Megazyme

www.megazyme.com

# ETHANOL

## ASSAY PROCEDURE

K-ETOH 08/18

(*60 Manual Assays per Kit) or
(600 Auto-Analyser Assays per Kit) or
(600 Microplate Assays per Kit)

** The number of tests per kit can be doubled if all volumes are halved*

## INTRODUCTION:

Ethanol is ubiquitous in its natural occurrence, and thus its quantitative determination is not only important in the manufacture of intoxicating wines, beers and spirits, but also for low-alcohol and non-alcoholic beverages, fruit juices and a range of other foodstuffs, including chocolates, sweets, jam, honey, vinegar and dairy products. A large range of non-foods also contain significant quantities of ethanol, such as cosmetics and pharmaceuticals.

## PRINCIPLE:

The quantification of ethanol requires two enzyme reactions; in the first reaction catalysed by alcohol dehydrogenase (ADH), ethanol is oxidised to acetaldehyde by nicotinamide-adenine dinucleotide ($NAD^+$) (1).

$$\text{(1)}\quad \text{Ethanol} + NAD^+ \xrightarrow{\text{(ADH)}} \text{acetaldehyde} + NADH + H^+$$

However, since the equilibrium of reaction (1) lies in favour of ethanol and $NAD^+$, a further reaction is required to "trap" the products. This is achieved by the quantitative oxidation of acetaldehyde to acetic acid in the presence of aldehyde dehydrogenase (Al-DH) and $NAD^+$ (2).

$$\text{(2)}\quad \text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{(Al-DH)}} \text{acetic acid} + NADH + H^+$$

The amount of NADH formed in this reaction pathway is stoichiometric with twice the amount of ethanol. It is the NADH which is measured by the increase in absorbance at 340 nm (Figure 1, page 12).

## SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The order of addition of reagents during the assay removes any possible interference from aldehydes and ketones. Methanol is not converted due to the unfavourable $K_m$-values of the enzymes used. The assay is optimised for ethanol, however quantitative conversion of *n*-propanol and *n*-butanol is also achieved. Higher primary alcohols react at a significantly reduced rate and, where present, can lead to a sample-dependent creep reaction.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.023 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.093 mg/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 0.25 to 12 µg of ethanol per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.00 mL, this corresponds to an ethanol concentration of approx. 0.023 to 0.046 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

## INTERFERENCE:

Alcohols present in the distilled water and buffers used for the assay, or in the air, can result in increased blanks or in creep reactions, respectively. Thus, it is necessary to cover the cuvettes during assay.

If the conversion of ethanol has been completed within the time specified in the assay (approx. 5 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding ethanol (~ 5 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding ethanol to the sample in the initial extraction steps.

## SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

## KITS:

Kits suitable for performing 60 assays in manual format (or 600 assays in auto-analyser format or 600 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

**Bottle 1:** Buffer (15 mL, pH 9.0) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

**Bottle 2:** $NAD^+$.
Stable for > 5 years below -10°C.

**Bottle 3:** Aldehyde dehydrogenase solution (3.25 mL).
Stable for > 2 years below -10°C.

**Bottle 4:** Alcohol dehydrogenase suspension (1.3 mL). Stable for > 2 years at 4°C.

**Bottle 5:** Ethanol standard solution (5 mL, 5 mg/mL). Stable in a well-sealed container (as supplied) for > 2 years at 4°C.

## PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

**1.** Use the contents of bottle 1 as supplied. Stable for > 2 years at 4°C.

**2.** Dissolve the contents of bottle 2 in 12.4 mL of distilled water. **Stable for > 1 year at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).

**3 & 4.** Use the contents of bottles 3 and 4 as supplied. Before opening for the first time, shake the bottles to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position. **Swirl the bottle to mix contents before use.** Stable for > 2 years at 4°C (ADH) or below -10°C (Al-DH).

**5.** Dilute 0.5 mL of the contents of bottle 5 to 50 mL with distilled water. Store in a well-sealed Duran® bottle. When diluted, this solution is stable for 2 days at 4°C.

**NOTE:** The ethanol standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of ethanol is determined directly from the extinction coefficient of NADH (page 4).

## EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL and 100 mL).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® [to dispense 0.2 mL aliquots of buffer (Bottle 1) and $NAD^+$ solution].
   - with 25.0 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Whatman No. 1 (9 cm) filter papers.

## A. MANUAL ASSAY PROCEDURE:

**Wavelength:** 340 nm
**Cuvette:** 1 cm light path (glass or plastic with cap)
**Temperature:** ~ 20-25°C
**Final volume:** 2.57 mL
**Sample solution:** 0.25-12 µg of ethanol per cuvette (in 0.10-2.00 mL sample volume)

**Read against air** (without a cuvette in the light path) or against water

| Pipette into cuvettes | Blank | Sample |
|---|---|---|
| distilled water (~ 25°C) | 2.10 mL | 2.00 mL |
| sample | - | 0.10 mL |
| solution 1 (buffer) | 0.20 mL | 0.20 mL |
| solution 2 ($NAD^+$) | 0.20 mL | 0.20 mL |
| solution 3 (Aldehyde dehydrogenase) | 0.05 mL | 0.05 mL |
| Mix* and read the absorbances of the solutions ($A_1$) after approx. 2 min and start the reactions by addition of: | | |
| suspension 4 (Alcohol dehydrogenase) | 0.02 mL | 0.02 mL |
| Mix* and read the absorbances of the solutions ($A_2$) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 1 min intervals until the absorbances increase constantly over 1 min**. | | |

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 4.

## CALCULATION:

Determine the absorbance difference ($A_2$-$A_1$) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{ethanol}$. The value of $\Delta A_{ethanol}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of ethanol can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 2} \times \Delta A \quad [g/L]$$

**where:**
V = final volume [mL]
MW = molecular weight of ethanol [g/mol]

$\varepsilon$ = extinction coefficient of NADH at 340 nm
= 6300 [l x $mol^{-1}$ x $cm^{-1}$]
d = light path [cm]
v = sample volume [mL]
2 = 2 moles of NADH produced for each mole of ethanol

***It follows for ethanol:***

$$c = \frac{2.57 \times 46.07}{6300 \times 1.0 \times 0.10 \times 2} \times \Delta A_{ethanol} \quad [g/L]$$

$$= 0.09397 \times \Delta A_{ethanol} \quad [g/L]$$

***It follows for ethanol in (v/v) terms:***

$$c = \frac{2.57 \times 46.07}{6300 \times 1.0 \times 0.10 \times 2} \times 0.1266 \times \Delta A_{ethanol} \quad [\% (v/v)]$$

$$= 0.01190 \times \Delta A_{ethanol} \quad [\% (v/v)]$$

**where:**
0.1266 = factor to convert g/L to % (v/v), taking the density of pure ethanol to be 0.79 g/mL.

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

**Content of ethanol**

$$= \frac{c_{ethanol}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

**NOTE:** These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

## B. AUTO-ANALYSER ASSAY PROCEDURE:

**NOTES:**

1. The Auto-Analyser Assay Procedure for ethanol can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of ethanol **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

### Preparation of R1:

| Component | Volume |
|---|---|
| distilled water<br>solution 1 (buffer)<br>solution 2 ($NAD^+$)<br>solution 3 (Al-DH) | 20.85 mL<br>2.4 mL<br>2.4 mL (after adding 12.4 mL of $H_2O$ to bottle 2)<br>0.6 mL |
| Total volume | 26.25 mL |

### Preparation of R2:

| Component | Volume |
|---|---|
| distilled water<br>suspension 4 (ADH) | 3.2 mL<br>0.25 mL |
| Total volume | 3.45 mL |

### EXAMPLE METHOD:

| | |
|---|---|
| **R1:** | 0.200 mL |
| Sample: | ~ 0.01 mL |
| **R2:** | 0.025 mL |
| **Reaction time:** | ~ 5 min at 37°C |
| **Wavelength:** | 340 nm |
| **Prepared reagent stability:** | > 2 days when refrigerated |
| **Calculation:** | endpoint |
| **Reaction direction:** | increase |
| **Linearity:** | up to 110 mg/L of ethanol using 0.01 mL sample volume |

## C. MICROPLATE ASSAY PROCEDURE:

**NOTES:**

1. The Microplate Assay Procedure for ethanol can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of ethanol **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

**Wavelength:** 340 nm
**Microplate:** 96-well (e.g. clear flat-bottomed, glass or plastic)
**Temperature:** ~ 25°C
**Final volume:** 0.257 mL
**Linearity:** 0.1-1.2 µg of ethanol per well (in 0.01-0.20 mL sample volume)

| Pipette into wells | Blank | Sample | Standard |
|---|---|---|---|
| distilled water | 0.210 mL | 0.200 mL | 0.200 mL |
| sample solution | - | 0.010 mL | - |
| standard solution | - | - | 0.010 mL |
| solution 1 (buffer) | 0.020 mL | 0.020 mL | 0.020 mL |
| solution 2 ($NAD^+$) | 0.020 mL | 0.020 mL | 0.020 mL |
| solution 3 (Al-DH) | 0.005 mL | 0.005 mL | 0.005 mL |
| Mix* and read the absorbances of the solutions ($A_1$) after approx. 2 min and start the reactions by addition of: | | | |
| suspension 4 (ADH) | 0.002 mL | 0.002 mL | 0.002 mL |
| Mix* and read the absorbances of the solutions ($A_2$) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 1 min intervals until the absorbances increase constantly over 1 min**. | | | |

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 4.

## CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L\ standard \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

## SAMPLE PREPARATION:

### 1. Sample dilution.

The amount of ethanol present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.25 and 12 µg. The sample solution must therefore be diluted sufficiently to yield a concentration of between 0.01 and 0.12 g/L.

**Dilution Table**

| Estimated concentration of ethanol (g/L) | Dilution with water | Dilution factor (F) |
|---|---|---|
| < 0.12 | No dilution required | 1 |
| 0.12-1.2 | 1 + 9 | 10 |
| 1.20-12.0 | 1 + 99 | 100 |
| 12.0-120 | 1 + 999 | 1000 |
| > 120 | 1 + 9999 | 10000 |

If the value of $\Delta A_{ethanol}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

### 2. Sample handling.

**Since ethanol is volatile, all operations should, where possible, be performed in sealed Duran® glass bottles.**

It is also necessary to show great care in pipetting, diluting and filtering solutions. Plastic tips of dispensing pipettes should be rinsed 3 times with the solution before taking the aliquot. Cuvettes and plastic tips should be rinsed 3 times with ethanol-free distilled water and dried before use.

Ensure that reagent bottles (especially the distilled water container) are sealed immediately the required volumes are removed, to minimise absorption of alcohol from the air. When setting up the assays, **do not** employ the pipette that was used to aliquot the ethanol standard or other concentrated ethanol solution.

### 3. Sample clarification.

#### a. Solutions:

***Carrez I solution.*** Dissolve 3.60 g of potassium hexacyanoferrate (II) {$K_4[Fe(CN)_6].3H_2O$} (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

***Carrez II solution.*** Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) in 100 mL of distilled water. Store at room temperature.

***Sodium hydroxide (NaOH, 100 mM).*** Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

### b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

## 4. General considerations.

**(a) Liquid samples:** clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

**(b) Acidic samples:** if > 0.1 mL of an acidic sample is to be used undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 9.0 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

**(c) Carbon dioxide:** samples containing a significant amount of carbon dioxide, such as beer, should be degassed by increasing the pH to approx. 9.0 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

**(d) Coloured samples:** an additional sample blank, i.e. sample with no ADH, may be necessary in the case of coloured samples.

**(e) Strongly coloured samples:** if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of polyvinylpolypyrrolidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

**(f) Solid samples:** homogenise or crush solid samples in distilled water and filter if necessary.

**(g) Samples containing fat:** extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask at 60°C. Adjust to room temperature and fill the volumetric flask to the mark with distilled water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents.

**(h) Samples containing protein:** deproteinise samples containing protein with perchloric acid; alternatively, clarify with Carrez reagents.

**(i) Samples containing micro-organisms:** filter samples through a 0.2 micron filter using a syringe apparatus.

## SAMPLE PREPARATION EXAMPLES:

### (a) Determination of ethanol in wine.

The ethanol concentration of white and red wine can generally be determined without any sample treatment (except dilution according to the dilution table). *Typically, for wines with 10-15% (v/v) ethanol, a dilution of 1:1,000 and sample volume of 0.1 mL are satisfactory.*

### (b) Determination of ethanol in beer, cider and alcoholic fruit juices.

After removal of carbon dioxide by increasing the pH of the solution to approx. 9 with 2 M NaOH and gentle stirring, dilute the sample according to the dilution table and analyse. *Typically, for beverages of 3-8% (v/v) ethanol, a dilution of 1:500 and sample volume of 0.1 mL are satisfactory.*

### (c) Determination of ethanol in "non-alcoholic" and "low alcohol" beers and other beverages.

After removal of carbon dioxide by increasing the pH of the solution to approx. 9.0 with 2 M NaOH and gentle stirring, dilute the sample according to the dilution table and analyse. *Typically, a dilution of 1:50 and sample volume of 0.1 mL are satisfactory.*

### (d) Determination of ethanol in spirits (whisky, brandy, etc.).

The ethanol concentration of spirits can generally be determined without any sample treatment (except dilution according to the dilution table). However, care should be taken as two dilution steps will generally be required. *Typically, for spirits of 30-60% (v/v) ethanol, a dilution of 1:10,000 and sample volume of 0.1 mL are satisfactory.*

### (e) Determination of ethanol in fruit juice, concentrates and related beverages.

The ethanol concentration of clear, neutral solutions can generally be determined without any sample treatment (except dilution according to the dilution table). When used undiluted, the pH of acidic solutions should be increased to approx. 9.0 with 2 M NaOH. Turbid liquids generally only require filtering before the dilution step. Coloured solutions are usually suitable for analysis after dilution to an appropriate ethanol concentration. However, if coloured solutions require analysis undiluted, they may need decolourising as follows: treat the solution with polyvinylpolypyrrolidone (PVPP) or activated charcoal at 2 g/100 mL and filter through Whatman GF/A glass fibre filter paper. *Typically, no dilution is necessary and sample volumes up to 0.5 mL will be required.*

### (f) Determination of ethanol in solid foodstuffs (such as liqueur chocolates).

Homogenise solid foodstuffs (~ 10 g) using a mortar or homogeniser if necessary. Add 2 g of representative material to 50 mL of ethanol-free water and stir for 30 min in a sealed Duran® bottle (heat at 60°C if necessary). Cool the extract (if necessary) and quantitatively transfer to a 100 mL volumetric flask. Dilute to the mark with ethanol-free water. Filter the turbid solution, dilute if necessary (according to the dilution table) and analyse. *Typically, a dilution of 1:10 and sample volume of 0.1 mL are satisfactory.*

### (g) Determination of ethanol in vinegar.

Generally, the analysis of vinegar only requires filtration and dilution before assay. However, the pH of samples to be analysed undiluted should be increased to approx. 9.0 using 2 M NaOH before filtration. *Typically, a dilution of 1:20 and sample volume of 0.1 mL are satisfactory.*

### (h) Determination of ethanol in jam.

Accurately weigh approx. 5 g of representative material into a 100 mL Duran® bottle and extract with 50 mL of ethanol-free water with agitation for 30 min at 60°C (with the bottle sealed). Cool the extract and, if acidic, adjust the pH to 9.0 using 2 M NaOH. Quantitatively transfer the solution to a 100 mL volumetric flask and adjust the volume to the mark with ethanol-free water. Filter turbid solutions and dilute if necessary according to the dilution table. *Typically, no dilution is necessary and sample volumes up to 0.5 mL will be required.*

### (i) Determination of ethanol in honey.

Accurately weigh approx. 10 g of representative material into a 100 mL volumetric flask containing 40 mL of ethanol-free water and stopper the flask immediately. Dissolve the honey by slight agitation at 60°C for 10 min. After cooling, adjust the volume to the mark with ethanol-free water. Filter the turbid solution and dilute if necessary according to the dilution table. *Typically, no dilution is necessary and sample volumes up to 0.5 mL will be required.*

### (j) Determination of ethanol in dairy products.

Accurately weigh approx. 10 g of representative material into a 100 mL Duran® bottle and extract with 50 mL of ethanol-free water with agitation for 30 min at 60°C (with the bottle sealed). Cool the extract and quantitatively transfer the solution to a 100 mL volumetric flask and adjust the volume to the mark with ethanol-free water. Filter turbid solutions and dilute if necessary according to the dilution table. *Typically, no dilution is necessary and sample volumes up to 0.5 mL will be required.*

**(k) Determination of ethanol in raw unpasteurised kombucha.** Remove micro-organisms by filtering through a 0.2 micron filter using a syringe apparatus. Remove residual gas from the sample by mixing on a vortex mixer for approx. 30 s. *Typically, a dilution of 1:200 and a sample volume of 0.1 mL are satisfactory.*

## REFERENCE:

Beutler, H. O. (1988). Ethanol. "*Methods of Enzymatic Analysis*" (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VI**, pp. 598-606, VCH Publishers (UK) Ltd., Cambridge, UK.

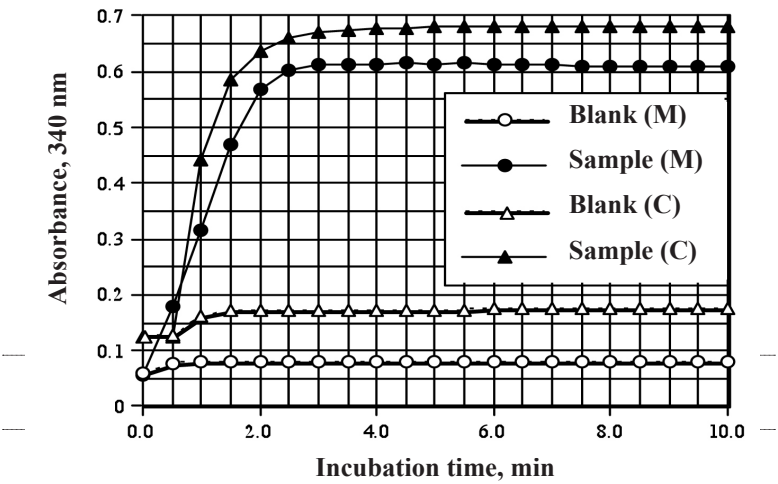


***Figure 1.*** Increase in absorbance at 340 nm on incubation of 5 µg of ethanol with alcohol dehydrogenase and aldehyde dehydrogenase in the presence of $NAD^+$. (M) Megazyme kit; (C) competitor kit.

## NOTES:

## NOTES:

## NOTES:


**Bray Business Park, Bray,**
**Co. Wicklow,**
**A98 YV29,**
**IRELAND.**

**Telephone: (353.1) 286 1220**
**Facsimile: (353.1) 286 1264**
**Internet: www.megazyme.com**
**E-Mail: info@megazyme.com**