WP2: Generation of an *in vitro* database (P8, P14, P15, P23 as well as IIVS).

Six basal cytotoxicity assays

In WP2, six cytotoxicity was used for testing;

- the 3T3 Neutral red uptake (P8, P15, IIVS),
- the NHK Neutral red uptake assay (P8, P15, IIVS),
- the HL60 ATP assay (P8, P23),
- the Fa32 Neutral red uptake (P14),
- the Fa32 fluorescent total protein assay (P14), and
- the HepG2 fluorescent total protein assay (P14, P23).

During the first 3 months of the project, P8 prepared and circulated the SOP's and the Excel and PRISM^{TM} templates for each of the 6 assay methods. These were then checked by P14, 15 and 23, in particular for the identification or repeat outliers within the 6 or 7 replicated for each test concentrations and for the final acceptance criteria, by which the assay run was deemed to have passed and thus be of sufficient quality to be accepted into the database.

In addition record sheets for each assay which recorded the person conducting the assay, the cell details in terms of line and passage number etc, the solvent and dilution procedure, the quality control for the equipment used etc, i.e. the type of documentation required within a GLP compliant laboratory was also circulated. For the 3T3 and NHK NRU assays all these record sheets and templates were based on those employed in the NICETAM/ECVAM study (3T3 NRU Anon 2006, NHK NRU Anon 2006, NICETAM ECVAM study).

The Excel templates for the evaluation of the raw data and the Prism Hill function analysis, were those used in the NICETAM/ECVAM validation study. The acceptance criteria were modified for the particular assay as were the criteria for the identification and removal of outlier wells within the replicates employed for each concentration tested. Therefore the Excel templates ensure that all the raw data was recorded, the outliers identified, that date and the cell line, passage number, medium and serum batch number, chemical identity and CAS number and compliance with the acceptance criteria were recorded. The inhibitor concentration (IC) that reduced the control by 20, 50 and 80%, the IC20, 50 and 80, and then automatically calculated by linear extrapolation and also the data is converted for incorporation into the PRISM template that calculates the IC20, 50 and 80 from the Hill function curve. It is the results from the PRISM template that is used in the final analysis.

Each completed Excel template for each run for each chemical and for each assay endpoint was submitted to the WP2 leader for checking before being entered into the running database (Table in Appendix I) and then finally transferred into the AcutoxBase. The Excel templates were also made available to other participants in the study.

To test the robustness of the NHK NRU assay approach, and due to some problems with the NHK medium supplier stipulated in the NICETAM/ECVAM validation study, the supplier for this study was changed. Also not only passage 1 but also passage 2 cells were used from more than one donor and using NHK medium additives from different batches. The results of the positive control SLS, did show that these changes did not result in a significant variation

in results obtained in this study or the NICAETM/ECVAM validation study results obtained by P8.

Automation of the 3T3/NRU protocol

WP2 leader also liaised with WP3 over the transfer of the 3T3/NRU protocol and data analysis to the automated robotic system. Direct comparisons were made between the results obtained with the *in vitro* from the WP2 partners and the robotic results.

Solubility protocols

The solubility protocol used in the NICETAM/ECVAM study was used with minor adjustments and for all the chemicals. The results of this testing, as well as the solubility protocols were made available for all the participants.

Results from testing of the 97 reference chemicals in the WP2 assays

Only the assay runs that passed the acceptance criteria were employed in determining the IC_{50} means and standard deviations. To ensure comparability between the chemicals tested in the NICETAM/ECVAM validation study by P8 and the results obtained in the 3T3/NRU assay in this study, the SLS positive control was rerun together with other chemicals selected due to both high and low cytotoxicity. Prior to running test chemicals, P15 and P23 performed repeated runs with the SLS positive control to set their laboratory acceptance standard for the SLS positive control run with all test sets of chemicals. These were found to be very similar to those obtained by P8 both in this and the NICAETM/ECVAM study.

Two problems can be experienced with the NRU assay. One is the precipitation of the Neutral Red (NR) that results in lance shaped crystals that were almost impossible to remove. The second is the so called “ring of death”. This is observed in a plate where there is a ring of cells in the base of the well that does not take up any NR.
The precipitation problem can arise if the NR concentration is too high and also if the concentrated solution is not stored correctly. Being aware of that this can occur, the NR solution is filtered before use and the plates scanned for crystals. Precipitation also tends to occur more frequently when the NR concentrate is made up from the NR powder rather than purchasing a stock solution.

As for the ring of death, this occurs where the plates have had the medium aspirated of their normal medium and prior to adding the test chemical concentration. Thus if a number of plates are tested at one time they may be left for too long without added medium. Thus the remaining medium on the centre of the well and the meniscus around the edge keep the cells alive whilst a ring dries out and dies. The use of multichannel pipettes to add the test chemical dilutions can reduce significantly the time between aspiration and medium addition and reduce or even eliminate this problem.

For the ATP assay a luminometer was employed to read from the plate. It was found that a) luminometers had different sensitivities and b) that they could read from below or above the plates. Thus it was observed that P8 and P23 obtained different raw data reading for this assay because of the luminometers available.

The optical density reading from the spectrophotometers were far closer for the NRU assays even though different machines were used. For the fluorescence only P14 undertook the assay so it is not possible to say how reproducible results would be between different machines.

Whilst P8 and P14 laboratories were not GLP compliant to ensure comparability of results between laboratories undertaking the same assays, GLP procedures with regards to regular checking of the equipment used, along with documentation pertaining to all the aspects of each assay run were completed and retained.

3T3 and NHK/NRU assays

The SOP's were completed within the one month time frame. The running database was compiled by WP2 leader within an Excel spreadsheet. The transfer of the data to the AcutoxBase did take longer than expected due to the problems of direct transfer of the data from Excel sheets to the AcutoxBase, the systems not being directly compatible.

For the chemicals in the ACuteTox list that were also included in the NICETAM/ ECVAM study, the results from the three participating laboratories in this study were included (with permission of NICETAM), and the majority were not rerun.
The chemicals were ranked according to their mean IC_{50} generated from the Hill function curve (Table in Appendix I). Where the IC_{50} value was greater than the maximal testable concentration, then if toxicity occurred between the IC_{20} and IC_{50} values (without the IC_{50} being attained) the extrapolated IC_{50} was recorded. If this is not the case a greater then value was entered (Table in Appendix I). Thus as far a possible and at least for the first set of 47 chemicals they were all tested in at least 2 laboratories independently P8 and P15 where they were not tested in the NICETAM/ECVAM study. For the second set up to 97 chemicals some were tested in only one laboratory (P8 or IIVS) to meet the time restriction on this phase of the study. IIVS helped in this latter phase of the study being another of the laboratories that took part in the NICETAM/ECVAM study. Ninety four chemicals were tested in the 3T3/NRU assay and 88 for the NHK NRU assay (Table in Appendix I) (Clothier *et al* 2008).

ATP assay

For this assay all the data was generated within this study by P8 and P23. Only the reference chemicals the first 47 were tested in this assay. This is due to the fact that during the first 18 months there was a three month period when the assay kits were not available due to quality control problems during manufacture. Thus, it was decided after this wait to move to a different supplier and thus a different modified protocol. To test that the results between the two assays were comparable some rerunning of chemicals was required. It also became apparent that the results being obtained between the two laboratories involved, gave very different raw data. So it was decided that rather than attempt to extend the timing for the testing it was sensible to just test the first 47, as it was unlikely that this would be promoted as the basal cytotoxicity assay of choice.

Fluorescent total protein Fa32 and HepG2 and Fa32 NRU assays

The Fa32 assays were conducted in one laboratory that had previously published on these assays and employed them in the MEIC study (Dierickx 2003 and 2005). The HepG2 fluorescent protein assay was carried out in two laboratories (P14 and P23). 93 of the 97 chemicals were tested in the Fa32 assays and 58 in the HepG2 protein assay (Clothier *et al* 2008).

Analysis of data

Once the testing was completed, the data for the IC_{50} mean values was employed in a comparison with the *in vivo* data. This analysis was conducted independently initially by P26. All 6 basal cytotoxicity assays give similar results, which confirm the results from the MEIC study (Clemedson *et al* 1996 and 1998). The toxicity ranking of the compounds is the same when the cell types are compared, with the exception of colchicine, cyclohexamide, hexachlorobenzene, digoxin and 5-fluorouracil. Another statistical analysis of the 3T3 data was conducted at the end June 2010 (See presentation of results from WP9)

Discussions were entered into as to how to treat the greater than values for the comparative analysis. The question centered on using the top concentration tested as the actual value if it was not possible to obtain a reasonable extrapolated IC_{50}. A reasonable extrapolated IC_{50} was one where the highest toxic concentration was between the IC_{20} and IC_{50}. In this case the extrapolated result was employed. However, if no toxicity was seen then if the solubility problems did not allow a high enough concentration to be tested, then entering the top concentration testable was considered misleading.

Hence, in the *in vivo-in vitro* comparisons only the chemicals for which both *in vivo* and *in vitro* data were available, were examined (Sjöström *et al* 2008). Since there was a large database of results with the 3T3/NRU assay, and since there were 12 chemicals of the 88 examined with the NHK when compared with the 3T3/NRU, it was considered that the 3T3 NRU assay should be employed as the "gold standard" basal cytotoxicity assay for the prediction of the GSH and EU classes. Furthermore, the 3T3/NRU assay has been formally validated in the NICETAM/ECVAM study.

The NHK NRU assay was considered for the other aim that was to predict human toxicity effects. It was not thought as the gold standard for the GSH and EU classification, prediction since there are in addition concerns about metabolic effects in these early passage cells from different donors. This was a concern in that there is the potential for metabolic activation due to skin exposure to chemicals during the lifetime of the donor.

Whilst no noticeable difference was observed between the two different donors deliberately chosen even when tested in different laboratories, but sine the chemical list is limited the concern remains. Greater experience is required for the thawing and culturing to prevent premature differentiation down the squamous pathway. Thus, whilst human and the advantages that this may give, the disadvantages outweigh this benefit.