit for use on multiple milk types.
(Data not shown)

Conclusions

Performance

When compared side by side, ZymoSnap performed as well as Fluorophos for accurately detecting ALP in dairy products. Detection was linear and aligned with each dilution of ALP tested. Results clearly show that ZymoSnap ALP can be used interchangeably with the reference method for ALP detection in a variety of milks, including flavored milks and creams.

Additional Advantages

In addition to performance, ZymoSnap ALP outperforms the reference method in numerous ways. First, ZymoSnap does not require the placement of a permanent instrument in the lab, which takes up critical lab space. Second, ZymoSnap ALP is a simple, all-in-one device with no need for addition of or reconstitution of other reagents. Once sample is added, activation is simple and incubation occurs directly in the device. Results determination is also performed using the same device. It is simply inserted into the hand-held EnSURE Touch luminometer, where results are displayed in 10 seconds. In addition, the data is stored on the device and/or in the cloud (SureTrend® Cloud) for later access for analysis or trending over time. No paper is needed for later access to the data. No glass is used either, making it safer for use.

In addition, other testing data can be stored in the same location. For example, environmental monitoring in the dairy facility can be captured using UltraSnap®, SuperSnap® or AquaSnap® devices using similar, rapid methods with easy-to-interpret results. The facility can also test for indicator organisms using MicroSnap® devices – this data can also be stored in the same cloud-based system.

Recommendation

Overall, ZymoSnap ALP performs equivalently to the reference method. Due to its ease of use, rapid time to results and ability to store other facility results in the same system, any facility should see improved turn-around time, reduced costs and simplified workflows when using ZymoSnap ALP. For these reasons, ZymoSnap should be the preferred choice.