

## DRUG TEST CONFIRMATIONS

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<b>HISTORY</b>		
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1	26/10/2005	New Edition
2	28/9/2007	Laboratory move to KCH, change to Pathnet
3	1/12/2008	Change method to LCMS
4	18/5/2009	Change LC to Agilent pumps/Aria OS

<b>CIRCULATION LIST</b>		
<b>Copy No</b>	<b>Location</b>	<b>Name</b>
1	Toxicology Laboratory	Andrew Marsh

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## PURPOSE OF THE TEST

The first stage of a drug screen is to use an immunoassay method to screen samples. This method can identify samples that are positive for a class of substances but cannot distinguish between different drugs within the class, for example morphine and codeine for opiates or amphetamine and ecstasy for the amphetamine group.

In the second stage of a drug screen, all samples that are positive for a specified class of drugs are confirmed by Liquid Chromatography-Mass spectrometry (LCMS). This procedure uses a different type of method from the screening process to identify the main classes of drug. The use of a different type of method allows non-specific interference to be reduced so that the accuracy of the final result is assured.

The predominant opiate found in the laboratory is morphine, which can either be present as the primary drug or more probably as a metabolite of heroin. Other main opiates detected are codeine and dihydrocodeine. There are a variety of opiate class drugs, some of which are available over the counter, some are prescription only medicines, and some are controlled or illicit drugs.

The main amphetamines found in the laboratory are amphetamine and ecstasy (methylene dioxy methyl amphetamine, MDMA), but there are also a large number of immunoassay positive results from interfering substances, principally mebeverine (Colofac<sup>®</sup>)

Other drugs that can also be confirmed using this method include the benzodiazepines, methadone and cocaine metabolites.

## PRINCIPLE OF THE TEST

Urine samples that require confirmation are spiked with internal standard solution before being aliquoted into a HPLC vial, which is sealed and loaded onto the HPLC system. An increasing gradient of acetonitrile in water separates the components of the urine sample, such that they are eluted into the source of the mass spectrometer at known retention times.

The eluant from the Jasco HPLC system is introduced into the LCQ through the divert valve and into the Electrospray Ionisation (ESI) probe. A combination of high voltage, heat and nitrogen gas drives off all of the solvent, leaving the ions to be drawn through the ion transfer capillary into the mass spectrometer. A series of lenses, including a quadrupole and octupole, guide the ions into the ion trap, where they can be collided or expelled and detected by the dynode

Each analytical cycle consists of a number of stages or Scan Events. Initially a full scan of parent ions is performed in positive mode. Parent ion masses are compared with the mass list, and if an ion is present within the specified time window, a data dependent scan can be performed, producing an MS<sup>2</sup> spectrum of the parent ion. This scan event can be repeated with several ions within the list, or dedicated scan events for specific ions. A negative ion mode can also be performed before the cycle starts a new first Full Scan.

Once the analytical run is complete, the chromatogram is analysed by the ToxID software for a specified set of analytes. The parent masses, retention times and ion intensities on the chromatogram are compared with the ToxID configuration file. If a match is found and the most intense daughter ion matches that in the configuration file, a library search is performed on that spectrum. A library match greater than the hit threshold set up in ToxID is considered a positive result for that particular analyte. The configuration file is compared with a calibrator sample on a daily basis to ensure that retention times and ion intensities do not shift significantly.

## SAMPLE COLLECTION AND PREPARATION

1. Urine is the only sample that is normally acceptable for drugs of abuse screening
2. Urine should be collected into a universal container without preservative
3. The volume of sample required for the test is at least 1mL.
4. No specific patient preparation is required
5. No specific timing of sample collection is required
6. Pre-analysis: Urine samples are received from the Olympus AU640
7. Post-analysis: The samples are stored in TOX6 for one week after receipt after which they are disposed of as clinical waste.

## REAGENTS, MATERIALS AND EQUIPMENT

### Stock Reagents

#### *Rathburn* Methanol

Sprinter No. : 005737  
Cat. No. : RH1019  
Volume : 2.5L  
Contents : HPLC grade methanol  
Storage : Stored at room temperature  
Location : Stored in flammable cupboard in the Confirmation Laboratory, or in the outside solvent store

#### *Fluka* Formic Acid

Sprinter No. : 001344  
Cat. No. : 94318  
Volume : 250 mL  
Contents : Formic acid  
Storage : Stored at room temperature  
Location : Stored in flammable cupboard in the Confirmation Laboratory, or in the outside solvent store

#### *Fluka* Ammonium Formate

Sprinter No. : 001344  
Cat. No. : 70221  
Volume : 100 g  
Contents : Ammonium formate  
Storage : Stored at room temperature  
Location : Stored in chemicals cupboard in the Confirmation Laboratory

#### *Rathburn* Acetonitrile

Sprinter No. : 005737  
Cat. No. : RH1016  
Volume : 2.5L  
Contents : HPLC grade acetonitrile  
Storage : Stored at room temperature  
Location : Stored in flammable cupboard in the Confirmation Laboratory, or in the outside solvent store

**Reagent Preparation**

Preparation of reagents must be recorded in the reagent file in the confirmation laboratory, and the date of preparation should be written on the bottle.

*HPLC Solvents*

## i) 1M Ammonium Formate

Preparation : Add 10.61g of ammonium formate to 150mL of deionised water in a 200ml volumetric flask. Fill to the mark.  
Stability : Stable refrigerated for 6 months from date of preparation  
Storage : Stored in TOX5 refrigerator  
Labelling : Label with Harmful hazard label

## ii) Mobile Phase A

Preparation : Add 1mL of formic acid to approximately 900mL of deionised water in a 1L volumetric flask. Add 10mL of 1M ammonium formate and fill to the mark with deionised water. Filter before use using the HPLC eluant filtration apparatus  
Stability : Stable for 2 months from date of preparation  
Storage : Stored at room temperature  
Labelling : Label with Harmful hazard label

## iii) Mobile Phase B

Preparation : Add 1mL of formic acid to approximately 900mL of acetonitrile in a 1L volumetric flask and fill to the mark with acetonitrile. Filter before use using the HPLC eluant filtration apparatus  
Stability : Stable for two months from the date of preparation.  
Storage : Stored at room temperature  
Labelling : Label with Harmful Flammable hazard label

**Materials**

## HPLC Crimp Vials

Supplier : Esslabs  
Sprinter No. : 007523  
Cat. No. : 5181-3375  
Contents : 100 glass vials (32x11.6mm)  
Storage : Stored at room temperature  
Location : Stored in cupboard in Mass Spec Laboratory

## HPLC Caps

Supplier : Esslabs  
Sprinter No. : 007523  
Cat. No. : 5818-1210  
Contents : 100/box  
Storage : Stored at room temperature  
Location : Stored in cupboard in Mass Spec Laboratory

## Membrane filters (0.45 µm)

Supplier : Phenomenex  
Sprinter No. : 001441  
Cat. No. : Nylon 66 AFO-0504 or equivalent  
Contents : 100/pack  
Storage : Stored at room temperature  
Location : Stored in cupboard in Confirmations Laboratory

#### PFP Gold HPLC Column

Supplier : Thermo Fisher Scientific  
Sprinter No. : 023596  
Cat. No. : 25405-052130 (50 x 2.1)  
Contents : One PFP Gold HPLC Column  
Storage : Stored at room temperature  
Location : Stored in cupboard in Confirmations Laboratory

#### Strata X Cartridge

Supplier : Phenomenex  
Sprinter No. : 001441  
Cat. No. : 25405-052130 (50 x 2.1)  
Contents : Strata X cartridge 10mg pipette mass  
Storage : Stored at room temperature  
Location : Stored in cupboard in Confirmations Laboratory

#### LCQ Fleet Consumables

Supplier : Thermo-Fischer  
Sprinter No. : 023596  
Cat. No. : See various in Equipment Folder or Desktop under LCQ Consumable

#### Plastic Pasteur pipettes

Supplier : Elkay  
Sprinter No. : 000248  
Cat. No. : 127P511000  
Contents : 100 pipettes/box  
Storage : Stored at room temperature  
Location : Stored in cupboard in Confirmations Laboratory

#### Cutoff Calibrator

To make up cut-off calibrator; add 20ml Biorad C2 and 20ml Biorad C3 plus 10 µl of 2.5mg/mL DHC (Dihydrocodeine), in a 50 ml volumetric flask.

Cut-off Calibrator is stable for 30 days at 2-8°C after opening or until the expiry date on the package, whichever comes first.

Validate Cut-off Calibrator by running alongside existing Cut-off Calibrator and ensuring that retention times are sensitivities are similar. Record details of preparation of the Cut-off Calibrator in the reagent log, stored on the shelves in the confirmation laboratory.

#### Equipment

HPLC Eluant filtration apparatus

Crimper for lids of HPLC vials

**COSHH ASSESSMENT**

Substance	Data Sheet Reference	Handling requirements	Risk Rating
Methanol	VWR 1015800	Toxic and Flammable. Wear gloves when handling. Obtain medical attention on eye contact or after ingestion.	M
Acetonitrile	VWR 1038600	Toxic and Flammable. Wear gloves when handling. Obtain medical attention if ingested.	M
Ammonium Formate	Sigma-Aldrich 55674	Irritant. Wear gloves when handling. Obtain medical attention if ingested.	L
Formic acid	Sigma-Aldrich 94318	Corrosive and Flammable. Toxic by inhalation. Wear gloves when handling. Obtain medical attention on eye contact or after ingestion.	M
Standard Solutions	Cerilliant various	Flammable, Toxic and Irritant	M

**HEALTH AND SAFETY PRECAUTIONS**

Laboratory coats must be worn when performing any part of this procedure

Gloves and goggles must be worn at all times while handling urine samples, methanol or solvents.

The BIORAD urine controls are lyophilised material of human source, which have been tested for hepatitis B surface antigen (HBsAG), antibody to hepatitis C (HCV) and antibody to HIV and found to be non-reactive. However as no method can offer complete assurance as to the absence of infectious agents, this material and all patient samples should be handled as though capable of transmitting infectious disease.

**QUALITY CONTROL**

Quality control samples are run with each batch of samples.

*Bio Rad* DAU Negative Control

Sprinter No. : 000434

Cat. No. : 460

Volume : 10x20mL

Contents : Drug free human urine

Storage : Stored at 2-8°C

Location : Stored in fridge TOX5

Preparation : Control is supplied ready to use

Stability : 30 days at 2-8°C after opening or until the expiry date on the package, whichever comes first

*Bio Rad* DAU Toxicology Control C2

Sprinter No. : 000434  
Cat. No. : 442  
Volume : 10x20mL  
Contents : Human urine containing drugs and metabolites  
Storage : Stored at 2-8°C  
Location : Stored in fridge TOX5  
Preparation : Control is supplied ready to use  
Stability : 30 days at 2-8°C after opening or until the expiry date on the package, whichever comes first

*Bio Rad* DAU Toxicology Control C3

Sprinter No. : 000434  
Cat. No. : 469  
Volume : 10x20mL  
Contents : Human urine containing drugs and metabolites  
Storage : Stored at 2-8°C  
Location : Stored in fridge TOX5  
Preparation : Control is supplied ready to use  
Stability : 30 days at 2-8°C after opening or until the expiry date on the package, whichever comes first

*Bio Rad* DAU Toxicology Control C4

Sprinter No. : 000434  
Cat. No. : 444  
Volume : 10x20mL  
Contents : Human urine containing drugs and metabolites  
Storage : Stored at 2-8°C  
Location : Stored in fridge TOX5  
Preparation : Control is supplied ready to use  
Stability : 30 days at 2-8°C after opening or until the expiry date on the package, whichever comes first

## QUALITY ASSURANCE

### Internal Quality Assessment

When samples run for analytes where no IQC is available, a sample known to contain the analyte will be run as an IQA sample.

### External Quality Assessment

Three external quality assurance samples are received every three months.

Results are faxed or e-mailed to the organisers, and repeat samples may also be obtained from there.

Samples are received lyophilised and require reconstitution with 15mL of deionised water using a volumetric pipette. The samples are allowed to stand for 5 minutes before gentle mixing for at least 20 minutes. External Quality Assessment samples are analysed by ion trap LCMS, even if the Olympus screening results do not indicate that this is required. This is to ensure that all drugs that are looked for in the laboratory are also looked for in the EQA samples. Failure to follow this procedure may give a false negative result for weak positive morphine, quinine or cyclizine, among others.



## METHOD

### Worksheet Creation

- i) From Pathnet, type CB120 to open the worklist generator. Fill in the Workcentre (538), Testing Site (2) and Detail test code (LCMS)
- ii) Run the Worklist by double clicking on the icon to download batch file, FTP\_LCMS stored at G:\Chem\Toxicology\Charts & Forms\Drugs\FTP
- iii) Double click on the LCMS worklist generator to open, "LCMS Sequence Import.XLS" stored in the same directory.
- iv) Select the "FULL WORKLIST" button to import the worklist into Excel. Respond to the prompts as appropriate. Print when complete.
- v) When complete, the list may be edited before selecting the "Export Sequence" button.
- vi) Input the number of the first vial in the autosampler tray when prompted, and save the sequences produced. Save the sequence file Xcal\_LCMSXXXX, with XXXX being the sequence number, to a memory stick for transfer to the LCMS

### Sample Preparation

- i) Pipette approximately 1mL of cut-off calibrator, QC or urine sample into a glass HPLC vial. If the sample has a large number of particulates, centrifuge or allow sediment to settle and pipette from the clear top layer.
- ii) Cap the HPLC vial.
- iii) Load vials into the appropriate position in the autosampler tray. Repeat for all samples.

### Run Preparation (Xcalibur)

- i) Select Xcalibur from the Start menu or desktop
- ii) From the Xcalibur Roadmap, select SEQUENCE SETUP.
- iii) Select FILE and IMPORT from the drop down menu. The shortcut for this is CTRL+I
- iv) Select the created sequence file Xcal\_LCMSXXXX on the memory stick
- vii) Once imported, the sequence file can be edited if required.
- viii) Ensure that the Instrument Method is set to "OPIATES" for a full length run, or "BARTS" for a short run, and that the Processing Method is set to ToxID. Save the sequence in the C:\LCMS Data\YEAR\MONTH\RUN folder, as above. The sequence name should match the Work list name, with a .SLD extension.

### Run Preparation (Xcalibur)

- i) Open Aria and select "Batch Editor"
- ii) From the File menu, select Import and Xcalibur SLD. Select the file just created.
- iii) Ensure that the LC Method and Detector Method are both set correctly in the header, and that the autosampler method is AS SPE.
- iv) Edit the tray and vial positions for each sample as appropriate. Fill down and Increment can be used to make this stage quicker. Select Vial Type VT54 and Injector LX. The columns for these may need to be selected from the Edit menu.
- v) When editing the sequence is complete, save the batch template as LCMSXXXX in the correct folder as specified at stage viii) above.
- vi) Select Submit (or Submit Selected). Check that the path is correct for the data files and amend if necessary. Check that the Batch number is correct. If this is the second time part of this batch has been run, a suffix will be needed to allow submission.

**Starting the Run**Xcalibur:

- i) Check that the Convector gauge pressure under the VACCUUM tab is greater than 1

Source	Vacuum	Syringe	Analog	State
Ion Gauge Pressure (E-5 Torr):				1.00
Convector Gauge Pressure (Torr):				1.24
Turbo Pump:				
Life (hours):				4293
Speed (Hz):				750
Power (watts):				62
Temperature (°C):				57

- ii) Check the plumbing of the HPLC system. The red tubing from the LX injector should be routed via valve B to the bottom port (6) of the Divert valve. The SPE cartridge should be attached to ports 1 & 5, and the analytical column should be on port 4, with the outflow of the analytical column connected through the grounding bar to the ESI probe. The ESI probe should be set at position C.

Aria

- i) Select the Pumps On option from the LC Method menu.
- ii) After 20-30 seconds, the pumps will have stabilised. Click on the Start Analysis button from the Batch Tab. Check that the autosampler drawers are closed and select OK.

**ToxID software**

ToxID software is used to identify and report drugs on the ion trap. This identifies a drug based on the parent ion mass, the principle daughter ion intensity and the library match scores. The output is a .PDF file and a .CSV file for each sample.

ToxID can be accessed manually from the start menu, and has a number of options:

<b>Source File</b>	This is the .RAW data file that is to be processed
<b>Config File</b>	This file configures ToxID with general and drug specific parameters. See below for operation
<b>Summary Report</b>	Selecting short report gives an abbreviated report with up to 8 analytes per page. This is the one used by default in the Toxicology Unit. There are 2 options within this, whether to View or Print the .PDF file created. Leaving both checkboxes blank will create the .PDF & .CSV files only
<b>Long report</b>	The long report gives a single page report for each analyte, containing the chromatogram, acquired and library spectra, difference spectrum and library structure (if known). As for the short report, there are checkboxes for viewing and printing reports
<b>Save</b>	Selecting this button saves the current setup without processing the .RAW file
<b>Process</b>	This button causes ToxID to process the .RAW file using the specified configuration file.



### Configuration File

The ToxID configuration file is a .CSV file that is best edited in Excel. The top 19 lines contain required general information, such as the laboratory name & logo, the mass and retention time windows to use, search libraries and the header row for the analyte list.

The rows below the header row should contain the following information for each drug:

<b>Analyte</b>	The analyte list shows each drug name with identification parameters for each. To enable correct operation, the drug name must match that found in the MS spectra library.
<b>Parent Mass</b>	This tells ToxID which MS2 scan filter to search
<b>Id</b>	Parent drug, Internal Standard or Metabolite
<b>Product mass</b>	This tells ToxID the mass of the most intense daughter ion, used to draw the chromatogram and use for the intensity measurement
<b>Retention Time</b>	The retention time $\pm$ the window specified in the header identifies where in the chromatogram to look for a peak
<b>Intensity</b>	Only if the daughter ion intensity is above the specified intensity threshold will a positive result be recorded
<b>SI</b>	The Search Index is a calculated score of the similarity of the acquired spectrum with the library spectrum. The specified value must be exceeded for a positive result to be recorded
<b>RSI</b>	The reverse search index is the similarity between the library spectrum and the acquired spectrum. This value must be exceeded for a positive result to be recorded

### Processing LCMS Data

- i) Select Xcalibur from the Start menu
- ii) Select the SEQUENCE VIEW 
- iii) Select FILE – OPEN – select the last sequence file YEAR/MONTH/DAY/worklist print time
- iv) Select BATCH PROCESS . Select the required lines to reprocess, and select the “Programs” checkbox.
- v) Select OK to finish.

## REQUESTING AND REPORTING OF RESULTS

### Interpreting results

ToxID gives a summary report for each patient sample. Negative results are not shown, and all identified drugs or metabolites listed should be considered as potentially positive.

Examine the results from the cutoff calibrator and the QC samples. This should give an estimate of the intensity threshold for positive results.

For each result in the ToxID report, check that the following applies:

- i) The intensity of the analyte is similar to or above the intensity of the cutoff calibrator
- ii) The chromatogram shows a peak, and is not either flat or random noise
- iii) The SI/RSI values are similar and at least 400 each

In general, a drug is considered POSITIVE if the parent drug or primary metabolite pass the above criteria, and the immunoassay result (if available) gives a positive result.

A result should be considered NEGATIVE if the only metabolite identified is the glucuronide, and that is only found in the MS2 spectrum.

For example, morphine glucuronide (MS2) may be reported as positive if one of the following analytes is also detected: Morphine, morphine glucuronide (MS3), acetyl-morphine

Extra certainty about a result may be achieved through identification of several metabolites, for example, Cocaine, Cocaethylene and benzoylecgonine may all be present after cocaine use.

**Entering Results**

- i) Log on to the laboratory information system (LIS) and enter the result under the laboratory accession number.
- ii) LCMS results are recorded under the detail code "LCMS". Record the results from the Worklist. This line is for internal lab use only, so acceptable shorthand is used. Always record Morphine results as present or absent, and any other significant spots if present.  
e.g. Mo+ Me+ cod + Morphine Positive, Methadone Positive, Codeine +  
Mo- Me+ coc + Morphine Negative, Methadone +, cocaine +
- iii) Enter the longhand morphine result on the Morphine result line. Only 3 codes are used, and these are displayed on screen (POSITIVE, NEGATIVE and N/A)
- iv) Repeat this for the codeine, 6-monoacetyl morphine and any other significant analytes.
- v) Comments are added by selecting F11 (Comment). Chartable comments that are visible with the results are placed above the line, and should be signed by the person entering the comment. Non-chartable internal comments are placed below the line.

All patient results are entered onto the LIMS system, including research work and external quality assessment. Quality control results are recorded on the LCMS, and this data must be kept for at least 10 years.

**REFERENCE RANGES**

There is no numerical reference range for drug screening, as the results are reported as positive or negative and will vary according to the patient's medication. However, drug users tend to keep taking the same drugs, and where a result does not fit in with previous drug use, immunoassay results, or where the clinical details do not match the results, extra care should be taken in interpreting the results, or repeat sample analysis to check.

**LIMITATION OF THE ASSAY**

Many different compounds will be detectable by LCMS, and their identities may only be safely determined with reference to a matching standard. The retention time windows and library searching should ensure that only correct analytes are reported.

The nature of the samples being tested and the reasons for the testing mean that it is probable that some patients will try to adulterate their samples to give false results. Any sample which does not look or behave as expected for a urine must be considered suspect and be brought to the attention of senior staff.

The cutoff levels are more sensitive than those used in the immunoassay, and are set at the following approximate values.

Morphine/Morphine glucuronide	500ng/mL
Codeine/ Codeine glucuronide	500ng/mL
Dihydrocodeine	500ng/mL
Cocaine metabolite	150ng/mL
Methadone	200ng/mL
Methadone metabolite	200ng/mL
Amphetamine	250ng/mL
Metamphetamine	250ng/mL

## INTERPRETATION OF RESULTS

Drugs will remain detectable in the urine for a varying period of time depending on the drug, the amount taken and a variety of other factors.

As a general guide, morphine, codeine and cocaine metabolites can usually be detected in urine for 3-5 days following use, although this gives no indication about the amount of drug taken.

Morphine is the main active metabolite of heroin, but is also prescribed and used itself. It is also a minor metabolite of codeine and pholcodine

6-monoacetyl morphine is the primary metabolite of heroin, but has a short half life and can normally only be detected in the urine for 12-24 hours after heroin use. Heroin (diacetyl morphine) has a half life in blood of around 2 minutes, so is very unlikely to be detected.

Codeine can be present when heroin is taken, as codeine is a common adulterant or contaminant, but it can also be taken as a medicine, available over the counter without prescription.

Dihydrocodeine is not metabolised to morphine, and the presence of morphine in a client given dihydrocodeine is an indication of extra use of opiates. Dihydrocodeine is now available as an over the counter medicine.

## ANALYTES SEEN IN QUALITY CONTROL MATERIAL

ANALYTE	Negative	C2	C3	C4
Amphetamine	NEG	POS	POS	POS
Metamphetamine	NEG	POS	POS	POS
MDEA	NEG	NEG	NEG	POS
MDMA	NEG	NEG	NEG	POS
Cocaine-Benzoylcegonine	NEG	POS	POS	POS
Methadone	NEG	NEG	POS	POS
Codeine	NEG	POS	POS	POS
Morphine-3-glucuronide	NEG	POS	POS	POS
PPX	NEG	NEG	POS	POS

## REFERENCES

Clarke's Analysis of Drugs and Poisons 3<sup>rd</sup> Ed. (2004) Ed. AC Moffat, MD Osselton, B Widdop

Xcalibur software manual

## PROCEDURE AMENDMENT FORM

Number	Date	Page No	Amendment	Authorised by:
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

- The amendment must be authorised by the author of the procedure
- The amendment must be underlined and an asterisk written in the margin along side the change
- Up to ten minor amendments can be made before the procedure is revised
- Major changes must result in the immediate review of the procedure

