ETHANOL
(LIQUID READY™)

VALIDATION REPORT

SKU: 700007710
K-ETOHLQ

09/25

Neogen Corporation, 620 Lesher Place, Lansing, MI 48912 USA | neogen.com

INTRODUCTION:

The Ethanol Assay Kit (Liquid Ready) is designed to be a robust, quick and easy method for the measurement of Ethanol 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 analysers.

- This test should only be carried out by trained laboratory employees. The product instructions must be followed to help ensure an accurate 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 of repetitive pipettor is recommended to reduce the risk of pipetting error.
- The reagent blank value must be determined once for each set of determinations 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 assay is very sensitive.** Ethanol from air (e.g. disinfection or cleaning agents) causes creep reactions and may impact results. It is necessary to run the assay in an ethanol-free environment, or to work with closed cuvettes.
- The Carrez Clarification kit (K-CARREZ; 700004270) is recommended for fat removal and deproteinization if necessary.
- Use separate pipette tips for each sample extract and control solutions to reduce the risk of cross-contamination. Additionally, pre-flush the tip before pipetting.
- When testing solid samples, ensure a representative portion is homogenized before weighing.

EQUIPMENT (RECOMMENDED):

1. Positive displacement pipettor (e.g. Eppendorf Multipette® with Combitip®) or micro-pipettors (e.g. Gilson® Pipetman®).
2. Macro-cuvettes (e.g. BRAND® PMMA, 1 cm pathlength).
3. Cuvette stoppers/caps or sealing film (e.g. Parafilm™).
4. Heat block incubator (or equivalent) for cuvettes (not required if ambient temperature is in the range of 20-37°C).
5. Spectrophotometer capable of reading at 340 nm.
6. pH-meter.
7. Syringe filters (0.2 micron, e.g. AGILENT® Nylon or equivalent).
8. Beaker (e.g. BRAND 50 mL capacity).
9. Glass rod (e.g. Aldrich® stirring rods).
10. Glass Test Tubes (e.g. Pyrex® 16 mm x 100 mm).
11. 2M Sodium Hydroxide (e.g. Sigma-Aldrich® Cat. No. P4494).

SUMMARY OF PERFORMANCE DATA:

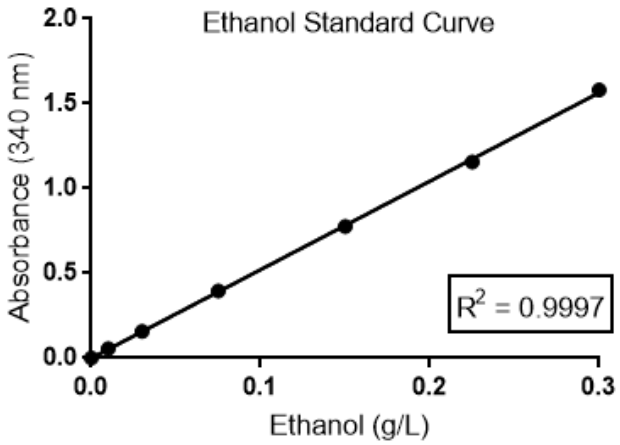
All testing was performed using the standard manual assay described in the product instructions. Results are summarized in the table below:

Recommended working range	0.010 g/L – 0.300 g/L
Limit of Detection (LOD)	0.0014 g/L
Limit of Quantification (LOQ)	0.0044 g/L
Limit of Precision	0.010 g/L
Specificity	Ethanol
Bias %	-0.9% to 1.7%
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	< 7 mins to reach completion at 20^{o}C, 25^{o}C and 37^{o}C
Repeatability	CV < 5% for pure Ethanol samples
	CV < 5% for a range of matrices tested (red wine, white wine, alcohol-free beer, ginger beer, alcohol-free gin, tomato juice and kombucha).
Selectivity/Cross-reactivity	No interferents found
Matrix Interference	Recovery between 95% - 105% in red wine, white wine, alcohol-free beer, ginger beer, alcohol-free gin, tomato juice and kombucha.

LINEARITY AND WORKING RANGE:

The recommended linear measurement range is 0.010 g/L to 0.300 g/L. Samples containing analyte concentrations above this range should be appropriately diluted with distilled water prior to analysis to support accurate quantification. Samples containing less than 0.010 g/L Ethanol will fall outside the validated range and will not meet the acceptance criteria.

Ethanol (g/L)	$\Delta_{absorbance}$ (340 nm)	Recovery (%)
0.000	0.000	-
0.010	0.055	101.44
0.030	0.158	100.36
0.075	0.395	100.15
0.150	0.775	98.21
0.225	1.156	97.68
0.300	1.580	100.15



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 $\Delta A_{Limit\ of\ Detection}$ is calculated as 3.3 x s'0; where s'0 is the standard deviation of a number of samples $\Delta_{Absorbance}$ 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 $\Delta A_{Limit\ of\ Quantification}$ is calculated as kQ x s'0; where s'0 is the standard deviation of a number of samples $\Delta_{Absorbance}$ reading. The IUPAC default value for kQ is 10.

The Limit of Precision, expressed in g/L, refers to the lowest analyte concentration at which acceptable recoveries (±5%) are consistently achieved under routine conditions. This parameter was determined experimentally by analyzing decreasing concentrations of the analyte, beginning at the limit of quantification (LOQ). The assessment focused on identifying the lowest concentration level that still met the predefined recovery criteria.

$\Delta A_{Limit\ of\ Detection}$	Limit of Detection (g/L)	$\Delta A_{Limit\ of\ Quantification}$	Limit of Quantification (g/L)	Precision Limit (g/L)
0.008	0.0014	0.023	0.0044	0.010

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 Ethanol Assay Kit (Liquid Ready) was evaluated using validated aqueous standards. Trueness was assessed by comparing the mean result (X), obtained through the standard manual protocol, with a suitable reference material.

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

Reference material (g/L)	Replicates, n	% CV	% Recovery	% *Bias*
0.010	16	1.79	101.7	1.69
0.750	16	0.80	101.1	1.08
1.500	16	0.83	99.1	-0.91

The recovery of the sample solutions fell within the acceptance criteria of 100 ± 5%, with a coefficient of variation (%CV) below 5%. The calculated bias for the method ranged from -0.91 % to 1.69 %, indicating acceptable agreement with the reference standard.

INTERFERENCE AND SELECTIVITY:

Selectivity

The selectivity of the method for Ethanol was evaluated in the presence of potential interfering compounds. This was achieved by spiking a fixed concentration of each interfering agent into a validated aqueous standard containing a known concentration of Ethanol and assessing the recovery.

NOTE: 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 D-Glucose to the sample in the initial extraction steps.

Compound tested	Concentration of interferent in assay (g/L)	Recovery of Ethanol (%)
D-Glucose	200	101.1
D-Fructose	200	101.0
Sucrose	200	99.6
Lactose	20	100.2
Glycerol	10	99.9
Sorbitol	200	99.9
L-Malic acid	10	100.9
L-Tartaric acid	10	98.7
Citric acid	5	99.0
Ascorbic acid	50	101.6
Galacturonic acid	10	98.8
D/L-Lactic acid	10	98.4
Acetic acid	50	98.6

The concentrations of the interfering agents were selected to reflect levels likely to be encountered in relevant sample matrices. All recovery values fell within the acceptance criteria of 100 ± 5%, indicating that no significant cross-reactivity occurred with any of the tested substances.

Matrix Interference

Matrix-based interference for this method was assessed by spiking extracted samples from complex matrices with a known concentration of a validated aqueous Ethanol standard and measuring the recovery.

NOTE: All matrices were prepared according to the sample preparation methods described in the product instruction document which can be found on the product webpage.

Matrix tested	Replicates, n	Spike Recovery (%)
Red Wine	8	98.1
White Wine	8	97.8
Kombucha	8	99.0
Ginger Beer	8	99.6
Alcohol Free Beer	8	99.3
Alcohol free Gin	8	97.8
Tomato Juice	8	97.6

A total of seven matrices were evaluated, with all relevant samples demonstrating recoveries within the acceptable range of 95–105%. Additionally, the average coefficient of variation (%CV) for all spike recoveries was 1.10%, underscoring the method's precision and specificity.

ROBUSTNESS AND STABILITY

Storage Temperature

To evaluate the storage stability of the test kit components, all materials were stored at 4°C. Real-time performance and enzyme activity testing were conducted on a monthly basis. Slope-based trend analysis was employed to predict the shelf life of the product.

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 the kit was further evaluated through three freeze-thaw cycles. All kit components were frozen at -20°C overnight and allowed to thaw before testing. This process was repeated three times using the same reagents.

Ethanol Recovery (g/L)				
Expected Ethanol (g/L)	T0	1st Cycle	2nd Cycle	3rd Cycle
0.010	0.011	0.010	0.010	0.010
0.300	0.301	0.297	0.297	0.296

Sample recovery remained within the acceptance criteria of 100 ± 5%. A t-test analysis confirmed that there was no statistically significant difference in assay performance across the three freeze-thaw cycles, demonstrating the stability of the K-ETOHLQ reagents under these conditions.

Assay Temperature

Enzymatic test kits can be sensitive to environmental conditions, with temperature being a key factor influencing reaction rate and analyte recovery. The Ethanol (Liquid Ready) was evaluated at three different reaction temperatures: 20°C, 25°C, and 37°C. Recovery rates and reaction times were analyzed across these conditions.

	Ethanol Recovery (g/L)		
Expected Ethanol (g/L)	20°C	25°C	37°C
0.010	0.010	0.011	0.010
0.300	0.304	0.302	0.290
Reaction Time	7 min	5 min	4 min
Recommended time	*7 minute reaction time is recommended*		

A t-test analysis indicated no statistically significant difference in recovery between the temperatures tested. All recoveries fell within the acceptance criteria of 100 ± 5%. Notably, a faster reaction time was observed at 37°C. Based on these results, the recommended reaction time for K-ETOHLQ is 7 minutes.

PRECISION AND REPEATABILITY

Precision

Precision reflects the consistency of results obtained under varying conditions, including different days, analysts, and reagent lots. The precision of the Ethanol Assay Kit (Liquid Ready) was evaluated using validated aqueous standards.

Expected Ethanol (g/L)	Replicates, n	Mean (g/L)	Standard Deviation	%CV
0.010	16	0.010	0.0002	1.79
0.150	16	0.152	0.0012	0.80
0.300	16	0.297	0.0025	0.83

All recoveries met the acceptance criteria of 100 ± 5%, with coefficients of variation (%CV) consistently below 5%. These results demonstrate that the method is both precise and repeatable under typical laboratory conditions.

Matrix Repeatability

Matrix repeatability was assessed by a single analyst over three consecutive days using a range of selected complex matrices. Sample preparation was conducted daily to evaluate both the precision of analyte extraction and the repeatability of the assay method. All extractions followed the sample preparation protocol outlined in the product instructions.

Matrix	Replicates, n	Mean result (g/L)	Mean result (% *v/v*)	Standard Deviation	%CV
Red wine	6	78.6	9.95	1.754	2.231
White wine	6	86.6	10.97	1.334	1.539
Kombucha	6	0.79	0.10	0.009	1.137
Ginger beer	6	2.39	0.30	0.036	1.510
Alcohol Free Lager	6	2.61	0.33	0.046	1.754
Alcohol Free Gin	6	0.58	0.07	0.007	1.244
Tomato Juice	6	1.79	0.23	0.032	1.792

Across all matrices tested, the coefficient of variation (%CV) was consistently below 5%, demonstrating that the method is precise and repeatable when applied to complex sample types.

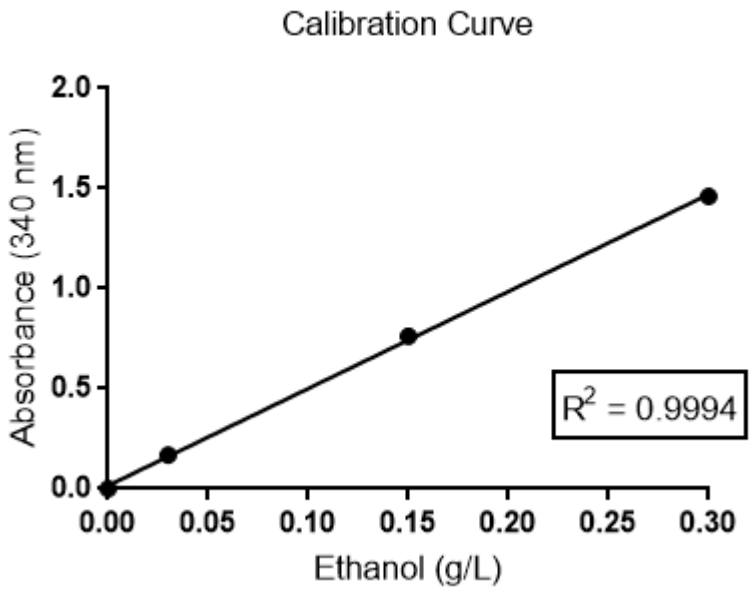
NOTE: Users should perform matrix validation work prior to routine use. This process will highlight any problematic matrices encountered. If you have questions about these or other matrices, please contact your local sales representative for support.

METHOD AUTOMATION

The Ethanol Liquid Ready Kit is specifically designed for use with auto-analyzers and can be readily adapted to a wide range of instrumentation. Quantification of Ethanol is achieved through a single-test format using a linear calibration fit. The performance data presented below were generated using a ChemWell-T analyzer operating at 37°C.

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

Ethanol (g/L)	Replicates, n	$\Delta_{absorbance}$ (340 nm)	Mean result (g/L)	Standard Deviation	%CV
0.030	4	0.169	0.029	0.001	0.029
0.150	4	0.763	0.147	0.005	0.147
0.300	4	1.461	0.284	0.005	0.284



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 Instructions
- Quick Reference Guide
- Mega-Calc™
- Safety data sheets (SDS)
- Certificate of analysis (CoA)

Contact us for more information: neogen.com/contact

Without guarantee

The information contained in this assay protocol is, to the best of our knowledge, true and accurate, but since the conditions of use are beyond our control, no warranty is given or is implied in respect of any recommendation or suggestions which may be made or that any use will not infringe any patents.

User Responsibility:

- Users are responsible for familiarizing themselves with product instructions and information. Visit our website at neogen.com, or contact your local Neogen® representative or authorized distributor for more information.
- When selecting a test method, it is important to recognize that external factors such as sampling methods, testing protocols, sample preparation, handling, laboratory technique and the sample itself may influence results.
- It is the user's responsibility in selecting any test method or product to evaluate a sufficient number of samples with the appropriate matrices and challenges to satisfy the user that the chosen test method meets the user's criteria.
- It is also the user's responsibility to determine that any test methods and results meet its customers' and suppliers' requirements.
- As with any test method, results obtained do not constitute a guarantee of the quality of the matrices or processes tested.

Terms and Conditions:

Neogen's full terms and conditions are available online.