Mutation Research 744 (2012) 36–41

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
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# Two new approaches to improve the analysis of BALB/c 3T3 cell transformation assay data

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ARTICLE INFO

*Article history:*
Received 30 November 2011
Accepted 1 December 2011
Available online 10 December 2011

*Keywords:*
BALB/c 3T3 cell transformation assay
Statistical analysis
Analysis programme
Non-monotone concentration response
(Negative binomial) Generalised linear model
William's-type protected tests

ABSTRACT

Validation activities of the BALB/c 3T3 cell transformation assay (CTA) – a test method used for the assessment of the carcinogenic potential of compounds – have revealed the need for statistical analysis tailored to specific features of BALB/c 3T3 CTA data. Whereas a standard statistical approach for the Syrian hamster embryo (SHE) CTA was considered sufficient, an international expert group was gathered by the European Centre for the Validation of Alternative Methods (ECVAM) to review commonly applied statistical approaches for BALB/c 3T3 CTA. As it was concluded that none of the commonly applied approaches is entirely appropriate, two novel statistical approaches were found to be recommended for the evaluation of BALB/c 3T3 CTA data accounting for possible non-monotone concentration–response relationship and variance heterogeneity: a negative binomial generalised linear model with William's-type downturn-protected trend tests and a normalisation of the data by a specific transformation allowing for application of a general linear model that estimates effects assuming a normal distribution with William's-type protected tests. Both approaches are described in this article and their performance and the quality of the results they generate is demonstrated using exemplary data. Our work confirmed that both approaches are suitable for the statistical analysis of BALB/c 3T3 CTA data and that each of them is superior to commonly used methods. Furthermore, a procedure dichotomising data into negatives and positives is proposed which allows re-testing in cases where inconclusive data are encountered. The scripts of the statistical evaluation programs written in R – a freely available statistical software – are appended including exemplary outputs (Appendix A).



## 1. Introduction

In validation of toxicological test methods, statistical analysis can facilitate data evaluation. However, when basing data analysis on adequate statistical inference, several aspects have to be considered. First of all, the statistical approach should appropriately address the purpose of the toxicological test method, taking into particular account the statistical characteristics of the measured variable(s)/endpoint(s) and data. Furthermore, it should be robust in the sense that it is applicable to most if not all data that can be expected to be generated by the test method in practice. Finally, when using the data for the intended purpose of the test method the statistical approach should provide results that support, or in the best case match, the biological relevance. Statistical methods with these properties should ultimately manifest a transparent and objective data analysis. They also should provide a sound basis for the interpretation of the test method data allowing the derivation of a prediction model, which is a key prerequisite for assessing the test's reliability and relevance.

For more than three decades, a variety of BALB/c 3T3 cell transformation assay (CTA) protocols have been applied mostly for research purposes, many of them referring to the pivotal work of Kakunaga [1]. Whilst they commonly have used the number of transformed foci type III per dish as endpoint, the protocols to obtain data for this endpoint differed, mainly with regard to the BALB/c 3T3 clone used, the set up of the

*Abbreviations:* CTA, cell transformation assay; ECVAM, European Centre for the Validation of Alternative Methods; GLM-NB, negative binomial generalised linear model; NT, Nishiyama transformation; SHE, Syrian hamster embryo; VMT, Validation Management Team.

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doi:10.1016/j.mrgentox.2011.12.002

experiments and the exposure conditions of the cells. Under those circumstances, it is likely that protocol specifications affected the statistical characteristics of that common endpoint. To our knowledge, however, the effect of different protocols on the number of transformed foci type III has never been investigated systematically.

Unfortunately, protocols available for the BALB/c 3T3 CTA do not address data analysis [2]. As a possible consequence of this, different approaches for data evaluation have been proposed. Some authors analyse the number of foci per dish, *e.g.* by means of linear models and analysis of variance methods (often combined with pairwise *t*-tests) after data transformation [3,4], by means of the non-parametric Mann–Whitney *U*-test [5] or by a parametric approach using a possibly modified Poisson distribution [6]. Others calculate the proportions of dishes showing foci and apply a test for proportions, *e.g.* Fisher's exact test [7,8].

When starting the prevalidation study of the BALB/c 3T3 CTA and the Syrian hamster embryo (SHE) CTA, appropriate statistical data analysis was required for both assays. The Validation Management Team (VMT) recommended to use the standard approach for the SHE CTA, *i.e.* Fisher's exact test or, if indicated, the Cochran-Armitage trend test, which is a widely accepted and routinely used test for linear trend [9,10]. For more detailed information on the prevalidation studies the reader is referred to Corvi et al. in this issue [11].

Regarding the BALB/c 3T3 CTA, the VMT consulted experts in the field of toxicological and pre-clinical statistics for advice. Based on the expert group's evaluation of the statistical appropriateness and applicability of commonly used approaches accounting for crucial statistical characteristics of the BALB/c 3T3 CTA data, it was concluded that none of those approaches suit these data. Therefore, two different methods, one based on a negative binomial generalised linear model (GLM-NB) [12] and another one based on the Nishiyama transformation (NT) [13] that allows the application of a general linear model estimating effects assuming a normal distribution, were recommended. As both were applied to the data generated in the BALB/c 3T3 CTA prevalidation study, we here present the two methods in detail, demonstrate their applicability to several examples of BALB/c 3T3 data and provide recommendations for potential users.

## 2. Materials and methods

### 2.1. Design of the BALB/c3T3 CTA and properties of the respective data

In order to be able to develop an appropriate statistical analysis of BALB/c 3T3 CTA data, their relevant properties needed to be identified. Based on available data for chemicals, both inducing and not inducing transformed foci type III, the following assumptions about the data to be analysed were made:

- The experiment is designed such that usually the responses of three to seven concentrations of a test chemical are compared against the response of a vehicle control.
- For each control and concentration 10 dishes will usually be used. At maximum, no more than one dish should be lost, *e.g.* due to bacterial contamination.
- Results are expressed as number of foci per dish.
- For focus-inducing chemicals, non-monotone concentration–response curves have to be expected, as at higher concentrations cytotoxicity may impair focus formation.
- The positive control serves for quality assurance purposes only and is not taken into account in data analysis.

A typical example of BALB/c 3T3 CTA data with seven concentrations is presented in Table 1.

Furthermore, the experts noted early on that all ten dishes of the control, but also those of a treatment concentration, may display the same number of transformed foci. Although this would in general occur in cases when no transformed foci were detected as it can been seen in the vehicle control and the second lowest concentrations of the example in Table 1, it could with some small probability also happen when only a few foci were observed. In such cases the empirical estimation of relevant distribution parameters could degenerate (*e.g.* with mean and dispersion estimated to be zero) which could seriously affect the outcome of any subsequent statistical inference.

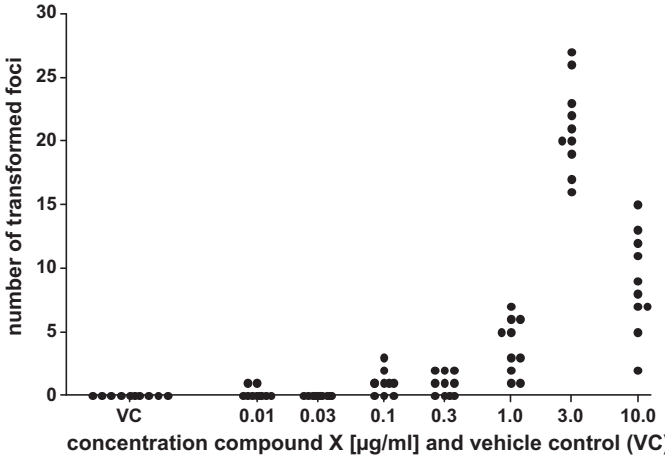



**Fig. 1.** Graphical representation of data from a BALB/c 3T3 cell transformation assay.

Taking these basic data characteristics into account, the expert group noted that the variability between dishes is an important feature of the response at each concentration and needs to be taken into account. Pooling the number of foci over the dishes may mask effects leading to an underestimation of variance, as the inter-dish variability would be assumed to be zero. Therefore, the dish was defined as the experimental unit (see also [14,15]).

Furthermore, it was agreed that concentrations, although they could be used in a quantitative manner (ranging from 0.01 to 10 μg/ml in the example of Table 1), should be considered as qualitative factor levels, *e.g.* as it is done when using ANOVA methods. Consequently, the concentration–count relationship should be simply assessed for the presence of potential trends using a qualitative approach and at this stage no more sophisticated quantitative dose–response modelling should be applied.

### 2.2. Recommended statistical data analysis

Based on the data properties outlined above (Section 2.1), several statistical evaluation methods were considered. It was observed that the variability between dishes can be well taken into account by a quasi-Poisson model developed for counts, where a dispersion parameter is estimated from the complete data [16]. This led to the agreement that a negative binomial generalised linear model (*i.e.* GLM-NB) with William's-type downturn-protected trend test [12] matched the particular statistical properties of the BALB/c 3T3 CTA well. In addition, a general linear model estimating the effects assuming a normal distribution of transformed data was proposed as an alternative analysis method. In this case, a general linear model with William's-type downturn-protected trend test is applied after normalisation of the data according to Nishiyama et al. [13] using the formula $y_{ij} = \sqrt{x_{ij}} + \sqrt{x_{ij}+1}$, where $x_{ij}$ represents the number of observed foci in dish $j$ of treatment/control $i$ and where $y_{ij}$ denotes the respective transformed data.

In contrast to commonly used multiple tests, like Dunnett's test [18], the William's-type protected tests were recommended because they were considered as being especially suited for non-monotone dose–response curves as typically observed in CTAs. A downturn in the response at high concentrations because of cytotoxicity is frequently observed, as can be seen in Fig. 1, which displays the number of transformed foci of Table 1.

Fig. 1 highlights that the highest concentration (10 μg/ml) of compound X induces less transformation than the preceding concentration (3 μg/ml), up to which a monotone concentration–response curve was observed. Such downturn effects are handled well by the William's-type downturn-protected trend test [12]. In order to demonstrate the aspect of downturn effect in more detail, a simplified case with three treatment concentrations T1, T2 and T3 and a vehicle control only was considered. In this case, the result can be associated with one of six possible types of concentration–response curves, which are schematically presented in Fig. 2. Note that this representation of curves only discriminates between concentrations resulting in responses similar to that of the vehicle control and concentrations resulting in responses higher than that of the vehicle control. For example, Fig. 2(a) reflects the case in which the first two concentrations T1 and T2 are similar to the vehicle control and where T3 gives a higher response. Amongst the six possible curves, downturn effects are present in Fig. 2(d), (e) and (f).

It was noted that the William's-type downturn-protected trend test is a multiple test procedure such that a conventional significance level to control the type I error rate, as *e.g.* 0.05, may not be applicable. Therefore a lower significance level will be required. It was proposed to lower the significance level to a default value of 0.01 to adjust to some degree for multiple comparisons.

**Table 1**
Example of data from a BALB/c 3T3 cell transformation assay displaying the number of type III foci per dish.

| Treatment | Dish number | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Vehicle control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Positive control | 12 | 17 | 16 | 8 | 16 | 21 | 15 | 13 | 9 | 10 |
| Compound X: 0.01 μg/ml | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Compound X: 0.03 μg/ml | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Compound X: 0.1 μg/ml | 0 | 1 | 0 | 1 | 2 | 1 | 3 | 1 | 1 | 0 |
| Compound X: 0.3 μg/ml | 1 | 1 | 1 | 2 | 0 | 2 | 2 | 0 | 0 | 0 |
| Compound X: 1 μg/ml | 3 | 7 | 1 | 1 | 3 | 2 | 5 | 6 | 6 | 5 |
| Compound X: 3 μg/ml | 21 | 16 | 26 | 19 | 22 | 23 | 20 | 17 | 27 | 20 |
| Compound X: 10 μg/ml | 7 | 5 | 7 | 13 | 9 | 11 | 8 | 2 | 15 | 12 |

The issue of distribution parameters estimated equal to zero was approached pragmatically. In case the location parameter estimate would be zero, *e.g.* as in the case of the vehicle control in Table 1, one focus is simply artificially added to one arbitrarily selected dish out of the ten dishes. This is assumed to be a minor data manipulation which can be expected to have no substantial effect on the statistical property of subsequent data analysis. The same manipulation would resolve issues arising from an estimated dispersion parameter of zero, *e.g.* in the case when all dishes of a concentration have the same number of transformed foci.

Finally, it has to be noted that most available computational implementations of the proposed approach required a balanced design. Consequently, the case of lost dishes, *e.g.* due to bacterial contamination, could not be accommodated. As a solution it was proposed to replace the missing outcome by the median of the remaining values. Although such a plug-in procedure potentially reduces the between-dish variance, a possible bias from this ad hoc missing value imputation method can be expected to be negligible.

Recognising the aim of the series of papers presented in this special issue and the readership we refrain here from going into the more technical statistical details. However, we annexed scripts of both statistical approaches in R, a statistical software freely available at http://www.r-project.org/. Some guidance on how to use these scripts, *e.g.* regarding proper data input, and how to interpret the output is also provided there.

## 3. Results

The performance of the two recommended methods, the GLM-NB and the NT, is demonstrated using data from three BALB/c 3T3 CTA experiments generated in the prevalidation study. As demonstrated in Fig. 3, experiment A shows only a few transformed foci, experiment B exhibits a monotone concentration–response curve, and experiment C suggests a downturn of response at higher concentrations. In order to facilitate representation we use here only five concentrations per experiment by excluding concentrations not contributing to the overall shape of the concentration–response curve. The respective complete experimental data can be found in the paper of Tanaka et al. in this issue [19].

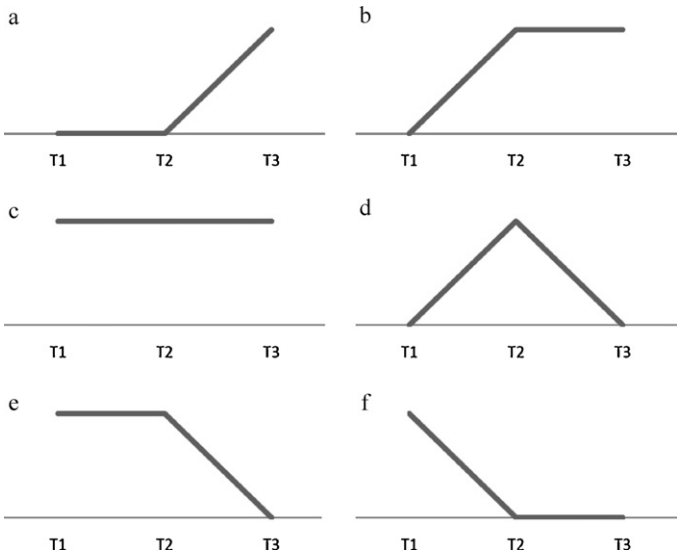



**Fig. 2.** Schematic representation of six concentration dose–response curves (a–f) addressed with William's-type downturn-protected trend test for an experiment with three treatment concentrations (T1, T2 and T3).

For each experiment 15 different concentration–response curves were evaluated by William's-type protected tests. The schematic shapes of these curves are shown in the second column of Table 2. Table 2 also lists the *p*-values resulting from the application of the two chosen statistical analyses to the experimental data.

For experiment A, where no increase of the number of foci is obvious, all *p*-values, both for GLM-NB and NT, are larger than 0.01 indicating no statistically significant effect according to the default selected above.

When analysing experiment B, it has to be noted that in one dish with the concentration 25 μg/ml a marked increase of the number of foci was observed. As this response pattern is typical, although rare, for the BALB/c 3T3 CTA, all dishes were considered to be relevant for the analysis. Applying the NT-approach, the four concentration–response curves C1–C4 resulted in statistically significant *p*-values < 0.01 (Table 2). Amongst them, the curve C1, *i.e.* the curve describing an effect of the highest concentration (150 μg/ml) only, was the most significant one. In contrast, the GLM-NB-approach resulted in a total of eight statistically significant curves (C2–C5, C8, C9, C11, C12), of which C4, *i.e.* the curve describing an effect of the four highest concentrations (25–150 μg/ml), had the lowest *p*-value.

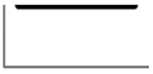
Analysing experiment C, which by visual inspection suggests the presence of a downturn at the highest concentration, both approaches were concordant and resulted in *p*-values < 0.01 for the same concentration–response curves, *i.e.* C1–C11. Furthermore, in both analyses curve C7, which describes an effect of the third and fourth and a downturn of the fifth concentration, was the most significant curve. However, the *p*-values of the GLM-NB tended to be lower than those of the NT.

## 4. Discussion

The outcome of the application of the NT- and the GLM-NB-approach to the data of three representative experiments largely met the expectations from the visual evaluation of the graphical presentation of the data. Data with apparently no treatment effects as well as different effects resulting in apparently monotone and non-monotone concentration–responses could be adequately addressed. The *p*-value of 0.01 discriminated reasonably well between presence and absence of effects. The GLM-NB tended to result in lower *p*-values and may be the more powerful method, at least for data such as those examined in this work.

The results for experiment B suggested that the GLM-NB-approach might better reflect concentration–response curves. In this example, the GLM-NB resulted in the lowest *p*-value for the curve describing effects starting at 25 μg/ml, whereas the

**Table 2**
*p*-Values obtained with both the Nishiyama transformation (NT) and negative binomial generalised linear model (GLM-NB) methods for three different BALB/c 3T3 cell transformation experiments (as displayed in Fig. 3), including schematic representation of the 15 concentration–response curves (C1–C15) addressed with William's-type protected tests. In case of *p*-values < 0.01, the smallest *p*-value is in bold type.

| Concentration–response curves | | Experiment A: *p*-values | | Experiment B: *p*-values | | Experiment C: *p*-values | |
|---|---|---|---|---|---|---|---|
| Name | Shape | NT | GLM-NB | NT | GLM-NB | NT | GLM-NB |
| C1 |  | 0.989635 | 0.985702 | **0.002121** | 0.038473 | 0.004477 | 0.007328 |
| C2 |  | 0.947399 | 0.887324 | 0.004242 | 0.004954 | 0.000046 | 0.000005 |
| C3 |  | 0.976099 | 0.941133 | 0.008362 | 0.000839 | 0.000008 | <0.000001 |
| C4 |  | 0.943290 | 0.893715 | 0.008246 | **0.000032** | 0.000103 | 0.000001 |
| C5 |  | 0.965500 | 0.927190 | 0.037675 | 0.000107 | 0.001173 | 0.000025 |
| C6 |  | 0.772007 | 0.666449 | 0.064612 | 0.099603 | 0.000021 | 0.000101 |
| C7 |  | 0.947396 | 0.887325 | 0.053771 | 0.013335 | **0.000004** | **<0.000001** |
| C8 |  | 0.886826 | 0.821479 | 0.032713 | 0.000773 | 0.000087 | 0.000002 |
| C9 |  | 0.943290 | 0.893709 | 0.119477 | 0.001611 | 0.001917 | 0.000110 |
| C10 |  | 0.989640 | 0.985701 | 0.146344 | 0.109839 | 0.000045 | 0.000183 |
| C11 |  | 0.924887 | 0.887321 | 0.054190 | 0.005480 | 0.001761 | 0.000714 |
| C12 |  | 0.967964 | 0.941135 | 0.213057 | 0.009760 | 0.024958 | 0.010054 |
| C13 |  | 0.668076 | 0.666445 | 0.065419 | 0.038615 | 0.187207 | 0.288580 |
| C14 |  | 0.924889 | 0.887325 | 0.356898 | 0.056673 | 0.409299 | 0.529274 |
| C15 |  | 0.989635 | 0.985701 | 0.889467 | 0.994610 | 0.781761 | 0.903086 |

NT detected an effect related to the highest concentration, *i.e.* 150 μg/ml, only.

With suitable statistical approaches both delivering a number of *p*-values for a single experiment, the interpretation of these data in biological terms can be addressed. As usual in this field the biological effect is dichotomously expressed as negative or positive; a simple interpretation procedure would be to call an experiment positive if at least one *p*-value would be <0.01, and negative otherwise. Applying this procedure to the outcome of the statistical methods, for both approaches experiment A would be negative and experiments B and C would be positive.

Alternatively and as proposed by the VMT, the well-established interpretation procedure of the SHE CTA could be adopted: if either two consecutive significant concentrations or one significant concentration with a positive trend [9,17] are present, then a positive, *i.e.* transformation-inducing effect of the treatment is concluded. Applying this interpretation procedure, experiment A would be called negative for both statistical approaches. Likewise, the effects

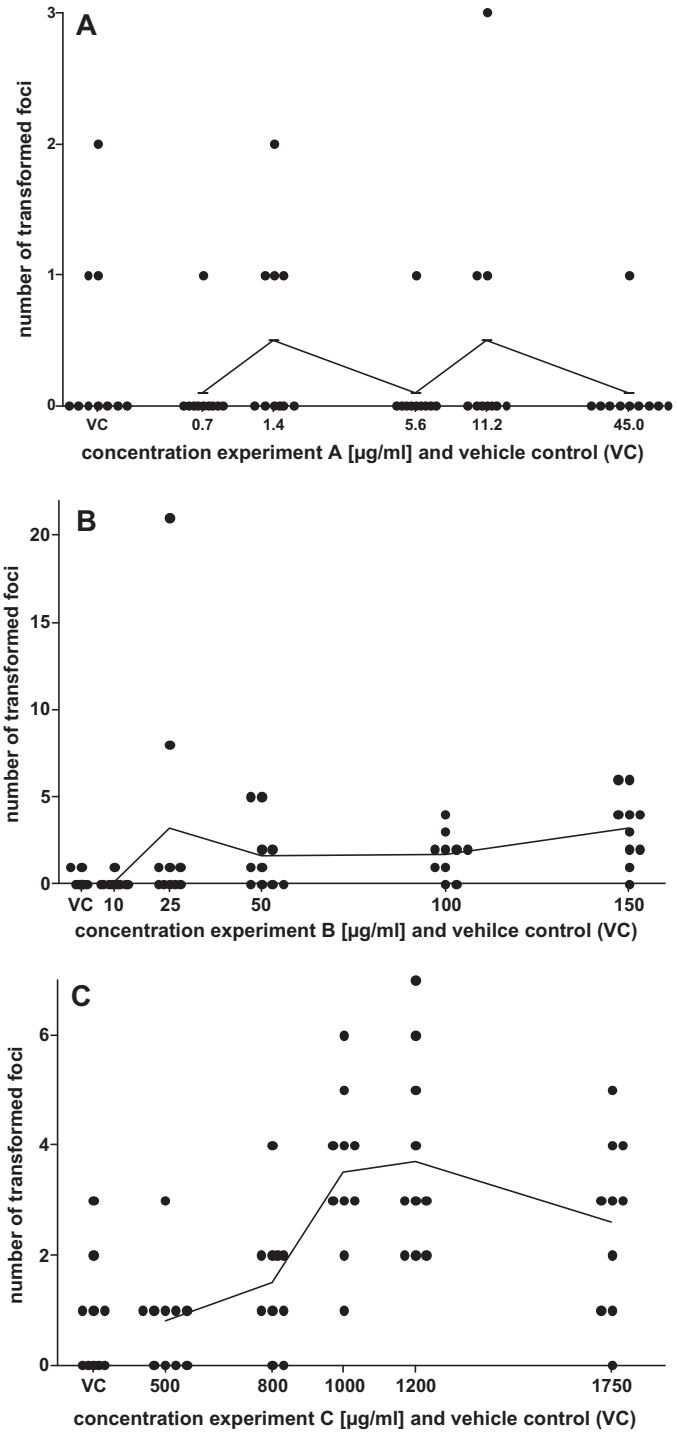



**Fig. 3.** Transformation data of three different BALB/c 3T3 cell transformation experiments (A, B and C). All replicates per treatment group are displayed and a line connecting the mean number of transformed foci for each test concentration is included.

observed in experiment C would lead to a positive call, as the curves showing the lowest *p*-value, *i.e.* curve C7, includes two effective concentrations. Considering experiment B, this type of interpretation would result in a positive conclusion for the GLM-NB-approach, because four effective concentrations are included in the curve with the lowest *p*-value. However, the NT, for which the most significant curve includes only one concentration, would result in a negative call. In such a situation, considering the treatment as negative is presumably inappropriate from a biological point of view. A viable option would be to interpret the experiment inconclusive and to perform a re-test, preferably with adjusted concentrations that provide a higher resolution around the concentration that induced an increase in foci.

## 5. Conclusions

In this study we applied two promising approaches, one based on a negative binomial generalised linear model with William's-type protected tests and a model based on a transformation proposed by Nishiyama et al. [13] also applying William's-type protected tests to real-life data sets. Minor shortcomings in the computational implementations of the approaches were overcome by negligible data manipulations. The respective scripts to be used in statistical freeware R, including guidance on use and interpretations, are provided in Appendix A.

In general, our results demonstrate the applicability of both approaches. Detailed and larger scale evaluation of the GLM-NB approach is needed to investigate its general suitability and usefulness for the BALB/c 3T3 CTA. In particular, more practical experience is required in order to optimise the analysis and to develop an improved scheme of interpretation, which would then also be able to identify inconclusive experiments.

## Conflicts of interest statement

None declared.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrgentox.2011.12.002.

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