

**L-MALIC ACID
(LIQUID READY™)
VALIDATION REPORT**

**SKU: 700007622
K-LMLQR**

11/24



INTRODUCTION:

The L-Malic Acid Assay Kit (Liquid Ready™) is a robust, quick and easy method for the measurement of L-Malic Acid in various matrices and is fully automatable for high throughput analysis of samples. Data presented in this report validates that this method is fit for the purpose intended.

RECOMMENDATIONS FOR ANALYSIS:

Please reach out to your local sales representative or to the technical team should you require any assistance, particularly in relation to assay troubleshooting, data analysis, additional matrix testing and application support in relation to automated analyzers.

- This test should only be carried out by trained laboratory personnel. The product instructions must be followed to ensure an accurate and robust result.
- Store the kit at 2 - 8 °C.
- Ensure all kit components come to room temperature 20 - 25 °C before use.
- Use the contents of bottles 1, 2 & 3 as supplied.
- Use a repetitive pipettor to add reagents to the cuvette for increased accuracy.
- The reagent blank value must be determined once for each run and subtracted from each sample result.
- Users should perform matrix validation experiments prior to routine use. This process will highlight any problematic matrices encountered.
- The Carrez Clarification kit (K-CARREZ; 700004270) is recommended for fat removal and deproteinization if necessary.
- Use separate tips for each sample extract and control solutions to avoid cross-contamination. Additionally, pre-flush the tip before pipetting.
- When testing solid samples, ensure a representative portion is taken and homogenized before weighing.

EQUIPMENT (RECOMMENDED):

Matrix Extraction

1. Syringe filters (e.g. AGILENT™ Nylon or equivalent, 0.2 micron); alternatively, eppendorf tubes and centrifuge.
2. Sartorius™ grade 292 filter paper or equivalent.
3. Blender (e.g. Nutribullet™).
4. Analytical balance and spatulas.
5. Wide-mouth bottles (e.g. Duran® bottles, 100 mL and 1 L).
6. Glass graduated cylinders (25 mL, 50 mL) and volumetric flasks (100 mL).
7. pH-meter or equivalent.
8. Refrigerator or ice bath (or equivalent).
9. Stir plate.
10. Glass rod.

SUMMARY OF PERFORMANCE DATA:

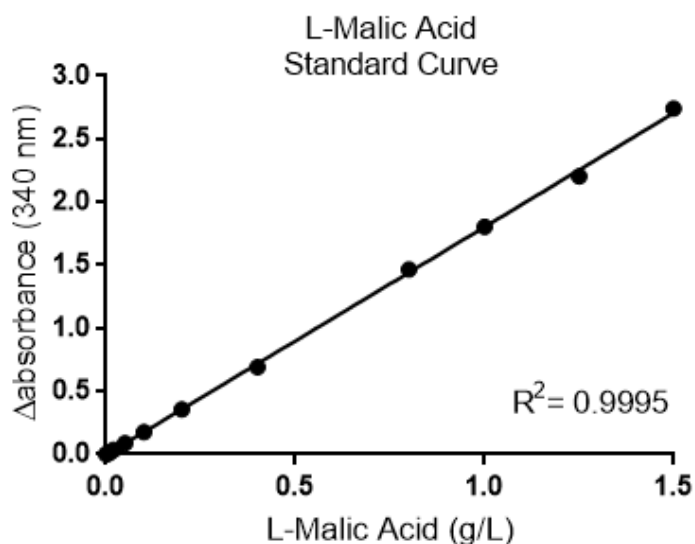
All testing was performed using the standard manual assay described in the product instructions. Results are summarised in the table below:

| | |
|----------------------------------|---|
| Recommended working range | 0.020 g/L – 1.000 g/L |
| Limit of Detection (LOD) | 0.005 g/L |
| Limit of Quantification (LOQ) | 0.016 g/L |
| Limit of Precision | 0.020 g/L |
| Specificity | L-Malic Acid |
| Bias % | -0.68% to 0.76% |
| Acceptable Recovery of Standards | 95% - 105% |
| Stability Studies | 24 months shelf life from date of manufacture, see product label for expiry |
| | Kit performance maintained after 3 freeze-thaw cycles |
| Robustness | < 5 mins to reach completion at 20°C, 25°C and 37°C |
| Repeatability | CV < 5% for pure L-Malic Acid samples |
| | CV < 15% for a range of matrices tested |
| Selectivity/Cross-reactivity | No cross reactivity observed with interfering agents tested |
| Matrix Interference | Recovery between 95% - 105% in red wine, white wine, apple juice, beer, sour jellies and Broccoli |

LINEARITY AND WORKING RANGE:

The recommended linear measurement range is from 0.020 g/L to 1.0 g/L of L-Malic Acid. Samples with concentrations up to 1.5 g/L can still be analyzed without dilution, however assay repeatability/robustness may be affected at concentrations above 1.0 g/L. Samples containing higher levels of analyte can be diluted with distilled water before measurement. Values below 0.02 g/L will not fall within the acceptance criteria.

| L-Malic Acid (g/L) | $\Delta_{\text{absorbance}}$ (340 nm) | Recovery (%) |
|--------------------|---------------------------------------|--------------|
| 0 | 0 | 0 |
| 0.005 | 0.005 | 56.40 |
| 0.01 | 0.016 | 85.77 |
| 0.02 | 0.035 | 98.11 |
| 0.05 | 0.088 | 97.50 |
| 0.1 | 0.176 | 97.55 |
| 0.2 | 0.355 | 98.21 |
| 0.4 | 0.691 | 95.66 |
| 0.8 | 1.466 | 101.4 |
| 1.0 | 1.803 | 99.75 |
| 1.25 | 2.204 | 97.55 |
| 1.5 | 2.740 | 101.1 |



LIMIT OF DETECTION, QUANTIFICATION AND PRECISION

The LOD is the lowest concentration of the analyte that can be detected by the method. This was determined by testing 20 replicates of the blank (i.e. adding 100 μL of water instead of sample). The $\Delta A_{\text{Limit of Detection}}$ is calculated as $3.3 \times s'0$; where $s'0$ is the standard deviation of a number of samples $\Delta_{\text{Absorbance}}$ reading.

The LOQ is the lowest level at which the kit's performance is acceptably repeatable. This was determined by testing 20 replicates of the blank (i.e. adding 100 μL of water instead of sample). The $\Delta A_{\text{Limit of Quantification}}$ is calculated as $kQ \times s'0$; where $s'0$ is the standard deviation of a number of samples $\Delta_{\text{Absorbance}}$ reading. The IUPAC default value for kQ is 10. Refer to the product instructions for (g/L) calculation.

The Limit of Precision (g/L) is the analyte concentration at which it was experimentally determined that acceptable recoveries ($\pm 5\%$) are achieved. It was assessed by measuring 12 replicates of the lowest analyte concentrations, starting at LOQ level.

| $\Delta A_{\text{Limit of Detection}}$ | Limit of Detection (g/L) | $\Delta A_{\text{Limit of Quantification}}$ | Limit of Quantification (g/L) | Precision Limit (g/L) |
|--|--------------------------|---|-------------------------------|-----------------------|
| 0.0093 | 0.005 | 0.0283 | 0.016 | 0.020 |

Note: The above detection limits were calculated based on assay concentration (i.e. samples post-extraction). The dilution used in pre-treatment must be accounted for when establishing the detection limits for specific samples.

TRUENESS AND BIAS

The Trueness of the L-Malic Acid Assay Kit (Liquid Ready™) was tested using validated aqueous L-Malic Acid standards. The *bias* is the comparison of the mean of the results (X) achieved using the standard manual protocol with a suitable validated material.

- Relative Bias is calculated in percent as: $b(\%) = (X - X_{ref}) / X_{ref} \times 100$

| Validated material (g/L) | Replicates, n | % CV | % Recovery | %Bias |
|--------------------------|---------------|-------|------------|--------|
| 0.020 | 12 | 3.017 | 99.30 | -0.679 |
| 0.500 | 12 | 0.456 | 100.8 | 0.760 |
| 1.000 | 12 | 0.904 | 100.4 | 0.648 |

Recovery of sample solutions fell within the acceptance criteria of $100 \pm 5\%$ and %CV below 5%. The *bias* for this method ranges from -0.679% to 0.648%.

INTERFERENCE AND SELECTIVITY

Selectivity

The assessment of the selectivity of this method towards L-Malic Acid in the presence of interfering compounds was tested by spiking a set concentration of interfering agent with a known concentration of validated aqueous L-Malic Acid standard and assessing the recovery. Concentrations of interfering agent were selected based on what were considered to be levels likely to be found in relevant samples.

NOTE: Interfering substances in the sample being analyzed 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 L-Malic Acid to the sample in the initial extraction steps.

| Compound tested | Concentration of interferent in assay (g/L) | Recovery L-Malic Acid (%) |
|-------------------|---|---------------------------|
| D-Glucose | 200 | 99.6 |
| D-Fructose | 200 | 103.1 |
| D-Galactose | 10 | 99.6 |
| Sucrose | 200 | 99.8 |
| Lactose | 20 | 99.6 |
| Glycerol | 10 | 100.1 |
| Sorbitol | 200 | 100.0 |
| L-Tartaric acid | 10 | 99.3 |
| Citric acid | 5 | 100.1 |
| Maleic acid | 5 | 100.9 |
| Succinic acid | 5 | 99.8 |
| Galacturonic acid | 10 | 99.9 |
| D/L-Lactic acid | 20 | 99.0 |
| Acetic acid | 50 | 99.5 |
| Ascorbic acid | 50 | 97.5 |
| Sodium chloride | 100 | 100.7 |
| Ethanol | 120 | 100.2 |

All recoveries were shown to be within the acceptance criteria of $100 \pm 5\%$. The analysis shows that there is no cross-reactivity found with any of the tested substances.

Matrix Interference

The assessment of the interference of this method when using specific complex matrices was tested by spiking extracted samples with a known concentration of validated aqueous L-Malic Acid standard and assessing the recovery.

NOTE: All matrices were prepared according to the sample preparation methods described in the product instruction disclosed in the product webpage

| Matrix tested | Replicates, n | L-Malic Acid Spike Recovery (%) |
|---------------|---------------|---------------------------------|
| Red Wine | 2 | 99.53 |
| White Wine | 2 | 98.45 |
| Beer | 2 | 98.32 |
| Apple Juice | 2 | 100.1 |
| Sour Jellies | 2 | 98.79 |
| Broccoli | 2 | 99.71 |

Five matrices were tested; all samples fall within 95-105% recovery. Furthermore, the average %CV for all spike recoveries was 0.66% which demonstrates the precision and specificity of this method.

ROBUSTNESS AND STABILITY

Storage Temperature

To assess the storage stability of the test kit components, all kit components were stored at 4°C. Real time monthly performance and enzyme activity testing was conducted, and slope predictions were used to estimate the product shelf life.

| Storage Temperature | Reagent Tested | Stability Data |
|---------------------|----------------|---|
| 4°C | Reagent 1 | 24 months shelf life from date of manufacture, see product label for expiry |
| | Reagent 2 | |

The storage robustness of this kit was also tested by performing three freeze-thaw cycles. All kit components were frozen at -20°C overnight and allowed to thaw before testing. This freeze-thaw cycle was repeated 3 times using the same components.

| L-Malic Acid Recovery (g/L) | | | | |
|-----------------------------|-------|-----------|-----------|-----------|
| Expected L-Malic Acid (g/L) | T0 | 1st Cycle | 2nd Cycle | 3rd Cycle |
| 0.020 | 0.019 | 0.020 | 0.020 | 0.020 |
| 0.500 | 0.509 | 0.521 | 0.512 | 0.514 |
| 1.000 | 1.016 | 1.035 | 1.011 | 1.003 |

Recovery of sample solutions was within the acceptance criteria of $100 \pm 5\%$. ANOVA evaluation of the freeze-thaw

cycles shows that there is no significant difference in assay performance over three consecutive freezing cycles of K-LMLQR reagents.

Assay Temperature

Enzymatic test kits are known to be subject to variation when tested under different environmental conditions; temperature being a key parameter that influences reaction rate and analyte recovery. The L-Malic Acid Assay kit (Liquid Ready™) was tested using different reaction incubation temperatures (20°C, 25°C and 37°C); recoveries and reaction times were analyzed.

| Expected L-Malic Acid (g/L) | L-Malic Acid Recovery (g/L) | | |
|-----------------------------|--|-------|-------|
| | 20°C | 25°C | 37°C |
| 0.020 | 0.019 | 0.020 | 0.019 |
| 0.500 | 0.509 | 0.511 | 0.510 |
| 1.000 | 1.016 | 1.011 | 1.012 |
| Reaction Time | 2 min | 2 min | 2 min |
| <i>Recommended time</i> | <i>5 minute reaction time is recommended</i> | | |

ANOVA evaluation of the effect of temperature in assay shows that there is no significant difference in recovery at the temperatures tested. All recoveries were found to be within acceptance criteria of $100 \pm 5\%$. The recommended reaction time for K-LMLQR is 5 minutes.

PRECISION AND REPEATABILITY

Precision

Precision is a measure of the variability in results, on different days and by different analysts, over period of time and using different lots of the test kit. The precision of the L-Malic Acid Assay kit (Liquid Ready™), was investigated using validated aqueous L-Malic Acid samples

| L-Malic Acid (g/L) | Replicates, n | Mean (g/L) | Standard Deviation | %CV |
|--------------------|---------------|------------|--------------------|-------|
| 0.020 | 24 | 0.020 | 0.0008 | 4.114 |
| 0.500 | 24 | 0.513 | 0.0032 | 0.633 |
| 1.000 | 24 | 1.017 | 0.0113 | 1.108 |

All recoveries were shown to be within acceptance criteria of $100 \pm 5\%$ and %CV below 5%. This demonstrates that the method is precise and repeatable when using validated aqueous L-Malic Acid standards.

NOTE: Users should perform matrix validation work prior to routine use. This process will highlight any problematic matrices encountered. If you have questions about these or other matrices, please contact your local sales representative for support.

Matrix Repeatability

Matrix repeatability was tested by one analyst over three days using a range of selected matrices. Sample preparation was performed daily to assess precision of analyte extraction as well as precision of the test method. All sample preparation were performed as per the extraction method stated in the product instruction.

| Matrix | Replicates, n | Mean result (g/L or g/100g) | Standard Deviation | %CV |
|--------------|---------------|-----------------------------|--------------------|-------|
| Red wine | 6 | 0.032 | 0.001 | 3.834 |
| White wine | 6 | 1.832 | 0.016 | 0.861 |
| Apple juice | 6 | 4.494 | 0.148 | 3.285 |
| Beer | 6 | 0.084 | 0.005 | 6.407 |
| Sour jellies | 6 | 0.264 | 0.030 | 11.27 |
| Broccoli | 6 | 0.114 | 0.005 | 3.960 |

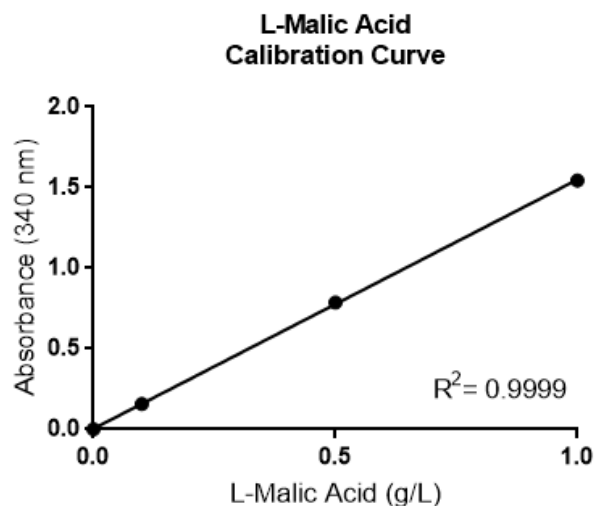
All sample analysis resulted in %CV values of <12%. Very low natural amounts of L-Malic Acid in matrices still resulted in good estimation for precision of extraction. This demonstrates that the method is precise and repeatable when testing complex matrices.

METHOD AUTOMATION:

The L-Malic Acid Liquid Ready™ kit was designed for biochemistry analyzers. It is easily adapted to any instrument. L-Malic Acid content can be determined in a single test using a cubic spline fit.

NOTE: For each batch of samples that is applied to the determination of L-Malic Acid, a calibration curve must be performed concurrently using the same batch of reagents.

| L-Malic Acid (g/L) | Replicates, n | Mean (g/L) | Standard Deviation | %CV |
|--------------------|---------------|------------|--------------------|-------|
| 0.1 | 4 | 0.099 | 0.0008 | 0.838 |
| 0.5 | 4 | 0.500 | 0.0009 | 0.173 |
| 1 | 4 | 1.001 | 0.0059 | 0.590 |



SERVICES AND TECHNICAL SUPPORT:

Please reach out to your local sales representative should you require any assistance, particularly in relation to:

- Troubleshooting
- Data analysis
- Additional matrix testing
- Application support in relation to automated analyzers.

Supporting documents can be found in the product page:

- Product Instruction
- L-Malic Acid Liquid Ready™ Quick Reference Guide
- Mega-Calc™ L-Malic Acid Liquid Ready™
- Safety data sheets (SDS)
- Certificate of analysis (CoA)



Contact us for more information: neogen.com/contact

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