

# A CRITICAL ASSESSMENT OF DOUBLE STAINING FOR FETAL SPECIMENS AS A ROUTINE IN PRENATAL DEVELOPMENT STUDIES

## UK Industrial Reproductive Toxicology Discussion Group (IRDG)

### INTRODUCTION

The morphology of the laboratory animal fetal skeleton has routinely been assessed by examination of the ossified areas of bone only. The harmonized OECD/EPA guidelines for Prenatal Development Toxicity have recommended additional examination of the fetal cartilage the bone precursor (US Environmental Protection Agency OPPTS guidelines 1998).

The most commonly used stains for fetal double staining are Alizarin Red S and Alcian Blue. Alizarin Red S binds to calcium and so stains selectively for the presence of ossified bone only. Alcian Blue stains by binding to mucopolysaccharides in the cartilage. The end product of this technique is a specimen in which the ossified portions of fetal bone are stained red, and cartilage portions are stained blue.

This document evaluates the benefits of performing routine double staining as a routine in regulatory prenatal development studies and presents the consensus of opinion reached by the Group.

### MATERIALS AND METHODS

Across the 7 participating UK laboratories 5 strains of rat and 4 strains of rabbit were used routinely. The days of pregnancy on which animals were routinely examined also varied between laboratories. For these investigations, the Wistar Han rat, on Day 21 of pregnancy, was selected for use in all laboratories to aid consistency in evaluation.

Species	Strain	Mean fetal weight	Mean litter size	Day of sacrifice*
Rat	Crj: WI (Glx/BRL/Han)BR	3.5g	11	Day 21 pregnancy
Rabbit	New Zealand White (4 suppliers)	35g - 38g	9 - 13	Day 29, 30

Day 1 pregnancy = day mating was recorded by positive smear in rats, day mating observed in rabbits

The following double staining methods were identified for evaluation: Kimmel (1981); Tyl (1996); Peters (1977); Whittaker and Dix (1979); Namiki (1992); adaptation of the Kimmel method; AstraZeneca Pharmaceuticals inhouse method. These methods included both consecutive (using 2 separate stain solutions) and combined (using one combined stain solution) double staining methods.

#### QUALITY STAINING

'Quality' cartilage staining was defined as pale, clear and consistent throughout the specimen, and reproducible across specimens. For particular areas of the skeleton this quality of stain was considered to be essential, and the selected staining methods were assessed against the quality of staining achieved in these areas, taking into account the simplicity of the method and the length of the staining schedule. The emphasis for the evaluation of the methods was on the quality of the cartilage stain.

The following listing contains the skeletal elements considered essential for quality cartilage stain:

#### SKULL

Periotic capsules, Mandibular articulation processes (rabbit only), Mandibular symphysis, Nasal cartilage, Hyoid, Occipital region, Palate [inner ear bones preferred]

#### VERTEBRAL COLUMN

Cartilaginous portion of centra, Lateral processes, Intervertebral discs,

#### RIB

Cervical supernumerary, Thoracolumbar supernumerary, Costal cartilage junctions, [rib/vertebra articulation in rabbits], [rib/vertebra articulations preferred, in rats]

#### STERNEBRA

Xiphoid cartilage, Intersternal cartilage, Cartilage of manubrium, Pectoral girdle

#### PECTORAL GIRDLE- FORELIMB

Scapula Blade, Joints, Carpals, Metacarpals, Phalanges, [scapula/clavicle/sternebralar articulation preferred, in rabbits]

#### PELVIC GIRDLE- HINDLIMB

Pubic symphysis, Cartilage of iliac crest, Ischial arch, Sacral vertebral fusion, Joints, Patella, Tarsals, Calcaneum, Astragalus, Metatarsals, Phalanges

### RESULTS

There was no notable difference between the results obtained using consecutive (2 separate stain solutions) or combined (one combined stain solution) double staining methods.

While some of the methods selected worked well within a particular laboratory, consistently reproducing quality specimens in different laboratories, or in the same laboratory, proved difficult.

Once a specimen has been 'overstained' with Alcian Blue, it is extremely difficult, or impossible, to remove the stain from the soft tissues sufficiently for examination of the skeleton form.

A quick and easy method of skin removal was not identified during these investigations. However, further work has shown that removal of fetal skin by immersion in a water bath at 50 degrees C for more than 10 minutes was very effective.

Alcian Blue stain will not penetrate skin. Slitting the skin down the vertebrae, across the occipital region and shoulders, down the limbs across the paws and down each digit does improve its penetration, but even this will not produce consistently quality results. It is also a difficult technique and can result in damage to the fragile skeleton.

The length of time that specimens are retained on ethanol prior to staining in Alcian Blue was identified as a factor which resulted in difficulties with clearing the stain from the soft tissues.

Any tagging attached to the fetus impedes cartilage stain penetration

All fat and visceral debris must be removed from the carcass to facilitate effective staining (see figure 1)



Figure 1. Lateral view of rat specimen (x8) showing poor staining around cervical and lumbar regions, resulting from inadequate removal of fat and visceral debris.

The rostral portion of the tongue must be removed to improve stain uptake in the palate. The occipital portion must be left in situ as it contains the hyoid bone.

Pale staining for areas of detail (eg joints) is much preferred to dark staining, as it greatly improves the clarity and detail of the processes and condyles

Slight alteration to the concentration of Alcian Blue stain does not appear to make an appreciable difference in the degree or depth of cartilage stain achieved.

It may be desirable to forego the Alcian Blue stain from some body areas in order to achieve ease and consistency of specimen preparation (see figure 2)



Figure 2. Lateral view of rat specimen (x8) showing well defined double staining, except in the skull paws and tail



Figure 3. Lateral view of rat specimen (x8) showing good stain uptake and clarity

#### PREFERRED TECHNIQUES

Rats: Whittaker and Dix method (Whittaker and Dix 1979) with Malls solution stage added after staining stage  
Namiki method using acetone for dehydration (Namiki 1992)

Rabbits: 1st day am specimens into 95% ethanol  
2nd day am into Alcian Blue stain (1.25g in 8 litres IMS, 2 litres acetic acid)  
3rd day am into 95% ethanol  
3rd day pm into 2% KOH and 0.002% aqueous alizarin red s  
5th day pm into 1:1 ethanol: glycerol

### DISCUSSION

All Alcian Blue staining techniques investigated revealed marked variation in the quality and consistency of stain uptake and soft tissue clearing. In some instances this was easily addressed by improving the specimen preparation, for example, removal of the tongue resulted in notable improvement of the quality of the Alcian Blue staining of the palate bones. However, some of the methods tested consistently produced poor staining in specific body areas.

The problem of stain penetration could be overcome by assessing cartilage formation at an earlier stage of development than is normally selected for skeletal assessment as effective penetration of Alcian blue stain is dependent on the amount of keratin in the skin ie on the age of the fetus. Alternatively, the decision to double stain specimens could be issue driven, performed as and when issues relating to bone formation arise. Once a potential problem has been identified using single Alizarin red s stain in a routine regulatory study, an additional study would be set up to address any specific issues. In these studies, isolated areas of the skeleton could be selected for staining, which would greatly improve the speed and quality of staining, and avoid the confusion and inconsistency of whole body cartilage preparation and examination

Before the decision is made to perform double staining routinely on regulatory studies, consideration should be given to what observations are to be recorded at the skeletal examination, and how the data are to be reported.

The structure and stage of development of both the cartilage and bone formation throughout the skeleton could be assessed and recorded.

To be able adequately and accurately to assess the relevance of the information recorded, it is necessary to collect an extensive amount of background data. These data will provide details of the range and occurrence of every variation in cartilage formation and every stage of development of each cartilaginous structure visible at the stage of development at which the fetuses are being examined.

It is difficult to envisage how any accurate evaluation of the effects of treatment on the developing fetus could be made without this detailed knowledge.

Only changes to the structure of the cartilage could be recorded; whereas structure and stage of development could continue to be recorded for bone formation

Using this approach for examinations would allow background information on bone formation to be used in assessing the relevance of the information collected, as any changes to the cartilage would be more obvious and less open to interpretation

Only changes in cartilage formation that are associated with ossification changes could be recorded.

The initial preference of the UK Industrial Reproductive Toxicology Discussion Group is that cartilage evaluation should be reserved as a secondary investigative tool once problems have been identified in the ossified bone, in initial investigations.