**Developmental toxicity**

The potential embryotoxicity of chemical substances in early stages of organogenesis can be assessed by the interference with differentiation and development of whole embryos in *in vitro* cultures.

**Objective & Application**

This protocol describes general conditions for dissection and culture of post-implantation rat and mouse embryos at early stages of organogenesis. The embryotoxicity testing in postimplantation rat whole-embryo culture is intended to identify substances, which induce malformation resulting in embryotoxicity.

The whole embryo culture technique possess features that are generally considered necessary in a teratogenicity screen, but due to the demanding procedure it might be unsuitable for routine use as a first step in a large scale screening program. However, the whole embryo culture has a valuable function in a tiered approach, at a second level of testing, when only a few compounds are to be considered and it can be employed to prioritise chemical compounds for further *in vivo* testing. There is also a possibility that this test could be used within ranking systems for families of chemicals. Furthermore, it has been used as an intermediate between screening and mechanistic studies. In this case, the *in vitro* test is used alongside *in vivo* studies (Brown *et al*., 1995).

According to the positive outcome of the recent ECVAM validation study, the Management Team concluded that, rather than representing a complete replacement, the test should be used in the context of testing strategies for the detection of embryotoxicants (Genschow *et al*., 2002).

Currently chemicals are tested for developmental toxicity with *in vivo* studies according to OECD Testing Guidelines (414, 415, 416, 421 and 422; OECD, 1983, 2001a,b, 1995 and 1996), EU test methods (B.31, B.34, B.35; EU, 2008) and segment 2 and 3 studies (teratogenicity and embryotoxicity; pre- and postnatal developmental toxicity) according to the ICH guidelines (ICH, 2005).

**Résumé**

Rat embryos with 1 to 5 somites are used in this method. In general, these embryos are relatively sensitive for xenobiotics as compared to older embryos. During 48 hours of culture, major aspects of organogenesis occur, including e.g. heart development, closure of the neural tube, development of ear and eye, brachial bars and limb buds. Interference during this period may lead to general retardation of growth and development or to specific malformations in one or several organ anlagen.

Embryos are cultured in rotating containers, thus facilitating the exchange of the culture medium with the gaseous phase above. The latter is essential for optimal oxygenation of the culture medium in view of the increasing demand for oxygen of the embryo. After culture, the morphology of the embryos is carefully assessed. Comparison of control embryos with exposed embryos forms the basis for conclusions regarding the embryotoxicity of tested compounds.

A comprehensive bibliographic review document "Post-implantation Whole Embryo Cultures (WEC) " is available as " Method Summary " in DB-ALM.

**Experimental Description**

**Endpoint and Endpoint Measurement:**

- **CELL VIABILITY:** Cell viability (heartbeat, yolk sac and allantoic circulation)
- **EMBRYO FUNCTIONALITIES (rat embryo):** Functionalities (heartbeat, yolk sac and allantoic circulation)
- **EMBRYO GROWTH (rat embryo):** Embryo growth (yolk sac diameter, crown-rump length, head length)
- **EMBRYO MORPHOLOGY (rat embryo):** Morphology (gross examination of embryo, including somite numbers, retardation of differentiation and specific malformations)

**Endpoint Value:**

- **IC\textsubscript{NOEC} FOR TMS:** maximum concentration that has no effect on the Total Morphological Score
- **IC\textsubscript{max} FOR MALFORMATIONS:** lowest concentration which shows a maximum rate of malformations
IC50 FOR MALFORMATIONS: concentration at which 50% of the embryos show malformations

Experimental System(s):

WHOLE EMBRYO CULTURE (rat)

Basic Procedure

Rat embryos are cultured on day 9.5 of gestation. To each embryo culture vessel 2 ml of rat serum is added. Compounds are dissolved and diluted in a proper solvent (e.g. Hanks' balanced salt solution, HBSS, DMSO or ethanol) and standard volumes of compound solutions are added to the culture serum in the culture vessel before addition of the embryo. The same volume of pure solvent is added to control cultures that is shown in prior experiments not to interfere with embryogenesis in culture. Embryos from each dam are distributed as evenly as possible over the control and various concentrations tested. Furthermore, the embryos are distributed so that the average initial somite number is nearly the same for each concentration.

Compound concentrations are always tested in the presence of a similar number of concurrent controls. After 48 hours culture, each embryo is transferred to a petri dish containing HBSS (37 ° C) and the embryos are scored in the same order in which they were put into culture. For details on the determination of the assay endpoints see section 2.4.

Data Analysis/Prediction Model

In the WEC assay an elaborate morphological scoring system was used to assess signs of malformation or retardation in embryos exposed over a 48-hour period as well as embryonic death. Before the ECVAM validation study the classification of the embryotoxic potential of a compound was mainly based on the nature of the effects observed and its potency was derived from the lowest effective concentrations necessary to elicit the effects. Since biostatistical evaluation had shown during the ECVAM validation study that the scoring system could not reproducibly be applied, two Prediction Models (PM1 and PM2) were developed. The second PM was created because the PM1 takes into account only parameters of differentiation and development. The PM2 incorporates cytotoxicity data for the differentiated mouse fibroblast cell line 3T3, which were derived from the Embryonic Stem Cell test (see section 3.3).

The PMs were applied to classify the embryotoxic potentials of the test chemicals of the validation study into three classes of embryotoxicity (non, weak and strong).

To compare the in vitro with the in vivo classifications, contingency statistics were obtained assessing the data accuracy, the method predictivity and the precision for all three toxicity classes of the test chemical. (Anon., 2002; Genschow et al., 2002)

For further details see section 3.3 "Prediction Model" of the attached Procedure Details.

An improved prediction model is under expert discussion. The use of this model would result in a reduction of the numbers of embryos compared to the prediction model described above (Chapin et al., 2008).

Test Compounds and Results Summary

Pharmaceuticals, consumer products, industrial and agricultural chemicals, food additives and contaminants (Brown, 2002; Piersma A.H. et al, 1995).

Discussion

An advantage of the embryo culture system over other culture assays is the fact, that many relevant developmental endpoints in the critical phase of organogenesis can be measured with this method. Results obtained so far with this protocol allow the identification of test compounds based on minimal effective doses tested and overall morphological appearance of the embryos. A limitation of the test system is the lack of biotransformation capacity (Hareng et al 2005). It has been reported that, non-specific and specific embryotoxic effects can principally be recorded with this assay. Inter-laboratories study showed good reproducibility and comparability of the test results (Piersma et al., 1995).

Furthermore, the ECVAM Validation Study has shown that the correlation between the in vitro data and in vivo data was good (accuracy 80%), according to the performance criteria defined, when including in the PM the cytotoxicity data obtained in the Embryonic Stem Cell Test (EST) (Prediction Model PM2).
The predictivity and precision for strongly embryotoxic test chemicals were reported to be excellent (100%). The predictivity for non-embryotoxic compounds (70%) and the precision for weakly embryotoxic compounds (65%) were considered sufficiently high (> l=65%). (Anon., 2002; Genschow et al., 2002).

A between-laboratory validation with the same substances used by Genschow et al. (2002) was performed by Piersma et al. (2008): they compared benchmark concentrations (BMC) derived from the individual dose-response curves for each substance (endpoints head length, crown-rump length and total morphological score) from 4 laboratories and found a high reproducibility (except for one laboratory, due to technical flaws). A comparison of in vitro with in vivo data for the same test substance did not reveal concordance between endpoints, because e.g. in vivo effects like delayed ossification and hydronephrosis cannot be scored in the in vitro test. The authors stated on the basis of a comparison of in vitro BMC and in vivo benchmark doses (BMD) that the replacement of in vivo animal studies by the whole embryo culture test is not yet feasible.

Two main innovations were considered to be valuable to be applied to the WEC test systems, namely the introduction of a metabolic system and gene expression profiling to study more subtle developmental changes, and were investigated during ReProTect project (July 2004-2009). A number of compounds which are known to change developmental toxicity potency were incubated in the presence of the different metabolic systems such as S9 fractions, human hepatocyte cultures and liver slices (Hareng et al. 2005).

A feasibility study was performed by Schenk et al. (2010) within the ReProTect project. They tested 10 chemicals with a test battery consisting of 14 in vitro tests. Developmental toxicity in vitro was examined by a combination of three test systems, including the WEC. The results from the WEC led to a correct prediction of the occurrence of in vivo observed developmental effects for 6 chemicals (4 correctly positive, 2 correctly negative) and to a false prediction for 3 chemicals (1 false positive, 2 false negative). One chemical gave ambiguous results in vivo and in vitro (Schenk et al., 2010).

Status

Participation in Evaluation Studies:

The in vitro rodent post-implantation embryo culture technique is employed in a large and still increasing number of laboratories. Various studies have addressed the possibility of using the embryo culture technique as an assay for embryotoxic potential of xenobiotic compounds. In an between-laboratory assessment reported by Piersma et al. in 1995, a good concordance of results across the laboratories was reported, the system showed a good sensitivity and specificity. A further assessment was realised (Piersma et al., 1996).

The Post-implantation whole embryo culture test as validated test system has subsequently been included in the “ReProTect” project (www.reprotect.eu, last accessed on 17.12.2010), a 5-years (July 2004-2009) integrated project funded by the European Commission under the EU 6th Framework Programme for Research, Technological Development and Demonstration (Hareng et al., 2005). The overall aim of the project was to develop a testing strategy to cover the entire mammalian reproductive cycle in the area of reproductive toxicity (Bremer et al., 2005; Hareng et al., 2005). The further development and validation of the WEC biotransformation system followed the modular approach presented by ECVAM in 2004 (Hartung et al. 2004). Within the frame of the ReProTect project, the aim for the WEC test system was to conclude module 2 (assessment of reproducibility of experimental data in same laboratory) by June 2007. However, the attempts to develop a metabolising system within the project have not been successful (Spielmann, 2009).

A feasibility study was performed by Schenk et al. (2010) within the ReProTect project (see chapter “discussion”). The results from the EST lead to a correct prediction of the occurrence of in vivo observed developmental effects for 6 out of 9 chemicals (Schenk et al., 2010).

Participation in Validation Studies:

The SOP presented in this DB-ALM protocol has participated in the international (ECVAM) embryotoxicity validation project: “In vitro tests for embryotoxicity: Prevalidation and validation of assays employing micromass cultures, rat embryo cultures and embryonic stem cells” (1996-2000).

Based on the successful outcome of the study, in October 2001 the ECVAM Scientific Advisory Committee (ESAC) unanimously endorsed the statement that the results obtained in the definitive phase
of the study with the Postimplantation Rat Whole-Embryo Culture assay were reproducible, the correlation between \textit{in vitro} and \textit{in vivo} data was good, and the test proved applicable to testing a diverse group of chemicals of different embryotoxic potentials.

ESAC therefore agreed with the conclusion of the Management Team of the study that the Post-implantation Rat Whole-Embryo Culture assay is a scientifically validated test which is ready to be considered for regulatory purposes. However, even if ESAC recognised that the three embryotoxicity methods evaluated do not represent replacements for current animal tests for reproductive toxicity as a whole, it has been underlined that they could provide suitable means for reducing and/or refining the use of animal procedures in the context of testing strategies (Anon., 2002).

\textbf{Regulatory Acceptance:}

Chapin \textit{et al.} (2008) stated that without supporting information from other test systems the whole embryo culture technique should not be used for human risk assessment purposes at the present time. Similarly, Piersma \textit{et al.} (2008) noted that the replacement of \textit{in vivo} animal studies by the whole embryo culture test is not yet feasible. More recently, Adler \textit{et al.} (2010) summarised that in spite of the standardisation and validation of the WEC the predictability and applicability domains are not sufficiently defined yet to allow regulatory implementation.

\textit{The protocol introduction has been updated in 2010 within the Thematic Review Project of European Partnership for Alternative Approaches to Animal Testing (EPAA).}

\textit{Last update:} December 2010
The protocol presents the standard operating procedure used in the validation study: "In Vitro tests for embryotoxicity: prevalidation and validation of assays employing micromass cultures, rat embryo cultures and embryonic stem cells".

This SOP, scientifically validated by the ECVAM Scientific Advisory Committee (ESAC) in October 2001 was included in the EU Integrated project "ReProTect" (www.reprotect.eu) for further development - introduction of a metabolic system and gene expression profiling.

*The DB-ALM is contacting the person responsible for the method to update or confirm the herewith included SOP. As soon as new information will become available this version will be updated.

Contact Details

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1. Materials

1.1 RAT STRAINS

Any rat strain that passes the quality checks with positive and negative control compounds and has a large number of embryos/litter may be used in this assay.

Rats are kept under spf conditions at 20-24 °C and at 50-70% humidity, and are allowed to feed and water ad libitum.

The health of the animals is checked daily from their general behaviour. Female rats of between 7 and 10 weeks old are acclimatised for at least 2 weeks before mating with males of at least 10 weeks old. For sera preparation, males of at least 250 g body weight are used.

1.2 TECHNICAL EQUIPMENT

Dissection microscope
Operation forceps and operation scissors
Pasteur pipettes
Petri dishes (diameter around 100 mm)
37 °C Incubator with rotation equipment (and microscope)
Rotation culture flasks (volume at least 10 ml)

1.3 CHEMICALS AND MEDIA

Hanks'balanced salt solution (HBSS) containing (g/l) CaCl2:: 0.185; NaCl: 8.0; KCl: 0.4; MgSO4: 0.2; NaHCO3: 0.308; glucose: 1.0; Na2HPO4: 0.075; KH2P04: 0.060; stored at 6 °C
DMSO
Ethanol p.a.
Ethanol 70%
T61 ® (Hoechst)
CO2 (technical grade)
Vacutainer tubes

1.4 SERA PREPARATIONS

Male rats are anaesthetized with CO2. The ventral body wall is opened and the aorta is prepared free. Blood is collected via an aorta punction using vacutainer tubes. Rat serum is centrifuged within one minute of collection during 5 minutes at 1800 rpm. Centrifuged serum batches are stored on ice. After
collection of all samples, sera are decanted and centrifuged once more for 3 minutes at 1800 rpm. Sera are then pooled and heat inactivated 30 minutes at 56 °C. Pooled sera are stored at -20 °C until use and are warmed to 37 °C just before use.

2. Methods

2.1 CONCENTRATION OF TEST CHEMICALS

To each embryo culture vessel 2 ml of rat serum is added. Compounds are dissolved and diluted in a proper solvent (e.g. HBSS, DMSO or ethanol) and standard volumes of compound solutions are added to the culture serum in the culture vessel before addition of the embryo. The same volume of pure solvent is added to control cultures, a volume that is shown in prior experiments not to interfere with embryogenesis in culture. Maximum solvent concentrations in culture are 1% aqueous medium (HBSS, aquadest), 0.125% DMSO, and 0.2% ethanol. Culture vessels are subsequently gassed before addition of embryos (see section 2.2.3).

Embryos from each dam are distributed as evenly as possible over the control and various concentrations tested. Furthermore, the embryos are distributed so that the average initial somite number is nearly the same for each concentration.

Compound concentrations are always tested in the presence of a similar number of concurrent controls.

2.2 ASSAY PROCEDURE

2.2.1 Mating procedure

Female rats are kept on a 12/12 hours light/dark cycle, with 6 a.m. to 6 p.m. designated as the dark period. Between 4 and 6 p.m., young adult nulliparous females in oestrus are allowed to mate with a male, and males and females are separated the next morning.

2.2.2 Embryo isolation

In the morning of day 10 of gestation (day of coitus is day 0), each dam is killed by an intracardiac injection with 0.2 ml T6 I®. The uterus is explanted via an incision in the ventral abdominal wall and transferred to an operation tablet. Conceptuses in their decidual tissue are removed from the uterus using sterile forceps and scissors. Isolated conceptuses are transferred to petri dishes containing sterile HBSS and the decidual tissue, Reichert membrane and parietal yolk sac of each embryo are carefully removed - leaving the visceral yolk sac intact- under a microscope using sterile microsurgical forceps. Embryos containing 1-5 somites will be cultured.

2.2.3 Culture conditions

Embryos are cultured under sterile conditions for 48 hours at 37 °C under continuous rotation at 20-40 rpm. Culture flasks are oxygenated twice daily for 30 seconds with increasing concentrations of oxygen. The oxygenation scheme in table 1 is given as an example, as optimal conditions may vary between individual laboratories. Embryo integrity is checked at 0-5-21-28-45 hours of culture - only if a microscope is present inside the 37 °C incubator - by the determination of caudal neural tube closure, embryo flexion, yolk sac circulation and heartbeat frequency (Appendix I). Time 0 hr is defined as the moment when the embryo is added to the culture flask.

Table 1: Oxygenation scheme

<table>
<thead>
<tr>
<th>Hours of culture</th>
<th>Concentrations of O2</th>
<th>CO2</th>
<th>N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>5</td>
<td>90</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td>21</td>
<td>20</td>
<td>5</td>
<td>75</td>
</tr>
<tr>
<td>28</td>
<td>20</td>
<td>5</td>
<td>75</td>
</tr>
<tr>
<td>45</td>
<td>40</td>
<td>5</td>
<td>55</td>
</tr>
</tbody>
</table>
2.3 DOSE-RESPONSE ASSESSMENT

Toxicity range finding is carried out using three embryos per compound concentration and using tenfold concentration intervals. Subsequently, taking TMS (see below) as the effect parameter, the highest ineffective concentration and a concentration which results in at least a 50% reduction of control TMS, as well as two intermediate concentrations are tested to a total of seven embryos per concentration. This test is in order to facilitate discrimination between specific embryotoxicity and general retardation. Concentrations tested may differ by a factor of 2 at minimum. The concentration of 1000 mg/l will be taken as the highest concentration to be tested for any compound. If at this concentration no effect occurs in seven embryos, no additional concentrations need to be tested.

2.4 ASSAY ENDPOINTS

After 48 hours culture, each embryo is transferred to a petri dish containing HBSS (37 °C), and its development is determined and described in detail (Appendix II). Embryos are scored in the same order in which they were put in the culture, with the aim to minimize the difference in culture duration between embryos.

Yolk sac diameter is measured under a microscope with an ocular micrometer and yolk sac vascularization, circulation and heartbeat are assessed. The yolk sac is then removed with microsurgical forceps and embryo crown-rump length, head length, the numbers of pairs of somites, embryo flexion and morphological development of all organ anlagen are determined. Somites are counted beginning at the forelimb bud in caudal direction, assuming that the somite located next to the middle of the forelimb bud is number 9. Morphological development of all organ anlagen are scored according to Appendix II as exemplified in Appendix IV (adapted from van Maele-Fabry et al., 1992). For intermediate stages of the development half points may be given, e.g. 3.5 if development is at a stage between 3 and 4. The total morphological score (TMS) is calculated as the sum of scores for all organ anlagen (A...R). Specific malformations defined as specific deviant morphology other than part of generalized delayed normal development are noted separately.

2.5 QUALITY CHECK OF THE EMBRYOS

Before culture, the integrity of the visceral yolk sac and the morphology of the embryo are carefully checked. To confirm normal development after culture, the development of concurrent control embryos, cultured in medium with added solvent only without the test compound, is assessed. Normality of control development is judged by comparison with historical controls from the same laboratory. The maximum acceptable rate of malformed embryos in the control group is 15% (1 out of 7).

2.6 QUALITY CHECK OF THE ASSAY

Before testing compounds of interest, the assay is checked using 5-fluorouracil (Sigma F6627) as a positive control at 0.03, 0.1 and 0.3, and 1 mg/l and penicillin G
sodium (Sigma PENN-NA) as a negative control at 1000 mg/l.

3. Evaluation: prediction of embryotoxic potential

3.1 DATA COLLECTION

Raw data are entered into the specific EXCEL spreadsheet developed for WEC data collection, using a separate file for each compound tested, and forwarded to the independent statistician.

3.2 ENDPOINTS

The end points scored in whole embryo culture can be divided into four groups (Appendix II). Growth parameters include yolk sac diameter, crown-rump length and head length. Functional parameters include heartbeat, yolk sac circulation, and allantoic circulation. General morphological parameters include final minus initial somite number, and morphological parameters A through R. Specific malformations may occur in any morphological parameter and are scored separately.

Assessment leads to assignment of each embryo to one of two categories, dependent on the endpoint affected as indicated below. General retardation is defined as a generalized effect on the embryo resulting in relatively small size and/or generalized retardation of differentiation in most or all organ anlagen in an otherwise proportionally normal embryo. Specific embryotoxicity is defined as a specific effect on one or a limited number of organ anlagen (malformation), likely to be ascribed to a selective action of the compound under study.

3.3 PREDICTION MODEL

Using linear discriminant analysis two PMs (PM1 and PM2) were developed for the WEC test from data of the preliminary phase of the ECVAM validation study.

Prediction Model 1

The test initially provided 7 different experimental endpoints which could contribute to distinguish among the three classes of embryotoxic chemicals non, weak and strong embryotoxic. The stepwise selection of variables was performed using analysis of discrimination.

Two endpoint values were accepted as function variables:

- $IC_{50, Mal}$ for malformations, which is the concentration at which 50% of all tested embryos of the group are malformed;

- $IC_{NOEC}$ for TMS, which describes the lowest concentration that has no effect on the Total Morphological Score.

<table>
<thead>
<tr>
<th>Function</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Function I</td>
<td>$18.08 \times \log (IC_{50, Mal}) - 11.56 \times \log (IC_{NOEC, TMS}) - 10.19$</td>
</tr>
<tr>
<td>Function II</td>
<td>$21.55 \times \log (IC_{50, Mal}) - 15.31 \times \log (IC_{NOEC, TMS}) - 10.65$</td>
</tr>
<tr>
<td>Function III</td>
<td>$8.70 \times \log (IC_{50, Mal}) - 8.53 \times \log (IC_{NOEC, TMS}) - 2.53$</td>
</tr>
</tbody>
</table>

Prediction Model 2

Since PM1 takes into account only parameters of differentiation and development, but no measure of cytotoxicity, this PM2 has been developed including cytotoxicity data provided by the cytotoxicity test with 3T3 cells, obtained in the Embryonic Stem Cell test evaluated in the same ECVAM validation study (see...
DB-ALM Protocol No.113).

The endpoint values measured in PM2 are:

- IC\textsubscript{max} for malformations, the lowest concentration at which a maximum malformation rate is obtained;

- the relative distance between IC\textsubscript{50} \textsubscript{3T3} (inhibition of the growth of 3T3 cells by 50\% of the control in the Neutral Red Uptake test) and IC\textsubscript{NOEC} for TMS.

<table>
<thead>
<tr>
<th>Function</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Function I</td>
<td>$0.21 \times (\text{IC}<em>{50}\text{ 3T3} - \text{IC}</em>{\text{NOEC}}\text{ TMS} / \text{IC}<em>{50}\text{ 3T3} ) \times 100 + 15.37 \times \log(\text{IC}</em>{\text{max}})$ - 23.58</td>
</tr>
<tr>
<td>Function II</td>
<td>$0.27 \times (\text{IC}<em>{50}\text{ 3T3} - \text{IC}</em>{\text{NOEC}}\text{ TMS} / \text{IC}<em>{50}\text{ 3T3} ) \times 100 + 17.71 \times \log(\text{IC}</em>{\text{max}})$ - 32.37</td>
</tr>
<tr>
<td>Function III</td>
<td>$0.093 \times (\text{IC}<em>{50}\text{ 3T3} - \text{IC}</em>{\text{NOEC}}\text{ TMS} / \text{IC}<em>{50}\text{ 3T3} ) \times 100 + 4.21 \times \log(\text{IC}</em>{\text{max}})$ - 4.23</td>
</tr>
</tbody>
</table>

**Chemicals Classification**

To precisely classify the chemicals according to the two PMs, the following procedure is applied: if the result of function I exceeds the results of function II and III, the chemical is classified non embryotoxic; if the result of function II exceeds the results of function I and III, the chemical is classified weak embryotoxic; finally, if the result of function III exceeds the results of functions I and II, the chemical is classified strong embryotoxic.
Appendix I

Development of embryos during culture

Project nr.:
Experiment nr.:
Date.:
Serum batch nr.:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMBRYO CODE</td>
<td></td>
</tr>
<tr>
<td>Initial somite number</td>
<td></td>
</tr>
<tr>
<td>Order of culture</td>
<td></td>
</tr>
</tbody>
</table>

**5 HRS**

<table>
<thead>
<tr>
<th>Neural tube</th>
<th>Cardiac frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yolk sac circulation</td>
<td></td>
</tr>
</tbody>
</table>

Flexion

Remarks

**45 HRS**

Cardiac frequency

Remarks

**21 HRS**

<table>
<thead>
<tr>
<th>Neural tube</th>
<th>Cardiac frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yolk sac circulation</td>
<td></td>
</tr>
</tbody>
</table>

Flexion

Remarks

**45 HRS**

Cardiac frequency

Remarks

Checklist for temperature and oxygenation

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Oxygen %</th>
<th>Time</th>
<th>Date</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 HRS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 HRS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 HRS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28 HRS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45 HRS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix II - Embryo scores after culture

<table>
<thead>
<tr>
<th>Growth parameters</th>
<th>Malformations (0 for normal/1 for malformed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yolk sac diameter (mm):</td>
<td>Yolk sac vessel defect</td>
</tr>
<tr>
<td>Crown-rump length (mm):</td>
<td>Allantois not fused with ectoplacental cone</td>
</tr>
<tr>
<td>Head length (mm):</td>
<td>Allantois large size</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Functional parameters (1 for normal/0 for abnormal)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yolk sac circulation</td>
<td>Heart ventrally turned</td>
</tr>
<tr>
<td>Allantois circulation</td>
<td>Posterior neuropore open</td>
</tr>
<tr>
<td>Heartbeat</td>
<td>Dorsal midline irregular</td>
</tr>
<tr>
<td>Somite development</td>
<td>Prosencephalon open</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Final somite number:</th>
<th>Mesencephalon open</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final-initial somite number:</td>
<td>Rhombencephalon open</td>
</tr>
</tbody>
</table>

Morphological scores (App.IV)

<table>
<thead>
<tr>
<th>A</th>
<th>Yolk sac blood vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Allantois</td>
</tr>
<tr>
<td>C</td>
<td>Flexion</td>
</tr>
<tr>
<td>D</td>
<td>Heart</td>
</tr>
<tr>
<td>E</td>
<td>Caudal neural tube</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Caudal neural tube</th>
<th>Otic vesicles deformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesencephalon narrow</td>
<td>Rhombencephalon narrow</td>
</tr>
<tr>
<td>Neural tube haemorrhagic</td>
<td>Rhombencephalon large and transparent</td>
</tr>
<tr>
<td>Craniocerebral appearance abnormal</td>
<td></td>
</tr>
</tbody>
</table>

© EURL ECVAM DB-ALM: Protocol
<table>
<thead>
<tr>
<th></th>
<th>Hind brain</th>
<th></th>
<th>Optic vesicles deformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>Mid brain</td>
<td></td>
<td>Branchial bars deformed</td>
</tr>
<tr>
<td>H</td>
<td>Fore brain</td>
<td></td>
<td>Maxillary process swollen</td>
</tr>
<tr>
<td>J</td>
<td>Otic system</td>
<td></td>
<td>Mandibular processes unapproached</td>
</tr>
<tr>
<td>K</td>
<td>Optic system</td>
<td></td>
<td>Mandibular process deformed</td>
</tr>
<tr>
<td>L</td>
<td>Olfactory system</td>
<td></td>
<td>Somites small</td>
</tr>
<tr>
<td>M</td>
<td>Branchial bars</td>
<td></td>
<td>Somites irregular</td>
</tr>
<tr>
<td>N</td>
<td>Maxillary process</td>
<td></td>
<td>Tail kinked</td>
</tr>
<tr>
<td>P</td>
<td>Mandibular process</td>
<td></td>
<td>Tail short and thickened</td>
</tr>
<tr>
<td>Q</td>
<td>Fore limb</td>
<td></td>
<td>Subcutaneous blisters</td>
</tr>
<tr>
<td>R</td>
<td>Hind limb</td>
<td></td>
<td>Haemmorhages</td>
</tr>
<tr>
<td></td>
<td>Total Morphological Score (TMS=S(A...R))</td>
<td></td>
<td>other</td>
</tr>
</tbody>
</table>

Date:  
Signature:
## Appendix III - Summary table per test compound

### COMPOUND:

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>CONCENTRATION in mg/l in culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Number of embryos cultured</th>
<th>Initial somite number</th>
<th>Final somite number</th>
<th>Yolk sac diameter</th>
<th>Crown-rump length</th>
<th>Head length</th>
<th>TMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mean ± S.E.M.</td>
<td>mean ± S.E.M.</td>
<td>mean ± S.E.M.</td>
<td>mean ± S.E.M.</td>
<td>mean ± S.E.M.</td>
<td></td>
</tr>
</tbody>
</table>

### NUMBER OF EMBRYOS WITH MALFORMATIONS

<table>
<thead>
<tr>
<th></th>
<th>Yolk sac</th>
<th>Allantois</th>
<th>Flexion</th>
<th>Heart</th>
<th>Neural tube</th>
<th>Hind, mid, fore, brain</th>
<th>Vesicular system</th>
<th>Facial processes</th>
<th>Somites</th>
<th>Others</th>
<th>Number of embryos with malformations</th>
</tr>
</thead>
</table>

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