



HIGH SENSITIVITY SALIVARY COTININE QUANTITATIVE ENZYME IMMUNOASSAY KIT

Item No. 1-2112, (Single) 96-Well Kit;
1-2112-5, (5-Pack) 480 Wells

Intended Use

The Salimetrics™ cotinine assay kit is a competitive immunoassay designed and validated for the quantitative *in vitro* measurement of the presence of cotinine in saliva samples. This kit may be used to measure primary or secondhand exposure to nicotine. Salimetrics has not validated this kit for use with serum/plasma. This kit is not intended to diagnose nicotine poisoning. A validated urine protocol is available upon request. Please read the complete kit insert before performing this assay. For further information about this kit, its application or the procedures in this insert, please contact the technical service team at Salimetrics or your local sales representative.

Introduction

Since primitive times, tobacco leaves have been processed and used by humans to deliver nicotine to the central nervous system. Historically, the preferred route of nicotine administration has varied from snuff, chew, and inhalation of smoke from burned tobacco leaves to contemporary methods such as transdermal patches, chewing gums, and smokeless inhalers. Regardless of delivery route, nicotine has addictive properties that cause the user to continue use despite efforts to quit. When tobacco leaves are smoked in cigarettes, nicotine is absorbed and distributed in the body within seconds. Metabolism is mainly by oxidation to cotinine and nicotine-N-oxide. Volumes of literature document the negative economic impact and health consequences of nicotine exposure. The costs to individuals and societies associated with nicotine exposure have led to widespread public health interventions to curb nicotine use.

The detection of exposure to tobacco smoke by measurement of cotinine is the preferred method. Nicotine is not considered a valid marker of smoking status due to its relatively short half-life (approximately 2 hours). By contrast, cotinine has an average half-life of 17 hours, and blood levels closely reflect the dose of nicotine absorbed from tobacco smoke. Saliva samples are easier to obtain, however, and saliva levels are highly correlated and used interchangeably with blood levels. (1)

Many of the commercially available assays for salivary cotinine are qualitative. They return “positive” or “negative” determinations with respect to tobacco/nicotine exposure. However, many studies show that levels of cotinine in saliva show large inter-individual differences. The sources of these differences include factors related to intrinsic and extrinsic predispositions that affect the physiology of nicotine metabolism, the dose of nicotine present in the source, and health behaviors relevant to how nicotine is used (e.g., cigarette vent blocking, duration, and frequency of puffs). (2) There is a clear advantage to providing inexpensive, accurate, quantitative, and noninvasive means of validating nicotine use, measuring the immediate physiological consequences of individual differences and intra-individual change in users’ behaviors, and determining secondhand tobacco smoke exposure. Salimetrics has designed this assay to provide the medical community with a highly sensitive and reliable means to do so.

Cotinine levels in biologic fluids have been measured by chromatographic (GC or HPLC – sometimes coupled with mass spectrometry) and immunoassay methods. Chromatographic methods have the advantage of higher specificity and sensitivity, (1) but EIA cotinine results have shown near perfect agreement with GC/MS confirmation of smoking status. (3) Immunoassay methods also use smaller sample volumes than chromatography methods and they do not require extractions or other manipulations of the samples, making them easier to use in large-scale epidemiological studies and avoiding the need for specialized laboratories. (4,5) Levels, however, may be higher with EIA since metabolites of cotinine, such as 3-OH-cotinine, are also measured. (1)

Test Principle

Standards and unknowns are added to a 96-well microtiter plate along with rabbit antibodies to cotinine and cotinine linked to horseradish peroxidase (conjugate). The cotinine in standards, unknowns, and the conjugate competes for the antibody binding sites. After incubation, unbound components are washed away. Bound conjugate is measured by the reaction of the peroxidase enzyme on the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with 2-molar sulfuric acid. Optical density is read on a standard plate reader at 450 nm. The amount of cotinine peroxidase detected is inversely proportional to the amount of cotinine present (6).

pH Indicator

A pH indicator in the assay diluent alerts the user to samples with high or low pH values. Acidic samples will turn the diluent yellow. Alkaline samples will turn the diluent purple. Dark yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Cotinine values from samples with a pH ≤ 3.5 or ≥ 9.0 may be artificially inflated or lowered.

Precautions

1. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in inaccurate values.
2. Do not mix components from different lots of kits.
3. Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month.
4. This kit uses break-apart microtitre strips. Unused wells must be stored at 2 - 8°C in the sealed foil pouch with desiccant and used in the frame provided.
5. Liquid stop solution is a 2-molar sulfuric acid solution. It is caustic; use with care.
6. Use care when covering and uncovering wells with plate sealer, to avoid spilling or cross contamination of wells.
7. Routine calibration of pipettes is critical for the best possible assay performance.
8. When using a multichannel pipette, reagents should be added to duplicate wells at the same time. Follow the same sequence when adding additional reagents so that incubation time with reagents is the same for all wells.
9. Pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate.
10. When running multiple plates, or multiple sets of strips, a standard curve should be run with each individual plate and/or sets of strips.
11. Laboratory temperature may affect assays. Salimetrics’ kits have been validated at 68 - 74°F (20 - 23.3°C). Higher or lower temperatures may affect OD values. Salimetrics cannot guarantee test results outside of this temperature range.
12. The quantity of reagent provided with this kit is sufficient for three individual runs. The volume of diluent and conjugate used for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
13. See ‘Material Safety Data’ at the end of procedure.

Storage

All components of this kit are stable at 2 - 8°C until the kit's expiration date.

Reagents and Reagent Preparation

- Microtitre plate:** A 96-well, high-affinity binding plate.
- Antiserum:** 15 mL of a solution of rabbit anti-cotinine antibody.
- Cotinine Standard:** 0.5 mL of cotinine in a saliva-like matrix with a non-mercury preservative, at a concentration of 200 ng/mL.
- Wash Buffer:** 100 mL of a 10X phosphate buffered solution containing detergents and a non-mercury preservative. Dilute only the amount needed for current day's use. Discard any leftover reagent. Dilute the wash buffer concentrate 10-fold with room temperature deionized water (100 mL of 10X wash buffer to 900 mL of deionized H₂O). (*Note: If precipitate has formed in the concentrated wash buffer, it may be heated to 40°C for 15 minutes. Cool to room temperature before use in assay.*)
- Assay Diluent:** 60 mL of a phosphate buffered solution containing a pH indicator and a non-mercury preservative.
- Enzyme Conjugate:** 75 µL of a solution of cotinine labeled with horseradish peroxidase. Dilute prior to use with assay diluent.
- Tetramethylbenzidine (TMB):** 25 mL of a non-toxic, ready-to-use solution.
- Stop Solution:** 12.5 mL of a 2-molar sulfuric acid solution.
- Cotinine Controls:** Two controls representing high and low levels of cotinine in a saliva-like matrix with a non-mercury preservative. Each vial contains 0.25 mL.

Materials Needed But Not Supplied

- Precision pipette to deliver 20 µL, 50 µL, and 100 µL
- Precision multichannel pipette to deliver 50 µL, 100 µL, and 200 µL
- Vortex
- Microplate incubator/shaker with 0.08-0.17 inch orbit
- Plate reader with a 450 nm filter
- Software for data reduction
- Deionized water
- Reagent reservoirs
- One 15 mL disposable tube
- Small disposable tubes
- Pipette tips
- 10 mL serological pipette

Specimen Collection

Collecting whole saliva samples from adults and children over 6 may be done by using the Salimetrics Oral Swab (SOS), Item No. 5001.02, or by unstimulated passive drool. Samples from younger children may be collected with the Salimetrics Children's Swab (SCS), Item No. 5001.06, and the Salimetrics Infant's Swab (SIS), Item No. 5001.08, may be used for children up to the age of 6 months. Collection protocols are available on request. Do **not** add sodium azide to saliva samples as a preservative. Samples visibly contaminated with blood should be recollected.

Avoid sample collection within 12 hours after consuming alcohol. Acidic or high sugar foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before sample is collected. Record the time and date of collection.

After collection it is important to keep samples cold, in order to avoid bacterial growth in the specimen; however, cotinine in saliva has been reported in the literature to be stable at room temperature for up to 12 days (7). If possible, refrigerate samples within 30 minutes, and freeze at or below -20°C within 4 hours after collection. (Samples may be stored at -20°C or lower for long term storage.)

Freezing saliva samples will precipitate the mucins. On day of assay, thaw completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. Avoid multiple freeze-thaw cycles. However, if samples have been refrozen, centrifuge again prior to assaying. Samples should be at room temperature before adding to assay plate. Pipette clear sample into appropriate wells. Particulate matter may interfere with antibody binding, leading to falsely elevated results.

Sample Preparation

Known smokers: Dilute saliva samples x 10 (10 µL saliva into 90 µL assay diluent), See "Calculations," Step 5 to determine resultant value.

Non-smokers: Run saliva sample straight.

Procedure

Bring all reagents to room temperature. A minimum of 1.5 hours is necessary for the 15 mL of assay diluent used in Step 5 (conjugate dilution) to come to room temperature. **Note:** It is important to keep the zip-lock pouch with the plate strips closed until warmed to room temperature as humidity may have an effect on the coated wells. Mix all reagents before use.

Step 1: Determine your plate layout. Here is a suggested layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	200 Std	200 Std	C-H	C-H								
B	66.7 Std	66.7 Std	C-L	C-L								
C	22.2 Std	22.2 Std	Unk-1	Unk-1								
D	7.4 Std	7.4 Std	Unk-2	Unk-2								
E	2.5 Std	2.5 Std	Unk-3	Unk-3								
F	0.8 Std	0.8 Std	Unk-4	Unk-4								
G	Zero	Zero	Unk-5	Unk-5								
H	NSB*	NSB*	Unk-6	Unk-6								

*NSB = Non-specific binding wells. These may serve as blanks. Use is optional.

Step 2: Keep the desired number of wells in the strip holder and return any remaining strips to the foil pouch. Reseal the zip-lock foil pouch with unused wells and desiccant. Store at 2 - 8°C.

Step 3:

- Label five small tubes, such as microcentrifuge tubes, with numbers 2 through 6.
- Pipette 100 µL of assay diluent in tubes 2 through 6. Serially dilute the standard 3X by adding 50 µL of the 200 ng/mL standard (tube 1) to tube 2. Mix well. After changing pipette tips, remove 50 µL from tube 2 to tube 3. Mix well. Continue for tubes 4, 5, and 6. The final concentrations of standards for tubes 1 through 6 are: 200 ng/mL, 66.7 ng/mL, 22.2 ng/mL, 7.4 ng/mL, 2.5 ng/mL and 0.8 ng/mL respectively.
- Pipette 15 mL of assay diluent into a disposable tube. (Scale down proportionally is not using the entire plate.) Set aside for Step 5.

Step 4:

- Pipette 20 µL of standards, controls, and unknowns into appropriate wells. Standards, controls, and unknowns should be assayed in duplicate.
- Pipette 20 µL of assay diluent into 2 wells to serve as the zero.
- If using NSB wells, pipette 120 µL of assay diluent into 2 wells to serve as the non-specific binding.

Step 5: Dilute the enzyme conjugate 1:300 by adding 50 µL of the conjugate to the 15 mL of assay diluent prepared in Step 3. (Scale down proportionally if not using the entire plate.) Conjugate in the microcentrifuge tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted conjugate solution and add 100 µL to each well using a multichannel pipette.

Step 6: Pipette 100 μ L of antiserum into all wells, except the nonspecific binding wells (if used), using a multichannel pipette.

Step 7: Cover the plate with a plate cover. Incubate the plate on a microplate incubator/shaker for 1.5 hours at 37°C with constant mixing at 500-600 rpm.

Step 8: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle or by pipetting 300 μ L of wash buffer into each well and then flipping the liquid into a sink. After each wash, the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the last wash.

Step 9: Add 200 μ L of TMB solution to each well using a multichannel pipette.

Step 10: Mix at 500 rpm for 5 minutes (or tap to mix) and incubate in the dark for an additional 25 minutes at room temperature.

Step 11:

- Add 50 μ L of stop solution using a multichannel pipette.
- Mix on a plate rotator at room temperature for 3 minutes at 500 rpm (or tap to mix). Be sure all wells have turned yellow. If green color remains, continue mixing until green color turns to yellow.
- Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry.
- Read in a plate reader at 450 nm. Read plate within 10 minutes of adding stop solution. (Correction at 630 is desirable.)

Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells (if used) from the average OD of the zero, standards, controls, and unknowns.
3. Calculate the percent bound (B/Bo) for each standard, control, and unknown by dividing the average OD (B) by the average OD for the zero (Bo).
4. Determine the concentrations of the controls and unknowns by interpolation using software capable of logistics. We recommend a 4-parameter curve fit.
5. When determining Cotinine levels for specimens that have been diluted, multiply the test result obtained by the dilution factor of the specimen.

Limitations

- Any samples registering a cotinine value greater than 200 ng/mL should be diluted with assay diluent and rerun for accurate results. See **Calculations**, Step 5.
- If any wells turn yellow or purple after first incubation period (Step 6), pH should be determined for the corresponding saliva samples. Samples with pH \geq 9.0 or \leq 5.0 should be recollected. See Specimen Collection section.
- See "Introduction" to review donor history effects on data generated.
- Samples collected with sodium azide are unsuitable for this assay.

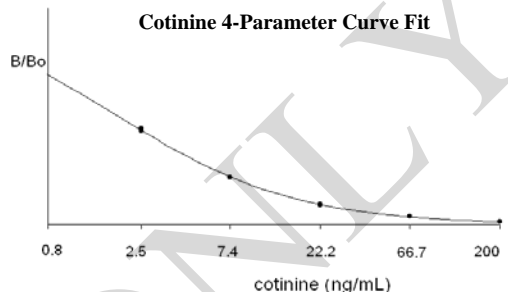
Quality Control

The Salimetrics' high and low salivary cotinine controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Sample	Average OD	B	B/Bo	Cotinine (ng/mL)
A1,A2	S1	0.045	0.037	0.022	200
B1,B2	S2	0.096	0.088	0.051	66.7
C1,C2	S3	0.222	0.214	0.125	22.2
D1,D2	S4	0.489	0.481	0.280	7.4
E1,E2	S5	0.893	0.885	0.516	2.5
F1,F2	S6	1.278	1.270	0.740	0.8
G1,G2	Bo	1.724	1.716	NA	NA
H1,H2	NSB	0.008	NA	NA	NA



Material Safety Data*

Hazardous Ingredients

Liquid stop solution is caustic; use with care. We recommend the procedures listed below for all kit reagents. Specific kit component MSDS sheets are available from Salimetrics upon request.

Handling

Follow good laboratory procedures when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using standard absorbent materials while wearing protective clothing. Follow local regulations for disposal.

Emergency Exposure Measures

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing, give oxygen and call a physician.

*The above information is believed to be accurate but is not all-inclusive. This information should only be used as a guide. Salimetrics shall not be liable for accidents or damage resulting from contact with reagents.

Performance Characteristics

A. Recovery:

Three saliva samples were spiked with known quantities of cotinine and assayed.

Sample	Endogenous (ng/mL)	Added (ng/mL)	Expected (ng/mL)	Observed (ng/mL)	Recovery (%)
1	3.26	5	8.26	8.22	99.6
1	2.96	50	52.96	60.45	114.1
1	3.22	100	103.22	102.27	99.1
2	0.00	500	500.00	470.32	94.1
3	17.02	5	22.02	20.69	94.0
3	17.02	50	67.02	64.77	96.6

B. Sensitivity:

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 10 sets of duplicates at the 0 ng/mL level. The minimal concentration of cotinine that can be distinguished from zero is 0.15 ng/mL.

C. Precision:

The intra-assay precision was determined from 10 samples each of four levels of cotinine.

Sample	N	Mean (ng/mL)	Std Dev (ng/mL)	Coefficient of Variation (%)
1	10	5.49	0.25	4.5
2	10	52.35	4.50	8.6
3	10	105.21	6.16	5.9
4	10	495.47	32.04	6.5

The inter-assay precision was determined from the mean of average duplicates for 8 separate runs.

Sample	N	Mean (ng/mL)	Std Dev (ng/mL)	Coefficient of Variation (%)
Low	8	6.07	0.55	9.04
High	8	102.23	4.30	4.21

D. Linearity of Dilution:

Two saliva samples were diluted with assay diluent and assayed.

Sample	Dilution Factor	Expected (ng/mL)	Observed (ng/mL)	Recovery (%)
Sample 1			120.27	
	1:2	60.14	56.87	94.6
	1:4	30.07	27.12	90.2
	1:8	15.03	14.00	93.1
	1:16	7.52	6.77	90.0
	1:32	3.76	3.50	93.2
	1:64	1.88	1.82	96.7
Sample 2			538.34	
	1:2	269.17	278.40	103.4
	1:4	134.59	139.68	103.8
	1:8	67.29	67.27	100.0
	1:16	33.65	33.34	99.1
	1:32	16.82	16.91	100.5
	1:64	8.41	9.24	109.8

E. Cross-Reactivity

Nicotine	0.0293%	Nicotinamide	ND
Nicotinic acid	ND	3-OH-cotinine*	24.82%

*3-OH-cotinine is a metabolite of cotinine.

F. Measurement of Salivary Cotinine in Smokers and Non-smokers Using the Salimetrics EIA

Group	N	Mean (ng/mL)	Std Dev (ng/mL)	Range (ng/mL)
Adult Smokers	21	206.33	123.47	47.87 - 586.39
Non-smokers	10	0	0	NA

The Salimetrics EIA is able to distinguish smokers from non-smokers with a high level of accuracy.

G. Salimetrics quantitative enzyme immunoassay for salivary cotinine discriminates smokers from non-smokers, and differentiates primary from secondary smoke exposure (8).

Table 1: Salivary cotinine levels (ng/mL) in smoking and non-smoking mothers and their 6-month old infants

Mother's Self-Reported Status: Smokers (n=27)			
Group	Mean	Std Dev	Ranges
Number of cigarettes smoked in prior 48 hours by mother	14.26	14.67	3 – 60
Mother's salivary cotinine (ng/mL)	241.27	176.67	0 – 565.36
Infant salivary cotinine (ng/mL)	9.59	8.58	0.91 – 33.19

Mother's Self-Reported Status: Non-smokers (n=20)			
Group	Mean	Std Dev	Ranges
Number of cigarettes smoked in prior 48 hours by mother	0.0	NA	NA
Mother's salivary cotinine (ng/mL)	2.15	5.45	0 - 23.9
Infant salivary cotinine (ng/mL)	2.12	3.54	0 - 12.34

Notes:

1. Smoking status determined by number of cigarettes smoked in the past 48 hours, "0" = non-smoker, "> 3" = smoker.
2. Independent sample t-test comparing smoking and non-smoking groups, $p < 0.001$.
3. Only 1 infant of a smoking mother had received breastmilk in prior 7 days.

Figure 1. Positive association between mother and infant salivary cotinine

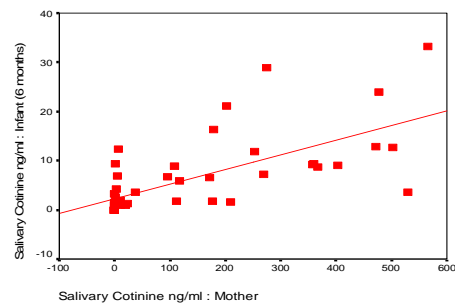
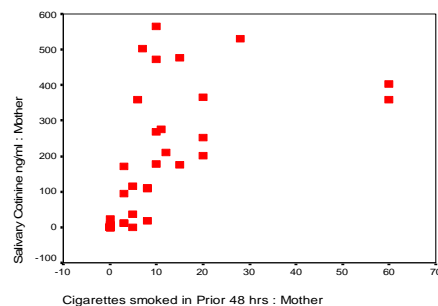


Figure 2. Positive association between number of cigarettes smoked in the prior 48 hours and salivary cotinine in mothers



H. Comparison of Cotinine Measurement by LC-ES/MS/MS to EIA (unpublished data)

(n=40)	Cotinine Results by LC-ES/MS/MS*	Cotinine Results by Salimetrics EIA
LC-ES/MS/MS Pearson Correlation (p-value)	--	0.90 (0.00)
Salimetrics Cotinine EIA Pearson Correlation (p-value)	0.90 (0.00)	--
Total Hrs. Exposed^ Pearson Correlation (p-value)	0.39 (0.01)	0.48 (0.00)

*Liquid Chromatography Electrospray Ionization Tandem Mass Spectroscopy

^ Self-reported hours of exposure to secondhand smoke

References

1. Benowitz, N.L. (1996). Cotinine as a biomarker of environmental tobacco smoke exposure. *Epidemiol Rev*, 18(2), 188-204.
2. Benowitz, N.L., Hukkanen, J., & Jacob, P. 3rd (2009). Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb Exp Pharmacol* 192, 29-60.
3. Alterman, A.I., Gariti, P., & Niedbala, R.S. (2002). Varying results for immunoassay screening kits for cotinine levels. *Psychol Addict Behav*, 16(3), 256-59.
4. Watts, R.R., Longone, J.J., Kinght, G.J., & lewtas, J. (1990). Cotinine analytical workshop report: Consideration of analytical methods fro determining cotinine in human body fluids as a measure of passive exposure to tobacco smoke. *Environ Health Perspect*, 84, 173-82.
5. Roche, D., Callai, F., Reungoat, P, & Momas, I. (2001). Adaptation of an enzyme immunoassay to assess urinary cotinine in nonsmokers exposed to tobacco smoke. *Clin Chem*, 47(5), 950-52.
6. Chard, T. (1990). *An introduction to radioimmunoassay and related techniques* (4th ed.). Amsterdam: Elsevier.
7. Foulds, J., Bryant, A., Stapleton, J., Jarvis, M.J., & Russell M.A. (1994). The stability of cotinine in unfrozen saliva mailed to the laboratory. *Am J Public Health*, 84(7), 1182-83.
8. Granger, D.A., Blair, C., Willoughby, M., Kivlighan, K.T., Hibel, L.C., Fortunato, C.K., Wiegand, L.E., & The Family Life Project Investigators (2007). Individual differences in salivary cortisol and alpha-amylase in mothers and infants: Relation to tobacco smoke exposure, *Dev Psychobiol* 49(7), 692-701 .

Seller's Limited Warranty

“Seller warrants that all goods sold hereunder will be free from defects in material and workmanship. Upon prompt notice by Buyer of any claimed defect, which notice must be sent within thirty (30) days from date such defect is first discovered and within three months from the date of shipment, Seller shall, at its option, either repair or replace the product that is proved to Seller’s satisfaction to be defective. This warranty does not cover any damage due to accident, misuse, negligence, or abnormal use.

It is expressly agreed that this limited warranty shall be in lieu of all warranties of fitness and in lieu of the warranty of merchantability. Seller shall not be liable for any incidental or consequential damages that arise out of the installation, use or operation of Seller’s product or out of the breach of any express or implied warranties.”

“European Authorized Representative”
Salimetrics Europe Ltd, Unit 7 Acorn Business Centre,
Oaks Drive, Newmarket, CB8 7SY, UK
(T) +44 (0) 1638782619

