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In Focus

Center of Comparative Medicine & Pathology
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The *Experimental Design Assistant (EDA)*: A Tool to Help Investigators Formulate an *In Vivo* Study Plan

High quality research is reproducible and translatable.

This article is part of series about available tools and resources for study reporting and experimental design[1,2,3,4,5,6]. View previous articles below.

Introduction to the ARRIVE Guidelines

Reporting of In Vivo Methods in Scientific Publications

Reporting on Animal Models in Scientific Publications

Planning Research and Experimental Procedures on Animals

The limited reproducibility of findings from preclinical animal studies has received considerable attention over the last few years because of its direct negative impact on translation, scientific progress, and the use of resources.[7] Poor reproducibility may be caused by flawed experimental design, inappropriate statistical analyses, and inadequate reporting. In an effort to increase *in vivo* research reproducibility and reliability, the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) developed a free web-based tool, the Experimental Design Assistant (EDA), to help researchers navigate and formulate the design of their animal experiments with specific methods to determine the minimum number of animals needed to reach their scientific objective, reduce subjective bias, and utilize appropriate statistical analysis.[7,8] The EDA's output includes a diagram that improves the transparency of the experimental plan. The EDA was created in collaboration with scientists and statisticians from academia and industry, and a team of software designers. It enables researchers to build a stepwise, schematic representation of an experiment—the EDA diagram—and uses computer-based logical reasoning to provide feedback and advice on the experimental plan. The system's main features are presented in Table 1.

Percie du Sert et al., (2017), explain that the EDA helps improve the reliability of experimental results and analysis by providing tools for randomization and blinding which ensures a key assumption of the statistical analysis, i.e., that different groups are drawn from the same background population using random sampling, is met.[7] Furthermore, applying this tool minimizes systematic differences between the treatment groups during study execution, assessment of the results, and data analysis. Such differences can be caused by researchers subconsciously influencing the animals' allocation to treatment groups, the animals' behavior, or data handling, e.g., removal of outliers. The EDA system generates a randomization sequence for the experiment, which takes into account any blocking factors included in the design and provides dedicated functionalities, such as support for blinding and sample size calculations, to assist researchers follow best practices (Fig. 1). A tailored critique provides suggestions on optimizing the experimental plan. For example, it helps researchers identify variables that could confound the outcome and provides advice on how to include them in the randomization and the statistical analysis. Another EDA feature relates to the power analysis conducted

Cont. on pg. 2

Table 1. Features of the Experimental Design Assistant (EDA).

Features of the EDA include the following:
• A computer-aided design tool to develop a diagram representing the experimental plan,
• feedback from an expert system on the experimental plan (the Critique),
• Analysis Suggestion,
• sample size calculation,
• randomisation sequence generation,
• support for allocation concealment and blinding,
• web-based resources to improve knowledge of experimental design and analysis.

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to determine the number of animals needed to yield dependable results and reliable conclusions. Finally, the system advises on which methods of statistical analysis are most appropriate. The EDA encourages researchers to consider the sources of bias at the experimental design stages. The EDA can also be used as a teaching resource, promoting a better understanding of the principles of experimental design at an early stage of the research training process.[7]

Given that there are no universally accepted standards for describing the different components of an experimental design, different terms are used to describe the same things, e.g., outcome measure vs. dependent variable.[7] EDA resolves this problem by helping the user generate unambiguous representations of these different designs using EDA diagrams (Fig. 2). Although the EDA is not designed to replace a statistician's advice, it can facilitate it by assisting the researcher identify much of the information that the statistician needs. The information is presented in a detailed standardized format, which can be made available to funding bodies, ethical review committees, journal editors, and peer reviewers. P-hacking and selective outcome reporting compromise the reliability of published results. These post hoc issues would be prevented by EDA diagrams which formalize a clear protocol and plan for the statistical analysis before collecting the data. Finally, EDA also helps to identify sources of variability by providing examples that are commonly encountered in animal research, such as

- Date of experiment (if spread over several days)
- Time of day when the experiment is performed
- Type of equipment used to record measurements
- Litter or cage mates
- Location of cages in the room
- Baseline variables (i.e., animal weight or locomotor activity

Cont. on pg. 3

p-hacking:
run multiple statistical tests on the same data and choose the one with the lowest *p* value

selective outcome reporting:
measure different outcomes, or the same outcome in different ways, and only report the ones that reach statistical significance.[7]

Fig 1. The Experimental Design Assistant (EDA) workflow:
1. The user draws a diagram (with nodes and links) representing the experiment they are planning. Examples, templates, and video tutorials provide for help.
2. Information is added into the nodes' properties, providing more details about each the specific step of the process represented by the node.
3. The "Critique" functionality (see Table 2) enables the researcher to obtain feedback on the diagram and the design it represents. The feedback might prompt a change in their plan or the addition of missing information.
4. Once feedback from the critique is addressed and the user is satisfied with the design, the system suggests an analysis method.
5. Depending on how the data will be analyzed, a suitable sample size is calculated within the system.
6. The EDA generates the randomization sequence. A spreadsheet detailing the group allocation for each animal can be sent directly to a third party identified by the user, thus blinding the allocation. This enables the researcher to remain unaware of the groups until the data has been collected and analyzed.
7. Diagrams can be safely shared with colleagues and collaborators at any stage of the process.
8. The user can export a report containing key information about the internal validity of the experiment, a summary of the feedback, and the EDA diagram.
9. Once the planning is complete, the experiment is carried out.
10. The diagram can be updated after data collection to enable the user to keep an accurate record (e.g., record the actual number of animals analyzed if some failed to complete the experiment or if data are missing for other reasons).

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Table 2. Feedback provided by the Experimental Design Assistant (EDA).

Aspect of experimental design covered by the feedback rules	High-level description of the type of feedback that the EDA can provide
Objective	Provides guidance to identify the null and alternative hypothesis, the effect of interest, and the effect size that is biologically relevant.
Randomisation	Detects when the allocation method is not specified. Highlights the importance of adequate randomisation, advises on randomisation procedures, and prompts users to consider different types of randomisation.
Blinding	Detects when there is no provision for blinding, explains why allocation concealment and blinding are important and the different stages of the experiment that can be blinded, and provides ways blinding can be achieved.
Groups and sample size	Provides guidance to identify the experimental unit(s) and determine suitable sample sizes.
Outcome measures	Highlights the implications of working with continuous and categorical data. Prompts user to identify the primary outcome measure.
Independent variables of interest	Detects when independent variables have not been identified or when they should be treated as continuous or categorical or as repeated factors. Detects when independent variables may be confounded.
Nuisance variables	Advises about nuisance variables commonly seen in animal experiments and how to account for them in the randomisation and analysis.
Statistical analysis	Suggests statistical analysis methods compatible with the design, along with software that can be used to perform these tests. Advises about parametric assumptions and data transformation. Suggests when the advice of a statistician should be sought.

In sum, the goal of the EDA is to promote a better understanding of experimental design and raise awareness about problems caused by a lack of randomization and blinding, underpowered experiments, or inappropriate statistical analysis.[7] The feedback provided by the system (Table 2) enables users to learn about the implications of different design choices and helps them make informed decisions about the most appropriate ones to adopt.

~Christopher Cheleuitte-Nieves, PhD, DVM, DACLAM, Senior Clinical Veterinarian

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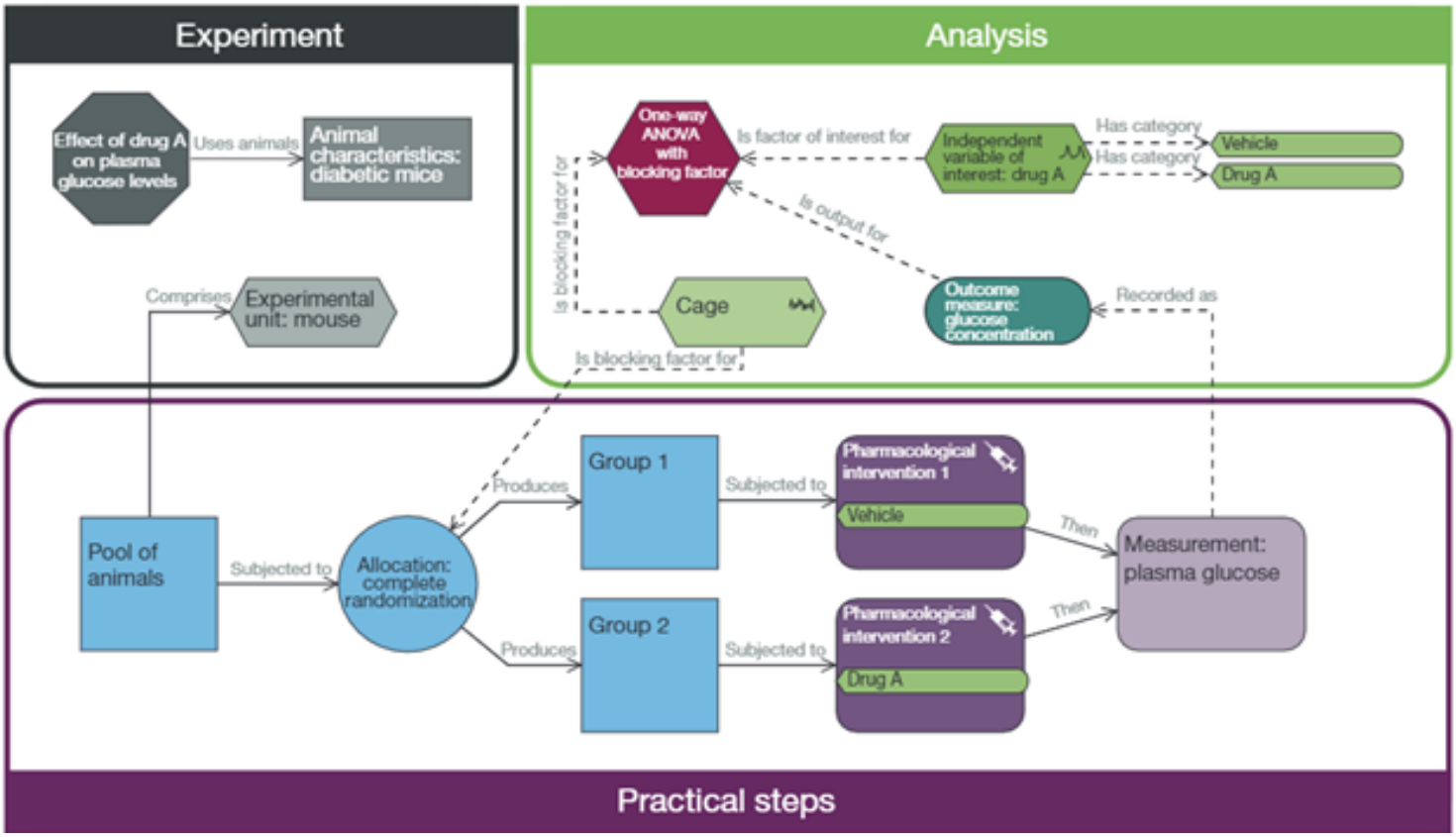

Fig 2. An example of an EDA diagram representing a two-group comparison in which each cage contains mice randomized to either of two treatments. Diagrams are composed of nodes and links to represent the entire experimental plan. The gray nodes contain high-level information about the experiment such as the null and alternative hypotheses, the effect of interest, the experimental unit and the animals' characteristics. The blue and purple nodes represent the practical steps carried out in the laboratory such as the allocation to groups, the group sizes and role in the experiment, the treatments and the measurements taken. The green and red nodes represent the analyses, the outcome measures and the independent variables of interest and nuisance variables (e.g., blocking factors). For more details, see https://eda.nc3rs.org.uk.

Using MALDI-TOF to Rapidly Speciate Bacteria and Fungi

The Laboratory for Comparative Pathology (LCP) has procured a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) instrument, which will provide RARC and investigative staff access to state-of-the-art technology for identifying bacteria and fungi. Speciation with MALDI-TOF is rapid and extremely accurate. The bacterial or fungal colony is removed from an agar culture plate, mixed with a UV-absorbing matrix and dried on small steel plates. In the process, the bacterial cell wall is dissolved and intracellular bacterial proteins are dispersed within the matrix. The matrix is dried and the preparation is then exposed to UV-laser pulses, resulting in the ablation and desorption of individual matrix-cell protein complexes. These complexes are then accelerated by an electric potential and separated by their mass/charge ratio. The resulting mass spectrographic profile is then compared to a database of profiles of known bacterial or fungal species and the best match identified. MALDI-TOF can accurately identify the species of a bacterial or fungal colony within seconds.

MALDI-TOF has become the primary tool for diagnostic microbiology laboratories to perform bacterial speciation and, to a large part, has replaced classical speciation techniques. These techniques, rely on biochemical characteristics or genotyping compared to the protein-based typing of MALDI-TOF. Biochemical testing applications such as API® or Vitek® have long been the mainstays of bacterial speciation; however, these methods are laborious, time consuming and often lead to nonspecific results for bacteria isolated from animals as they were developed for use in human clinical microbiology. In comparison, molecular techniques, including 16S rRNA and whole-genome sequencing, are direct, sensitive methods of speciating bacteria that are the gold standard of species classification. However, these methods are time consuming and expensive. In comparison, MALDI-TOF is extremely rapid, highly accurate and inexpensive when one excludes the instrument's acquisition cost. One limitation of MALDI-TOF is that since the technique is relatively new, not all bacterial species can be found in the database's reference collection and therefore cannot be differentiated from closely related species. However, the library of spectra is continuously expanding with new species added regularly.

Laboratories utilizing bacteria and fungi may benefit from MALDI-TOF identification. The importance of ensuring the correct speciation of bacterial organisms with MALDI-TOF is of increasing importance as new bacterial genera and species are continually being described, and existing taxa are routinely reclassified. The use of existing laboratory microbe strains that have previously been speciated using only phenotypic and biochemical test methods may benefit from more precise speciation using MALDI-TOF.

Please contact LCP@mskcc.org or LCP@med.cornell.edu if you are interested in using MALDI-TOF for your research.

~Dr. Juliette Wipf, DVM, PhD, FVH
LCP Laboratory Manager

~Dr. Amanda Carlson, DVM
Veterinary Postdoctoral Associate

The Clinical Pathology Team with the MALDI-TOF Biotyper. Left to right: Irina Dobtsis, Anapama Saha, Juliette Wipf, Joanna Nowak.

In Memory: Caroline Murray

Caroline's career in Laboratory Animal Research spanned over 30 years, starting as a veterinary technician, before becoming a veterinary technician supervisor, and then an instructor at LaGuardia Community College. LaGuardia is where she found her passion for teaching. She joined the Research Animal Resource Center in 2004 as an Education & Quality Assurance Specialist. In her role, she assumed varying responsibilities including training, participating on the IACUC and oversight of hazardous materials suites.

Caroline was first and foremost a teacher. She enjoyed sharing her knowledge of animals with new researchers, interns and students. Known affectionately as "the rat whisperer" she took pride in helping people foster a love for the highly social rodents with which she extensively worked. She was also active in educational outreach, teaching animal handling workshops and speaking at local schools instilling an interest in animal science in the next generation of scientists and technicians.

She will be remembered for her sunny disposition and delightfully corny jokes. She had a gift for making someone feel special and welcome in any environment, most importantly while she was teaching a class. Throughout her career, Caroline has had a significant impact on the lives of many people. She will be greatly missed by her colleagues who also considered her a friend.

CCMP welcomes Sebastian Carrasco, Comparative Pathologist and Assistant Professor of Pathology and Laboratory Medicine

Sebastian Carrasco DVM, MPVM, M.Sc., Ph.D. DACVP

The CCMP's Laboratory of Comparative Pathology is pleased to welcome Sebastian Carrasco DVM, MPVM, M.Sc., Ph.D., DACVP as Comparative Pathologist and Assistant Professor of Pathology and Laboratory Medicine. Dr. Carrasco is a veterinary pathologist - scientist with expertise in bacterial pathogenesis, macrophage immunology, comparative pathology, and the development and characterization of animal models of human disease. He received his DVM from Universidad Mayor Chile and obtained his Master degrees in preventive veterinary medicine and comparative pathology from the University of California Davis. He then completed his Ph.D. in microbiology and immunology at the Indiana University School of Medicine. After finishing his graduate studies, he pursued residency training in anatomic pathology with an emphasis on laboratory animal pathology at the UC Davis School of Veterinary Medicine. Prior to joining CCMP, he was a comparative pathologist - scientist in the Division of Comparative Medicine at Massachusetts Institute of Technology (MIT), where he provided diagnostic pathology services for a broad range of laboratory animal species. His research studies focus on understanding the role of novel *Borrelia burgdorferi* (*Bb*) virulence factors and macrophage scavenger receptor CD36 in the pathogenesis of arthritis and carditis in the mouse model of Lyme disease. His collaborative research focuses on comparative pathology and phenotyping in diverse translational research areas, including infectious diseases, immunology, aging, microbiome, toxicopathology, and cancer. He has participated in various teaching activities and mentored five postdoctoral fellows in laboratory animal medicine during his tenure at MIT. He also oversaw the lab animal pathology rotation for postdoctoral lab animal fellows at MIT. Dr. Carrasco is an active member in geropathology grading committees for laboratory rodents and common marmosets at the Geropathology Research Network and holds memberships in different professional organizations, including ASM, AAI, STP, DPA, and ACVP. Dr. Carrasco is dedicated to the field of comparative pathology and greatly enjoys working in academia as a veterinary pathologist, researcher, and teacher for the next generation of comparative pathologists and lab animal veterinarians. In his free time, he enjoys doing outdoor activities with his family and playing soccer with his son.

A Common Mouse Restraint Technique Causes Severe Cardiovascular Abnormalities

A recently published study by veterinarians at Cornell University, Ithaca reveals that a commonly used method of mouse restraint induces severe bradyarrhythmias. The restraint method, hereafter referred to as "two-finger" restraint, involves grasping the loose skin at the base of the mouse's head (scruff) between the index finger and thumb. The middle and ring fingers are subsequently used to grasp the remainder of the loose skin down the mouse's back. This technique creates a longitudinal fold of skin along the dorsum of the animals' neck which, if not performed properly, can result in significant and focal pressure on the ventral neck resulting in cyanosis, dyspnea, or even death secondary to airway occlusion. The Cornell study demonstrated that this restraint technique, even when applied without causing the aforementioned complications, induces significant bradycardia. This bradycardia is characterized by up to a 79% reduction in heart rate and affected male and female mice of multiple strains, including C57BL/6J, BALB/cJ, FVB/J, and DBA/2J. Arrhythmias were induced by this restraint technique in 58% of mice studied, and were characterized by marked bradycardia with irregular R-R intervals, ventricular escape complexes, and wide QRS complexes. Prolonged sinus pause persisted for an average of 4 minutes after release from two-finger restraint. Restraint induced bradycardia was attenuated by pre-treatment with atropine, suggesting a vagal-mediated mechanism for the bradycardia. During the study, one mouse restrained by the two-finger technique died during restraint secondary to severe bradyarrhythmia.

The alternative "three-finger" restraint technique modifies the two-finger method so that a transverse, rather than longitudinal, skin fold is created, which alleviates pressure on the mouse's ventral neck. Three-finger restraint is performed by first gripping the skin at the base of the head between the thumb and middle finger. The index finger then replaces the middle finger and the transverse skin fold is gently rolled between the index finger and thumb until the head is immobilized. The image panel below demonstrates the difference in dorsal and ventral skin tension between the two-finger (A) and the three finger (B) restraint methods. The latter technique was not found to induce bradyarrhythmias in mice when compared to non-immobilizing restraint.

The adoption of the three-finger restraint technique is a refinement in animal welfare and may improve study reproducibility. All investigative staff, in particular those studying cardiovascular physiology, should cease using the two-finger restraint method. Similarly, RARC is modifying its training policies replacing the two-finger with the three-finger restraint technique. A video is available through Norecopa to demonstrate how to perform this restraint method: www.vimeo.com/290857433 and RARC's Education and Quality Assurance staff are available to provide hands-on training.

Reference:

Labitt RN, Oxford EM, Davis AK, Butler SD, Daugherity EK. A Validated Smartphone-based Electrocardiogram Reveals Severe Bradyarrhythmias during Immobilizing Restraint in Mice of Both Sexes and Four Strains. J Am Assoc Lab Anim Sci. 2021 Mar 1;60(2):201-212. doi: 10.30802/AALAS-JAALAS-20-000069. Epub 2021 Feb 26. PMID: 33637137; PMCID: PMC7974811.

Image panel: (A) Two-finger restraint creates a dorsal longitudinal skin fold, a crease on the ventral neck (arrow) resulting in pressure on the ventral neck, and abduction of the forelimbs dorsally. (B) Three-finger restraint creates a dorsal transverse skin fold (arrow), absence of crease on the ventral neck, and forelimbs in a natural position.