Megazyme "Advanced" Wine Test Kits

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It is widely accepted that testing plays a pivotal role throughout the whole of the vinification process. Production of the best quality wine and minimisation of process problems, such as "stuck" fermentation or troublesome infections, ideally involves testing from before harvesting of the grapes, until bottling of the wine. Traditional methods of wine analysis, such as thin layer chromatography (TLC), distillation, and titration, can be expensive, time consuming, require elaborate equipment or specialist expertise, and frequently lack accuracy and/or specificity. However, enzymatic bio-analysis methods in the form of test kits have been developed from the 1970s onwards and today enable accurate measurement of the vast majority of analytes of interest to the winemaker (see Table 1), using just one piece of apparatus, the spectrophotometer. Grape juice and wine are ideal samples for this type of analysis, as they are homogenous liquids, are easy to manipulate, and can generally be analysed without significant preparation. Moreover, the past 20 years has seen the real price of both spectrophotometers and enzymatic bio-analysis reagents fall, while the expertise required to perform these tests has also become less demanding and at the same time more widespread. It is somewhat surprising then that since their introduction very little improvement has been made to the actual enzymatic bio-analysis reagents (i.e. the enzymes) themselves, and until recently many of the methods suffered from poor reagent stability, inconvenient reaction times, or the fact they were simply not developed for wine analysis in the first place.

Megazyme, the Company

Megazyme International Ireland Limited is a biotechnology company that has specialised in the development, manufacture, application, supply and support of enzymatic bio-analysis test kits and related products since 1989, and has always been at the forefront of this technology in the food, feed, dairy and beverage industries. In 2002, Megazyme decided to make a significant contribution to the wine industry by the development of a range of advanced enzymatic test kits. This task has now been successfully completed through the strategic and comprehensive process of identifying the limitations of all existing enzymatic bio-analysis test kits, and then using advanced techniques, such as molecular biology (Figure 1), to rapidly overcome them. Novel test kits have also been developed for analytes of emerging interest to the oenologist, such as yeast available nitrogen (YAN), or where previously enzymes were simply either not available, or were too expensive to employ, such as for D-mannitol analysis. Megazyme currently offers 64 advanced test kits, 30 of which have specific applications in the wine industry (Table 1), and the range is still growing. These new and advanced products now make enzymatic bio-analysis commercially viable for wineries of practically any size.

Figure 1. Production of recombinant enzymes via molecular biology.

Analyte	Cat. No.	Oenological Significance	Advantages of Megazyme Test Kits
Acetaldehyde	K-ACHYD	A sensory compound that adds flavour and complexity, but spoils wine at high concentrations	AlDH supplied as a stabilised suspension rather than a lyophilised powder, thus less wasted enzyme
Acetic Acid	K-ACET K-ACETAF K-ACETAK	A sensory compound that adds flavour and complexity in small amounts, but spoils wine at high concentrations. Produced naturally by yeast in small amounts and by spoilage organisms such as *Acetobacter aceti* in large quantities. This is the predominant of the acids comprising volatile acidity (VA)	All kits contain PVP to prevent tannin inhibition 1. K-ACET (manual, efficient) contains stable ACS suspension 2. K-ACETAF (auto) used to prepare very stable R1 and R2 3. K-ACETAK (auto) is a new, stable, and very rapid acetate kinase (AK) based kit with excellent linearity
Ammonia	K-AMIAR	Most important inorganic source of Yeast Available Nitrogen (YAN)	Novel enzyme employed is not inhibited by tannins, endpoint reaction time ~ 2 min. Ideal for manual and auto applications
L-Arginine	K-LARGE	Most important amino acid in grape juice with respect to YAN	Simple and rapid test kit gives sequential values for ammonia, urea and L-arginine. No tannin inhibition
L-Ascorbic Acid	K-ASCO	Present naturally in grapes and can be added as an anti-oxidant	Rapid reaction, stable reagents
Citric Acid	K-CITR	Naturally present in small amounts, large amounts indicate addition for acidification (EU limit is 1 g/L)	Contains PVP to prevent tannin inhibition. Ideal for manual and auto applications
Ethanol	K-ETOH	Produced during alcoholic fermentation. Amounts > 17.5 % (v/v) indicate supplementation	Rapid reaction, stable reagents (AlDH supplied as a stable suspension)
D-Fructose / D-Glucose	K-FRUGL K-FRGLMQ	Grape quality indicator. One of the two principle fermentable sugars of grape juice	Contains PVP to prevent tannin inhibition. Ideal for manual and auto applications. Stable reagents
Formic Acid	K-FORM	Minor wine acid	Rapid reaction, stable reagents
D-Gluconic Acid	K-GATE	Grape quality indicator for the production of certain wines	Rapid reaction, stable reagents
D-Glucose	K-GLUC K-GLUHKR/L	Grape quality indicator. One of the two principle fermentable sugars of grape juice	Choice of simple formats available, based either on glucose oxidase/peroxidase, or hexokinase/G-6-PDH
Glycerol	K-GCROL	Quality indicator of finished wine, important for “mouth-feel”	Novel tablet format offers superior stability, rapid reaction
D-Lactic Acid	K-DATE	Produced predominantly by lactic acid spoilage bacteria	Rapid reaction, stable reagents
L-Lactic Acid	K-LATE	Produced predominantly from L-malic acid during malolactic fermentation	Rapid reaction, stable reagents. Ideal for manual and auto applications
D-Malic Acid	K-DMAL	Only present in significant quantities in adulterated wine	D-MDH supplied as a stabilised suspension rather than a lyophilised powder, thus less wasted enzyme
L-Malic Acid	K-LMALR/L K-LMALAF K-LMALMQ	Grape quality indicator. Very important grape acid, converted to less acidic L-lactic acid during malolactic fermentation	All kits contain PVP to prevent tannin inhibition 1. K-LMALR/L (manual) rapid reaction 2. K-LMALAF (auto) rapid reaction, excellent linearity 3. K-LMALMQ (manual, colorimeter based)
D-Mannitol	K-MANOL	Produced by spoilage organisms from D-fructose, resulting in an undesirable “mannitol taint”	Novel kit, rapid reaction, stable reagents, simple format
Primary Amino Nitrogen (PAN)	K-PANOPA	Primary amino nitrogen (PAN) is the most important organic source of Yeast Available Nitrogen (YAN)	Novel kit, rapid reaction, stable reagents, simple format
D-Sorbitol	K-SORB	High levels indicate addition of fruit	Diaphorase supplied as a stabilised suspension rather than a lyophilised powder, thus less wasted enzyme
Succinic Acid	K-SUCC	Wine acid produced during fermentation	Rapid reaction (even at 25°C), stable reagents
Sucrose	K-SUFRG K-SUCGL	Added to increase the amount of alcohol. Use only permitted in certain situations	Choice of simple formats available, based either on glucose oxidase/peroxidase, or hexokinase/G-6-PDH
Urea	K-URAMR	Source of YAN and precursor of the carcinogen ethyl carbamate. Over-supplementation with DAP can result in elevated levels	Simple and rapid test kit gives sequential values for ammonia and urea. No tannin inhibition

Table 1. Advantages of Megazyme test kits in the measurement of key wine analytes.

The Megazyme Advantage

In general, Megazyme test kits are ideal for wine analysis because they have long shelf lives (spanning at least two vintages), rapid analysis times (often only a few minutes), simple formats, and very competitive pricing. Furthermore, each test kit is under continual scrutiny and re-evaluation by our very experienced R&D team. As all test kits have been developed by the company, our research scientists are ideally positioned to offer the very best and rapid customer support. Recently, with the customer in mind, a range of calculation aids (MegaCalc™) based on Microsoft EXCEL were developed, which enable hassle-free and rapid processing of raw absorbance data for all manual analysis test kits (Figure 2). These calculators are offered free of charge on the Megazyme website (www.megazyme.com), and can be downloaded for added convenience.

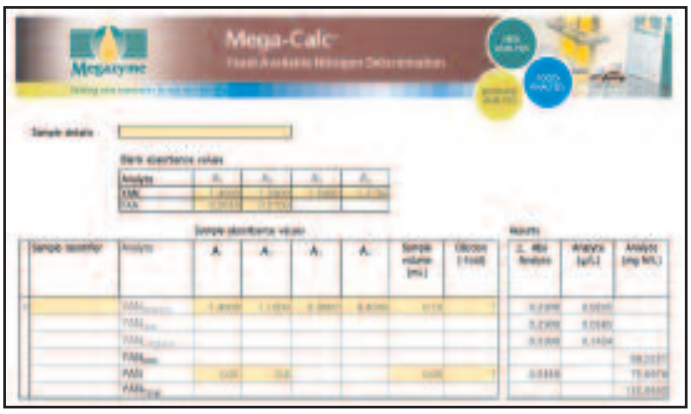

Figure 2. The **MegaCalc™** raw absorbance data processing aid for YAN determination using the L-arginine / urea / ammonia, and primary amino nitrogen test kits. Blank absorbance values are entered at the top of the page where indicated. After absorbance values are entered for the ammonia, urea, L-arginine and primary amino nitrogen reaction endpoints, the EXCEL based program automatically calculates values for these individual analytes in g/L and also a total YAN value in mg N/L.

What Laboratory Equipment is Required?

In addition to a spectrophotometer or in some cases a colorimeter, very little laboratory equipment is actually required. A set of pipettes with disposable plastic tips to accurately dispense volumes between 20 µL and 1000 µL is necessary, along with plastic cuvettes or glass test-tubes (depending on the absorbance measuring device used), and basic filtering or other simple sample treatment apparatus may also be necessary.

Advanced Features of Megazyme Test Kits

In addition to the general advantages of Megazyme test kits as detailed above, many products possess special features resulting from their custom optimisation for wine analysis. These features, briefly detailed in the last column of Table 1, are discussed in detail below:

1. Reagent Stability

(a) Stabilised Enzyme Suspensions: Although the shelf life of many test kits as purchased appears satisfactory, once used for the first time, certain enzyme components can be very labile. For example, all acetic acid kits except that supplied by Megazyme (K-ACET), employ a freeze-dried enzyme component, acetyl-coenzyme A synthetase (ACS). Once reconstituted, this enzyme can only be used within a very limiting time period, e.g. just 5 days in some cases, after which it must be discarded. This severe limitation was identified by Megazyme and an ACS identified that could be stabilised by the optimum and preferred technique of ammonium sulphate precipitation. Not only has this resulted in the Megazyme kit being more user friendly and flexible, but it also, and more importantly, gives the kit a much longer shelf life ***during use*** of > 2 years. The same stabilisation technique was also employed in the optimisation of the acetaldehyde (K-ACHYD), D-malic acid (K-DMAL), and sorbitol (K-SORB) kits.

(b) Reagent Tablets: Some test kits require the use of labile substrates such as ATP. The glycerol kit is a good example, and the stability of prepared reagent for some competitor kits is very poor, e.g. just 4 days. Megazyme's glycerol kit (K-GCROL), however, contains these labile components in a novel dry tablet form, and thus their stability is no longer an issue, as only sufficient reagent for the analyses in hand need be prepared. Additionally, in order to ensure maximum stability of the reagents, the tablets are enclosed within special "desiccant impregnated" vials. These vials, designed especially for this application, are also used with other reagent tablets, such as in the ammonia (K-AMIAR), citric acid (K-CITR), L-arginine / urea / ammonia (K-LARGE), primary amino nitrogen (K-PANOPA), and urea / ammonia (K-URAMR) kits.

(c) Stabilisation System: Acetic acid is a major quality indicator of wine and is monitored throughout the winemaking process. However, reagent prepared for auto-analyser applications using ACS based kits has very poor stability (as little as one day even when refrigerated), owing to an increasing absorbance value. This phenomenon was researched at Megazyme and a way found to overcome this major limitation (Figure 3), giving reagent R1 prepared from K-ACET or K-ACETAF a stability of up to 7 days at 4°C.

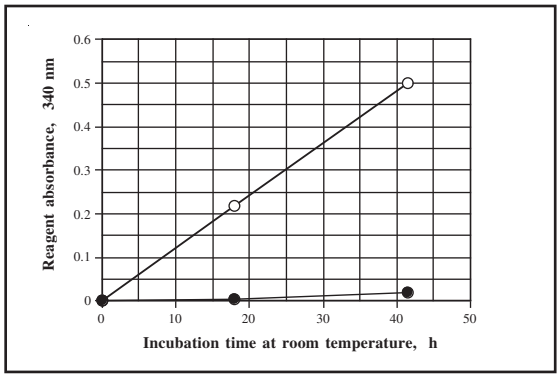

Figure 3. Stabilised advanced acetic acid kit reagent. Both basic (open circles) and stabilized (closed circles) acetic acid kit reagents were prepared for auto-analyer applications and incubated at room temperature (~ 22.5°C). At time points (0 h, 18 h and 41.5 h) the absorbance of the reagents was determined. Over this time period, equivalent to ~ 6 days at 4°C, the absorbance of the stabilized reagent at 340 nm only increased by 0.018, while that of the unstable basic reagent had increased by 0.495.

2. Sample Interference Prevention

(a) Enzyme Selection: Most competitor products are based on the use of enzymes, many of animal origin, that were developed in some cases as far back as the 1960s. Although such enzymes generally perform satisfactorily, problems can arise when analysing wine. One example is the use of beef liver glutamate dehydrogenase in the measurement of ammonia. This enzyme can be significantly inhibited by tannins leading to either long or incomplete reactions. Using the molecular biology approach, Megazyme rapidly screened many different recombinant (not of animal origin) enzymes in order to identify one that does not exhibit tannin inhibition. This approach was successful, and the result is a very rapid (~ 3 min; Figure 4), and reliable advanced ammonia test kit (K-AMIAR).

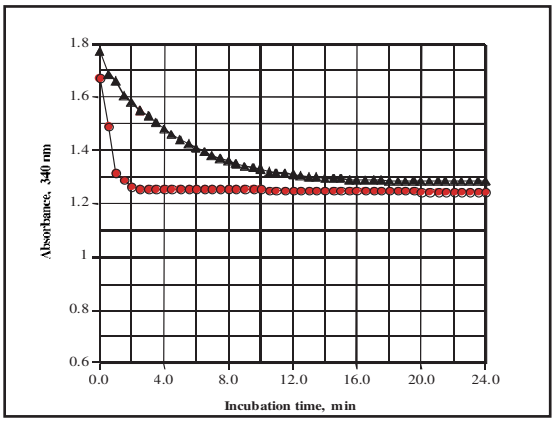

Figure 4. Analysis of red wine for ammonia using beef liver and recombinant GlDH. The same amount of red wine was analysed using a traditional ammonia test kit employing beef liver GlDH (closed black triangles) and a rapid advanced product from Megazyme employing a recombinant enzyme (closed red circles). The end of the reaction is indicated when no further fall in absorbance occurs, i.e. after only ~ 3 min with the Megazyme kit.

(b) Inclusion of Polyvinylpyrollidone (PVP):
It is well known that tannins can cause inhibition of test kit enzymes, and that this can affect the obtained results. It is also known that PVP can be employed to prevent/minimise such interference. However, other test kit manufacturers do not appear to include PVP in their products, and instead it is left to the customer to add it into the assay system. Thus for added convenience and assurance, Megazyme has formulated optimised quantities of PVP into the relevant wine test kits, namely the acetic acid

(K-ACET, K-ACETAF, K-ACETAK), L-malic acid (K-LMALR, K-LMALL, K-LMALAF), D-fructose/D-glucose (K-FRUGL), and citric acid (K-CITR) kits (Table 1).

3. Simplified Formats

Most test kit manufacturers offer just one single test kit for each analyte, e.g. for L-malic acid. However, there are actually many different types of user, each with very different requirements and available resources. Megazyme thus developed specific test kits with each type of end-user in mind, leading to simple and easy to use formats that require no adaptation by the customer:

(a) Manual Format Test Kits: This is the most popular method of analysis for the average winery possessing a standard spectrophotometer and a test kit is available for each analyte (Table 1).

Figure 5. The MegaQuant™ Meter and reagents for L-malic acid analysis.

(b) MegaQuant™ Format Test Kits: Testing by small wineries and grape growers was until recently very difficult, as such companies generally do not even possess a basic spectrophotometer, and thus must rely either on traditional techniques or central laboratories for their analytical requirements. However, in response to this pressing requirement, a novel product was recently launched called MegaQuant™, that allows rapid and specific measurement of both L-malic acid (K-LMALMQ, G-LMALMQ) and D-glucose plus D-fructose (K-FRGLMQ, G-FRGLMQ), without the requirement for an expensive spectrophotometer or other specialised laboratory apparatus and skills (Figure 5).

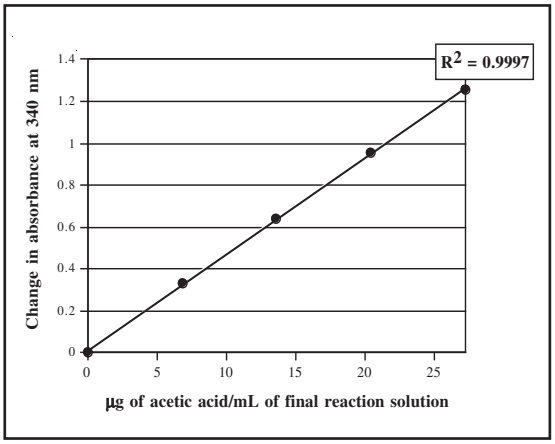

Figure 6. Linear calibration curves for Megazyme's stable acetate kinase format acetic acid kit. After incubation of prepared reagents for 40.5 h at room temperature (~ 22.5°C), Megazyme's advanced acetate kinase format acetic acid kit gave an essentially identical linear calibration curve to that obtained at time 0 h (graphs are superimposed).

(c) Analyser Format Test Kits: It is common practice for analysts to purchase standard test kits and adapt them to a particular auto-analyser. However, this takes time and the form and quantities of the individual components is often less than optimal, leading to significant waste or inconvenience. Megazyme thus developed optimised "analyser formats" for existing manual kits that are simple to use and result in no wastage of kit reagents. Where existing kits could not be employed, new kits were developed as necessary. Analyser formats are now available for acetic acid (K-ACETAF, K-ACETAK), ammonia (K-AMIAR), citric acid (K-CITR), D-fructose and D-glucose (K-FRUGL), glycerol (K-GCROL), L-lactic acid (K-LATE) and L-malic acid (K-LMALAF) analyses, and offer excellent prepared reagent stability and performance linearity (Figure 6). In addition to test kits, Megazyme also offers a range of individual kit components for those customers who wish to formulate their own reagents. This is becoming increasingly popular by the experienced auto-analyst, and rapid assistance is available from the technical support team at Megazyme for such custom method developments.

4. Novel Method Development

(a) Yeast Available Nitrogen (YAN): Before alcoholic fermentation can begin, the oenologist must establish the nutritional status of the juice, in order to assess whether supplementation is required. This very important process includes the estimation of YAN. This is perhaps the most crucial parameter measured during the vinification process,[1] as too little YAN may result in sluggish or stuck fermentation, with potentially disastrous consequences for wine quality, while unnecessary supplementation with di-ammonium phosphate (DAP), can result in a reduction in wine stability and the production, in certain cases, of the known carcinogen, ethyl carbamate.[2] YAN is comprised of three principle components, free ammonium ions, primary amino nitrogen (PAN, from amino acids) and the contribution made by the side-chain of L-arginine. To enable YAN to be quantified accurately, two test kits were developed; the primary amino nitrogen kit (K-PANOPA) is a simplified and optimised version of the original Dukes and Butzke method,[3] while the L-arginine / urea / ammonia kit (K-LARGE) is a rapid sequential method based on the novel glutamate dehydrogenase employed in the ammonia kit (K-AMIAR), that can be performed in < 16 min (Figure 7).

(b) Acetate Kinase Based Acetic Acid Kit: In response to calls from the wine industry, Megazyme developed an advanced and very rapid test kit for acetic acid based on the enzyme acetate kinase. As can be appreciated from Figure 6, this product, that has already become very popular, offers both excellent stability and linearity.

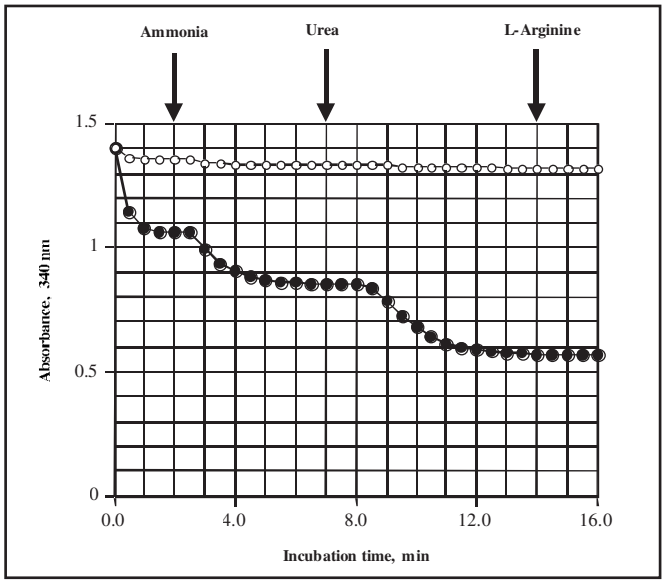

Figure 7. Performance of a sequential rapid enzymatic test kit for L-arginine, urea and ammonia. The sequential reaction shown demonstrates that L-arginine, urea and ammonia can be determined using the same cuvette (closed circles), with reference to a blank containing no sample (open circles). Individual endpoints for ammonia, urea and L-arginine, after the addition of recombinant GlDH, urease and arginase, respectively, are indicated by arrows. The total analysis time is < 16 min.

Future Test Kit Development

Megazyme remains committed to working with the oenologist to develop novel test kit solutions to emerging challenges presented by the modern wine industry.

References

1. Martin, O., Brandriss, M. C., Schneider, G. & Bakalinsky, A.T. (2003). Improved anaerobic use of arginine by *Saccharomyces cerevisiae*. *App. Env. Microbiol.* **69**, 1623-1628.
2. Butzke, C. E. & Bisson, L. F. (1997). USFDA Center for Food Safety and Applied Nutrition, "Ethyl Carbamate Preventative Action Manual".
3. Dukes, B. & Butzke, C. (1998). Rapid determination of primary amino acids in grape juice using an *o*-phthaldialdehyde / N-acetyl-L-cysteine spectrophotometric assay. *Am. J. Enol.Vitic.* **49**, 125-134.

April, 2006

Megazyme Assay Kits, Enzymes and Reagents for the Wine Industry

Since 1989, Megazyme has developed a world-wide reputation for the purity of the enzymes it supplies and the quality and innovative nature of the diagnostic kits it develops and supplies.

Megazyme now offers the largest range of enzymatic test kits for food, feed and beverage analysis, with the following kits, enzymes and reagents being specifically tailored for the wine industry:

DIAGNOSTIC KITS

Cat. No.	Analyte	No. of Assays
K-ACHYD	Acetaldehyde	50
K-ACET	Acetic Acid	53
K-ACETAF	Acetic Acid (AF)	550
K-ACETAK	Acetic Acid (AK)	550
K-AMIA	Ammonia	100
K-AMIAR	Ammonia (Rapid)	96
K-LARGE	L-Arginine/Urea/Ammonia (Rapid)	50
K-ASCO	L-Ascorbic Acid	40
K-CITR	Citric Acid	72
K-ETOH	Ethanol	60
K-FRUGL	D-Fructose/D-Glucose	110
K-FRGLMQ	D-Fructose/D-Glucose (MegaQuant™ Format)	60
K-GATE	D-Gluconic Acid	60
K-GLUC	D-Glucose (GOPOD)	600
K-GLUHK	D-Glucose (HK Format)	110 or 220
K-GCROL	Glycerol	70
K-ISOC	D-Isocitric Acid	100
K-DLATE	D-/L-Lactic Acid	100
K-DATE	D-Lactic Acid	50
K-LATE	L-Lactic Acid	50
K-DMAL	D-Malic Acid	100
K-LMAL	L-Malic Acid	58 or 116
K-MALAF	L-Malic Acid (AF)	1,380
K-LMALMQ	L-Malic Acid (MegaQuant™ Format)	60
K-MANOL	D-Mannitol/L-Arabitol	60
K-PANOPA	Primary Amino Nitrogen	100
K-SORB	D-Sorbitol/Xylitol	58
K-SUCC	Succinic Acid (Rapid)	20
K-SUFRG	Sucrose/D-Fructose/D-Glucose	100
K-SUCGL	Sucrose/D-Glucose	250
K-TSTA	Total Starch	100
K-URAMR	Urea/Ammonia (Rapid)	100

REAGENTS

Cat. No.	Product	Amount
R-ATP20	Adenosine 5'-triphosphate	20 g
R-ATP100	Adenosine 5'-triphosphate	100 g
R-NAD5	β-NAD^+	5 g
R-NAD25	β-NAD^+	25 g
R-NADP2	β-$NADP^+$	2 g
R-NADP10	β-$NADP^+$	10 g
R-NADH2	β-NADH	2 g
R-NADH10	β-NADH	10 g

ENZYMES

E-ACKEC	Acetate kinase	100,000 U
E-ACSBS	Acetyl-CoA synthetase	250 U
E-GPDH5	Glucose-6-P dehydrogenase	5,000 U
E-GPDH25	Glucose-6-P dehydrogenase	25,000 U
E-GLDHEC	Glutamate dehydrogenase (not inhibited by tannins)	10,000 U
E-GOTEC	Glutamate-oxaloacetate transaminase	5,000 U
E-HEX10	Hexokinase	10,000 U
E-HEX50	Hexokinase	50,000 U
E-HKGDH	Glucose-6-P dehydrogenase (210 U/mL) + Hexokinase (420 U/mL)	10 mL
E-DLDHLM	D-Lactate dehydrogenase	15,000 U
E-LMDHEC	L-Malate dehydrogenase	50,000 U
E-PGIEC	Phosphoglucose isomerase	10,000 U
E-PGIECB	Phosphoglucose isomerase	50,000 U

GENERAL

G-LMALMQ	MegaQuant™ Meter + L-Malic Acid reagents	(60 tests)
G-FRGLMQ	MegaQuant™ Meter + D-Glucose/D-Fructose reagents	(60 tests)
GC-GLY200	Glycylglcine buffer	200 g

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