# Methods for applying blinding and randomisation in animal experiments

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# Methods for applying blinding and randomisation in animal experiments

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**Abstract**

Blinding and randomisation are important methods for increasing the robustness of pre-clinical studies, as incomplete or improper implementation thereof is recognised as a source of bias. Randomisation ensures that any known and unknown covariates introducing bias are randomly distributed over the experimental groups. Thereby, differences between the experimental groups that might otherwise have contributed to false positive or -negative results are diminished. Methods for randomisation range from simple randomisation (e.g. rolling a dice) to advanced randomisation strategies involving the use of specialised software. Blinding on the other hand ensures that researchers are unaware of group allocation during the preparation, execution and acquisition and/or the analysis of the data. This minimises the risk of unintentional influences resulting in bias. Methods for blinding require strong protocols and a team approach. In this review, we outline methods for randomisation and blinding and give practical tips on how to implement them, with a focus on animal studies.

**Keywords**

Bias, blinding, experimental design, randomisation, robust design, techniques

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## Why blind and randomise?

Experimental research (e.g. an animal study) is often designed to give insight into the effects of an intervention on one (or more) specific variable(s). Randomisation and blinding ensure that findings within a study can be attributed to the tested intervention with a higher confidence, by reducing the risk of bias. This is done by ensuring that results are i) less influenced by known and unknown variables other than the intervention itself, or ii) less influenced by the researcher performing the experiments or analysing the data, respectively. In other words, randomisation and blinding increase the internal validity of a study.

In experimental research, the chance that differences between an intervention and a control group are observed by coincidence is determined in statistical tests, and is often summarised by a *p*(robability) value. Naturally occurring variation, as is even present in studies with inbred animals, can be accounted for by increasing the sample size, and can even form the rationale for performing a priori power calculations. Researchers typically consider differences between groups to be statistically significant (i.e. unlikely to be a coincidence) if the *p*-value is less than 0.05. However, this assumes an 'ideal' experimental setup, whereas in reality various sources of bias can introduce extra risk, meaning that the observed effects may not have been caused by the intervention itself. For example, factors that may introduce extra, or enhance existing, variation between experimental groups may be sources of bias, are often overlooked or remain unidentified. One example is the manual distribution of

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animals across experimental groups, where mice that are picked up by the researcher first are placed within the same experimental group, potentially causing the first group to contain the slowest mice. Randomisation can overcome this problem. However, even after mice have been randomly distributed over experimental groups at the start of an experiment, other factors like cage placement within a room may affect factors such as light intensity[1] and cage temperature,[2,3] thereby influencing the outcome of the experiment. Therefore, it is critical that randomisation is integrated in multiple aspects of the experimental design (Figure 1).

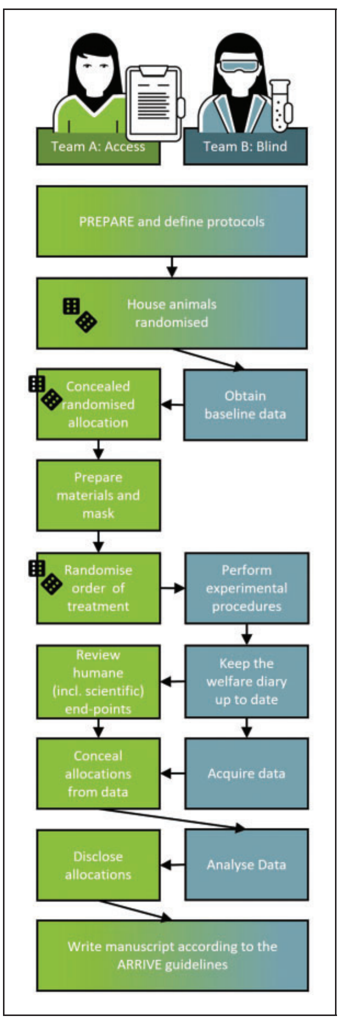


**Figure 1.** Visual overview of steps executed by team A(ccess) in green and team B(linded) in blue. In the design phase, in principle both teams start together with the initiation protocols and the PREPARE guidelines.[20] Then, teams disperse based on their allocation to access (Team A) or blinded (Team B) until the data analysis phase is complete. Both teams convene with reporting according to the ARRIVE guidelines.[24]

Although randomisation can minimise the risk of confounding, it does not take away the risk of bias, which can occur during the experimental phase (e.g. experimenter expectancy bias, observer bias, detection bias) as well as thereafter when analysing the results (e.g. confirmation bias). More specifically, when researchers acquire data, they will do so with a hypothesis in mind.[4,5] This introduces the risk of misjudgements or unintentional distortions by the researchers when making decisions. This example of experimenter expectancy bias was demonstrated in a famous experiment of Rosenthal and Fode in 1963,[6] where experimenters were shown to judge the behaviour of rats in a maze differently when they believed some were bred for superior intelligence, although they were in fact randomly selected. Related hereto, knowledge of treatment or group assignments may subconsciously or otherwise influence the approach or care for animals: one might unintentionally inject the compound of interest with more precision and care as compared to a vehicle control substance, through which the procedure itself differs between the experimental groups. Knowledge of treatment could also influence outcome assessments,[7] even in an automated measurement. One could, for example, unintentionally spend more time handling treated animals, causing them to become more tame, thereby affecting behavioural and/or stress-related outcomes.[6] In addition, decisions to withdraw animals from the experiment based on humane endpoints may be affected.[8] One might for example judge humane endpoints more rigorously in the untreated control animals, or vice versa. In addition, bias can occur during data analysis, where scientist may accept results without question if they are as expected, whilst submitting data to further scrutiny if they are unexpected or not in line with the hypothesis. The latter situation especially could cause additional bias, for instance when data is unnecessarily stratified (e.g. reporting bias), or when new exclusion criteria are introduced (e.g. attrition bias).[8] In order to mitigate the risk of such bias, researchers can be blinded during the execution of experiments as well as during the acquisition and analysis of the data. A systematic review of 250 trials in 33 meta-analyses reported a significant reduction of the effect of treatments in double-blinded clinical trials as compared to unblinded trials.[9] In human studies, the need for blinding is widely acknowledged and is now common practice in most clinical trials. However, while in animal studies the effects of bias caused by a lack of blinding are evident,[10,11] blinding often remains unreported in publications,[12,13] suggesting a lack of blinding in many experimental designs.

It should be clear by now that both randomisation and blinding are essential aspects of any empirical study. Both methods are crucial for safeguarding that the observed effects are caused by the intervention of interest. Experimental design in clinical trials, animal studies and non-animal methods should therefore include a well-conceived, -described and -executed plan for both randomisation and blinding to reduce unintended bias.

Effort and commitment are necessary in creating a robust design that includes both randomisation and blinding. However, we believe that every type of animal study could accomodate implementation of either one or both of the discussed methods, in some if not all parts of the design. In undertaking the current review, we therefore aimed to provide guidance for those performing animal research to implement randomisation and blinding in all studies.

## Methods of randomisation

Simple randomisation of experimental units – ranging from cells in culture dishes to animals and humans – can be achieved with the flip of a coin or the roll of a dice. Assuming a fair coin and a fair dice, this provides two easy, readily available methods that can be implemented with minimum effort. With larger set-ups, one might predetermine group allocation, for example by using spreadsheet software (e.g. with help of the RAND function in Excel). Alternatively, more extensive online tools can assist in this process (e.g. random.org or eda.nc3rs.org.uk[14]). Although simple randomisation would account for most unknown factors, by pure chance, differences between groups may be introduced nonetheless, particularly in experiments with small sample sizes, as is often the case in animal experiments for ethical reasons. Therefore, one might take randomisation one step further by taking known variables or covariates into account. For example, an experiment focussed on diet may take body weight into account in the randomisation to ensure equal starting weights. Methods for taking categorical variables into account include the use of a Latin square, that is, a two-dimensional array with each condition occurring exactly once in each row and column. Alternatively, one could rely on a randomised block design,[15] which involves the grouping of experimental units into blocks based on a known variable or covariate, followed by random assignment of the different treatments to the experimental units within each block (i.e. group allocation).[16,17] An example where a randomised block design can assist is a cohort study, as each cohort will inevitably differ from each other in various aspects, therefore each group should be equally represented within each cohort (i.e. each block). That way, differences between cohorts can be accounted for during analysis.[17] Even when a parameter is continuous as opposed to categorical, such a randomised block design can be applied by sorting the experimental units based on the variable or covariate of interest. In the example of body weight, the first block will then contain the experimental units with the largest body weights, and the last block those with the smallest body weights.

However, in cases where it is desired to balance multiple (continuous) variables or covariates into experimental groups, more specialised software will be required. To this end, we previously developed the open source software tool RandoMice,[18] which can be downloaded and installed free of charge. Although originally developed for mice, as implied by its name, the data of any experimental unit (ranging from *in vitro* culture dishes to laboratory animals) can be copied from spreadsheet software such as Excel. RandoMice can then be instructed to repeatedly randomly distribute the experimental units into blocks. Differences between blocks in terms of averages and dispersion are scored, ultimately allowing selection of a division of experimental units in which all provided variables and covariates are well balanced. Finally, groups can be allocated to the blocks using the same software.

## Methods for blinding

Blinding is an important method to reduce bias in research. It is desirable to implement blinding in each part of a study. In animal studies, this ranges from blinding for the treatment and follow-up of the animals, to blinding for data acquisition and analysis.[19] Well-executed blinding is dependent on clear rules for communication, team engagement, and team reliability. To introduce blinding as a routine procedure in a study design, we propose dividing the research team into two groups (Figure 1). Here, we distinguish Team B (Team Blinded), which is blinded as much as possible, and Team A (Team Access), which has insight into the treatment allocation. Researchers in Team B are responsible for executing experimental procedures, filling out animal welfare diaries, and acquiring and analysing data. In practice, Team B may consist of just one single researcher or multiple researchers. Members of Team A are preferably 'uninterested' colleagues with no direct dependence on the outcome, for example, they may be colleagues from a different lab. Team A can help in case of unexpected events (e.g. welfare-related issues) and with recoding labels and samples. In principle, a researcher will not be part of both Teams A and B, as this would introduce a breach in blinding, however roles might be switched for specific parts of a study.

Within an empirical cycle, blinding can be addressed and implemented separately in the various steps of an experiment. In the following sections we will distinguish the steps 'study design', 'study execution', 'animal welfare diary' and 'reporting', and describe the considerations for blinding.

### Study design

To ensure all researchers involved in a blinded study feel confident, and to diminish the risk of errors due to masking or coding errors, a good blinding plan is mandatory. A blinding plan describes the method of blinding, the person(s) involved in organising the blinding and the person(s) to be blinded. In rare cases knowledge of the study aim may affect the outcome. Therefore, part of the blinding plan may be that Team B remains uninformed of the study aim. In this case, Team A would be the designers of the study and Team B the 'uninterested' colleagues. An example is a study by Rosenthal and Fode where human–animal interaction was the topic of study.[6] However, in the majority of cases a blinding plan consists of a description of blinding methods during group allocation, treatments and acquisition of the data. The plan should additionally describe under what circumstances and in response to which unexpected occurrences blinding may be broken during the study.[21] A rule of thumb is that all data is consistently linked to the experimental unit, leading to a 'tidy data set'.[22] In practice, such a data set will have a column containing animal numbers (i.e. experimental unit identifiers), columns with information on the animals (e.g. starting body weight) and separate columns for each measurement (i.e. data points). Such a data set can be easily unblinded when the group allocation is also linked to each experimental unit. This can be done by adding a single column containing information on the group allocation.

### Study execution

During study execution, the success of blinding is dependent on a clear schedule and a team approach. This involves close collaboration between Teams A and B. For example, in an animal facility, Team B might weigh the animals, whereas Team A might subsequently fill the syringes based on body weight, and mask the content of the syringes with tape. Next, the contents of the syringes can be administered (blinded) by Team B.

Since the outcome of an experiment is dependent on the degree of blinding, it can be helpful to evaluate the quality of the blinding during study execution. This can be done by asking members of Team B to guess the group allocation, without revealing it. The initiation of discussions on 'how blind is blind?' has led to a greater focus on quality control and thereby improvements in blinding methods in human randomised control trials.[23] By similarly actively pursuing quality in animal studies, valuable information to improve the internal validity of the research could be afforded, for example, revealing confounding effects due to pattern recognition or identifying causes of accidental unblinding. Known challenges in blinding include visible traits that give away group allocation (e.g. genetically altered animals with different coloured fur) or treatment that may affect the physical appearance (e.g. body weight when testing an anti-obesity drug, or tumour growth). A possible solution is recoding, either after a procedure has been performed or after data has been acquired. Recoding can easily be delegated to Team A or carried out using automated systems in the case of file recoding. Alternatively, with some creativity, Team A could help mitigate or reduce these visible cues, for example by combining studies or having procedures performed by skilled personnel that are uninformed of the study aims. Study execution could be split into different parts: allocation, execution, outcome assessment and data analysis. In principle, Team B stays blinded to the allocation throughout the entire execution of the experiment including the assessment and analysis of the outcome, however tasks might be split between individuals within Team B.[10]

### Animal welfare diary

Unforeseen events can occur throughout a study, for example, problems with animal wellbeing due to unexpected side effects or fighting between animals. A clear welfare diary is therefore essential. A member of Team A may need to evaluate this diary, to appraise humane endpoints and monitor the group composition. A veterinarian could join Team A. In addition, a lack of power due to drop-outs and subsequent missing data may need the knowledge and mandate within Team A to decide whether the scientific aims can still be met or the -endpoint has been reached. Therefore a statistician might also join Team A.

### Reporting

Blinding methods, breaches in blinding and all (unforeseen) events related to welfare issues should be reported as part of adhering to the ARRIVE guidelines,[24] and in sufficient detail to allow for the blinding method to be implemented by others.

## To conclude

Implementation of both randomisation and blinding in animal research is feasible. Both methods require clear procedures, planning, team training, engagement and sometimes creativity. In the current review, we have aimed to provide guidance for the use of randomisation

**Table 1.** Resources for randomisation and blinding.

| Subject | Issue or item | Solution or tip |
|---|---|---|
| Team | Inform staff | - Be prepared by using frameworks on design, quality control, conduct and reporting<br>• PREPARE (norecopa.no)[20]<br>• EQIPD (paasp.net)[25]<br>• EDA (Experimental Design Assistant)[14]<br>• ARRIVE guidelines[24]<br>- Use checklist to address blinding and randomisation specifically (suggested: 2.1.7 Blinding EQIPD[25])<br>- Provide, if needed, study dossiers with masked study aim to Team B |
| | Team allocation | - Decide who will be join team A and who will join team B (decide based on practical skills, knowledge and expertise)<br>- Make roles in both team explicit to avoid unintentional unblinding |
| | Scarcity of staff | - Staff outside the research team can be included for tasks in team A (e.g. recoding, or masking)<br>- Use technical solutions to recode or mask with resources like: Blind analysis tool (ImageJ.net)[26] |
| | Trained staff | - Blinding and randomisation as core skills in experimental design<br>- Organise training and education<br>- Add training with available online tools and resources:<br>Reading:<br>• Chapter : Blinding and randomization (Springer)[27]<br>Video<br>• Blinding reduces bias in experimental design[28]<br>E-learnings<br>• ETPLAS.EU[29] suggested: EU-10: Design of procedures and projects<br>• EQIPD E-Learning (go-eqipd.org)[25] suggested: Experimental design Module 3 and Validity Module 4<br>• Experimental design E-Learning (BPS.ac.uk)[28] suggested: Blinding-reducing bias in experimental design |
| Plan | Risk of losing oversight | - Prepare and organise using:<br>• A check on the items of PREPARE (norecopa.no)[20]<br>• The diagram of the Experimental Design Assistant (EDA)[14] |
| Animals | Randomise at arrival | - Have a plan for housing the animals prior to arrival<br>- Randomise animals (team A or B) at arrival with dice, a list or software |
| | Genotype is the variable | - Mask genotype on the identification label |
| | Ungrouped allocation | - Use individual animal numbers instead of masked groups to avoid pattern recognition in data and welfare diary |
| | Randomise over cages | - Mark the animals individually and distribute them over cages when feasible for species or strain |
| | Randomise over location | - Handling scheme and cage location are part of the methods and design of the study |
| | Variables housed per cage | - Multiple cages per group<br>- Conceal Team B from experimental design and study aim |
| Procedures | Randomise handling schemes | - Add order of events, procedures, time of day randomised in your design |
| | Conceal recognisable compound | - Prepare syringes, equipment in advance by Team A. |

(continued)

**Table 1.** Continued.

| Subject | Issue or item | Solution or tip |
|---|---|---|
| | | - Use foil, paper or tape to cover the content |
| | Weight-based administration | - Weighing by Team B and preparation, masking and coding by Team A, followed by administration by Team B |
| | Variables affect environment | - Have Team A remove cage lid with food before animal procedures<br>- Transfer animals to neutral cages by Team A before procedures by Team B |
| | Recognisable phenotype or appearance | - Mask the study aim<br>- Automated measurement solution (e.g. video tracking)<br>- Combine multiple studies in the execution phase |
| | Blinding quality control | - Implement a four-eye-principle for crucial steps<br>- Implement allocation guessing for Team B to assess pattern recognition and blinding |
| Welfare diary | Welfare data | - Conceal or mask welfare data before analysing data |
| | Humane endpoints | - Keep humane endpoint decisions with Team B<br>- In case of missing data, have decisions on remaining power of an experiment for Team A (scientific endpoint) |
| Outcome measures | Sample recognition | - Recode samples, files and materials before storage |
| | Sample quantification | - Provide blinded study dossiers to staff |
| | Database manipulation | - Provide standard operating procedures for database manipulation<br>- Keep data blinded when executing database manipulations |
| Unblinding | Unforeseen event | - Unblind when risks arise for the health and safety of staff and animals (e.g. include veterinarian in Team A)<br>- Re-blind whenever possible |
| Data analysis | Evaluation of findings | - Add the allocation column to data set after data analysis |
| Reporting | Report randomisation | - Report all randomised entities separately (allocation, housing, treatment order, etc.)<br>- Report according to the ARRIVE guidelines[24] |
| | Report blinding | - Report all blinded parts of the study separately<br>- Include limitations in blinding and unforeseen events |
| | - Report according to the ARRIVE guidelines[24] | |

Resources for randomisation and blinding in the design, execution and analysis phases of the project. All references are provided with links to websites. Terminology: Team A has access to the group allocation and Team B is blinded for the group allocation.

and blinding in (animal) studies. More detailed, practical, tips, solutions and resources are presented in Table 1.

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## Ethical approval

Our study did not require ethical board approval because it did not contain human or animal trials.

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## Méthodes d'application de l'insu et de la randomisation dans les expériences animales

*Résumé*


L'insu et la randomisation sont des méthodes importantes pour augmenter la robustesse des études précliniques, car leur mise en œuvre incomplète ou incorrecte est reconnue comme une source de biais. La randomisation garantit que toutes les covariables connues et inconnues introduisant un biais soient réparties aléatoirement sur les groupes expérimentaux. Les différences entre les groupes expérimentaux sont ainsi diminuées, ce qui aurait autrement contribué à des résultats faussement positifs ou négatifs. Les méthodes de randomisation vont de la simple randomisation (par exemple lancer un dé) à des stratégies de randomisation avancées impliquant l'utilisation de logiciels spécialisés. L'insu, d'autre part, garantit que les chercheurs ignorent l'affectation des groupes lors de la préparation et du déroulement d'un essai, et de l'acquisition et/ou de l'analyse des données. Cela minimise les risques d'influences involontaires entraînant des biais. Les méthodes de mise en œuvre de l'insu nécessitent des protocoles solides et une approche d'équipe. Dans cette revue, nous décrivons les méthodes de randomisation et de mise en insu et fournissons des conseils pratiques sur la façon de les mettre en œuvre, en mettant l'accent sur les études animales.

## Methoden zur Anwendung von Verblindung und Randomisierung in Tierversuchen

*Abstract*

Verblindung und Randomisierung sind wichtige Methoden, um die Robustheit präklinischer Studien zu erhöhen, da ihre unvollständige oder unsachgemäße Durchführung als Quelle von Verzerrungen gilt. Die Randomisierung stellt sicher, dass alle bekannten und unbekannten Kovariaten, die zu Verzerrungen führen, per Zufall auf die Versuchsgruppen verteilt werden. Dadurch werden die Unterschiede zwischen den Versuchsgruppen verringert, die andernfalls zu falsch positiven oder negativen Ergebnissen geführt hätten. Die Methoden zur Randomisierung reichen von der einfachen Randomisierung (z. B. Würfeln) bis hin zu fortgeschrittenen Randomisierungsstrategien, die den Einsatz spezieller Software erfordern. Die Verblindung hingegen gewährleistet, dass die Forscher während der Vorbereitung, der Durchführung und der Erfassung und/oder Auswertung der Daten keine Kenntnis von der Gruppenzuordnung haben. Dadurch wird das Risiko unbeabsichtigter Einflüsse, die zu Verzerrungen führen, minimiert. Methoden zur Verblindung erfordern strenge Protokolle und einen Teamansatz. In dieser Übersichtsarbeit werden Methoden zur Randomisierung und Verblindung dargelegt und praktische Tipps zu ihrer Umsetzung gegeben, wobei der Schwerpunkt auf Tierversuchen liegt.

## Métodos para aplicar el enmascaramiento y la aleatorización en los experimentos con animales

*Resumen*

El enmascaramiento y la aleatorización son métodos importantes para aumentar la robustez de los estudios preclínicos, ya que su implementación incompleta o incorrecta se reconoce como una fuente de sesgo. La aleatorización garantiza que cualquier covariable conocida y desconocida que introduzca un sesgo se distribuya aleatoriamente entre los grupos experimentales. Así se reducen las diferencias entre los grupos experimentales que, de otro modo, habrían contribuido a obtener resultados falsos positivos o negativos. Los métodos de aleatorización van desde la aleatorización simple (por ejemplo, tirar un dado) hasta estrategias de aleatorización avanzadas que implican el uso de software especializado. El enmascaramiento, por otro lado, garantiza que los investigadores desconocen la asignación de grupos durante la preparación, la ejecución y la obtención y/o el análisis de los datos. De este modo se minimizan los riesgos de influencias involuntarias que puedan dar lugar a sesgos. Los métodos para el enmascaramiento requieren protocolos sólidos y un enfoque de equipo. En esta revisión esbozamos los métodos de aleatorización y enmascaramiento y damos consejos prácticos sobre cómo aplicarlos, centrándonos en los estudios con animales.