

# Non-Destructive and Rapid Skeletal Visualization in DART studies

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## Abstract

Developmental and reproductive toxicology (DART) studies are required for new chemical entities going to market to ensure that there are not adverse developmental consequences associated with the chemical. These studies involve dosing a pregnant female animal (e.g. mouse, rat, rabbit) with the compound and evaluating the effect of the chemical on the offspring. This evaluation involves the parallel processes of histopathology and gross morphological evaluation wherein the most time-consuming component of DART studies is the performance of gross skeletal evaluation. Since the introduction of Thalidomide into the marketplace, close attention has been paid to the impact of chemicals on skeletal development and DART studies require the visualization of gross skeletal morphology for abnormalities. The current methodology for conducting skeletal evaluation involves digesting soft tissue using a strong base (potassium hydroxide) and staining the bone with alizarin and cartilage in some cases with alcian. This methodology is primarily unchanged from the skeletal visualization method first presented by Alden in 1926<sup>1</sup>. However, the problem with this approach is that while it results in samples with bones that can be easily visualized, the process takes one to two weeks and requires constant oversight as it is a destructive process that can result in completely over digesting samples. Within the last several years, many tissue clearing approaches have been developed that render tissues transparent for molecular visualization in 3D. We have leveraged the learnings from these tissue clearing approaches to develop Visikol® TOX™ which is a tissue clearing based approach for skeletal visualization for use in DART studies. Instead of digesting tissues away for skeletal visualization, we render them transparent in a four step process that allows for facile skeletal visualization. This process results in reducing overall DART study time by up to 30%. Through the work described here, we conducted a 3<sup>rd</sup> party GLP study at Product Safety Labs (Dayton, NJ) to compare the Visikol TOX approach to the traditional KOH-based tissue processing methodology. It was demonstrated that both approaches allowed for skeletal evaluation, but that the Visikol TOX approach was considerably faster.

## Materials and Methods

To demonstrate the benefits of the Visikol TOX methodology, we conducted an equivalency study comparing the Visikol TOX methodology directly to the traditional KOH methodology for fetal mice, rats and rabbits. The 3<sup>rd</sup> party study was conducted by Product Safety Labs and focused on single staining with Alizarin red S.

### Animals

Two timed pregnant animals of each species (rabbits, rats and mice) were received on October 6, 2016, from Charles River Laboratories, Inc. Rabbits were received on GD 20 from Quebec City, Canada. Rats and mice were received from Raleigh, NC (USA), on GD Days 12 and 10, respectively. Following eight days of acclimation and a general health check, animals were euthanized. The rabbits were euthanized on GD 28; rats on GD 20; and mice on GD 18.

- **Rabbits:** Eleven fetuses were collected, with five used for controls and six for Visikol TOX.
- **Rats:** Sixteen fetuses were collected, with half used for controls and half for Visikol TOX.
- **Mice:** Fourteen fetuses were collected, with half used for controls and half for Visikol TOX.

### KOH Processing Method

Rabbit fetuses were skinned. All fetuses were processed according to the diaphonization procedure used for skeletal visualization with Alizarin S stain and KOH digestion as follows (times approximate):

- Dehydrated in 70% ethanol for 2 weeks and the eviscerated.
- Transferred to Alizarin S stain for 24 hours.
- Transferred to 1% KOH solution for 9 hours (mice), one day (rats) or three days (rabbits) – all times  $\pm$  20%.
- Transferred to 70% ethanol-glycerin mixture (2:1) for 1-2 Days.
- Transferred to 70% ethanol-glycerin mixture (1:1) for at least 1 Day.

### Visikol TOX Processing Method

Rabbit fetuses were skinned. All fetuses were processed according to the proprietary Visikol TOX skeletal visualization technique. The technique below was used as a default (times approximate):

- Eviscerated and placed whole in 70% ethanol and left overnight (>16 hours).
- Placed whole in pretreatment solution for 2 hours, but not exceeding 3 hours.
- Transferred directly to Alizarin S stain for 1-2 Days.
- Transferred to post treatment solution for 1-2 Days.
- Transferred to clearing treatment until clear ( $\leq$  1 Day).

### Tissue Evaluation Criteria

The Visikol and KOH diaphonization specimens were compared using skeletal visualization. The endpoints included (but were not limited to):

- C1-C8 cervical vertebrae for clarity and ability to distinguish important anatomical features.
- Sacral and caudal vertebrae for clarity and ability to distinguish.
- Clarity of lower mandible.
- Clarity of tarsals, metatarsals, carpals, metacarpals.
- Durability of specimens to physical manipulation.
- Flexibility of specimens to physical manipulation.

## Skeletal Processing Comparison

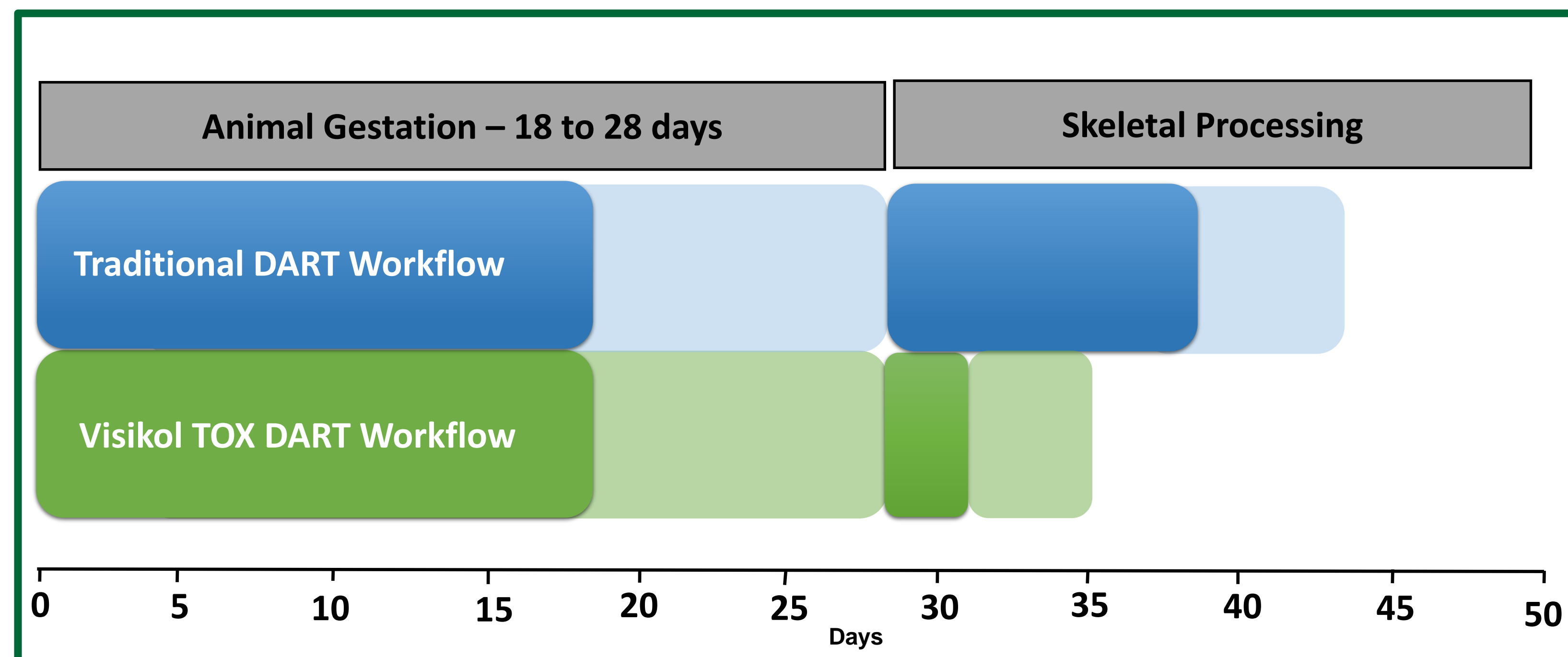


Figure 1. Overview comparing overall Visikol TOX skeletal processing timeline to traditional KOH based skeletal processing workflow.



Figure 2. Fetal animals that have been processed with the traditional KOH-based method. A) GD28 fetal rabbit, B) GD20 fetal rat, C) GD18 fetal mouse.



Figure 3. Fetal animals that have been processed with the Visikol TOX method. A) GD28 fetal rabbit, B) GD20 fetal rat, C) GD18 fetal mouse.

## Results and Discussion

The study demonstrated that specimen preparation and alizarin staining using the Visikol TOX approach was more rapid than the traditional skeletal processing technique. The Visikol TOX technique allowed for complete skeletal processing in as little as 48 hours. However, both processes resulted in the same end-point (Figure 2, Figure 3) wherein skeletal features were easily discernable.

## References

1. Alden BD: A note on the staining of the skeleton of cleared specimens with Alizarin red S: Stain Technology 1962; 1: 123-124.