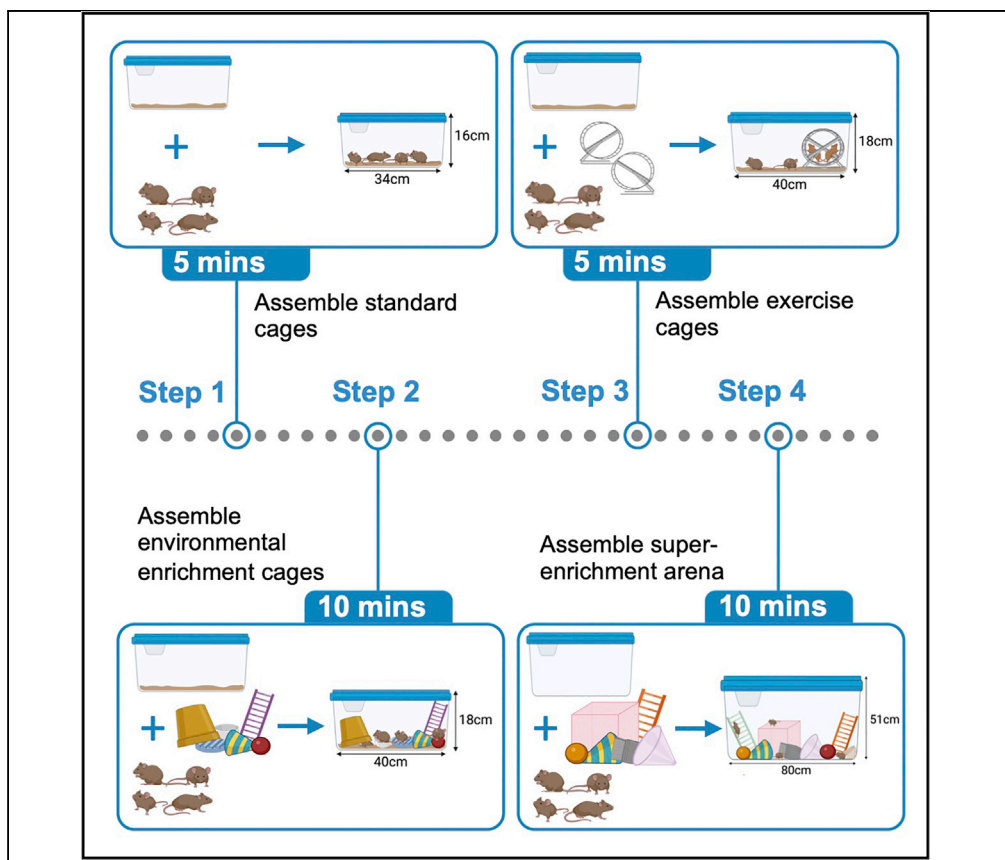


Protocol

Environmental enrichment and exercise housing protocols for mice



Here, we present a protocol that allows comparison of the effects of the standard home cage, environmentally enriched home cage with additional super-enrichment, and the exercise (running wheels only) home cage in laboratory mice. We first describe the steps to assemble these three types of cages, respectively. We then detail the assembly of super-enrichment arenas, which provide additional stimulation beyond that provided by home-cage enrichment. This protocol can help to improve reproducibility of results from studies involving environmental enrichment and exercise by offering consistent housing conditions between laboratories.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to modify levels of sensory, cognitive, and physical activity in laboratory mice

Steps to assemble standard cages, environmental enrichment cages, and exercise cages

Steps to assemble a super-enrichment arena

This protocol can be applied to other rodent models, including disease models

Protocol

Environmental enrichment and exercise housing protocols for mice

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SUMMARY

Here, we present a protocol that allows comparison of the effects of the standard home cage, environmentally enriched home cage with additional super-enrichment, and the exercise (running wheels only) home cage in laboratory mice. We first describe the steps to assemble these three types of cages, respectively. We then detail the assembly of super-enrichment arenas, which provide additional stimulation beyond that provided by home-cage enrichment. This protocol can help to improve reproducibility of results from studies involving environmental enrichment and exercise by offering consistent housing conditions between laboratories.

For complete details on the use and execution of this protocol, please refer to Gubert et al. (2021).

BEFORE YOU BEGIN

1. Sterilize the following items prior to assembly:
 - a. Cages.
 - b. Running wheels and stands.
 - c. All environmental enrichment items.

Note: All cages were sterilized using a commercial cage and rack washer and commercially available chemicals. The wash cycle runs for 240 s at 55°C using TP Alka detergent followed by a dripping cycle for 30 s and neutralization for 4 s using TP Acid. Neutralization is followed by a rinse cycle for 20 s at 82°C, steam sanitization and an exhaust cycle for 60 s at 45°C. Running wheels and environmental enrichment items are sterilized in a separate wash cycle to cages but using the same chemicals and process as above.

2. For experiments involving analysis of gut microbiota, co-house animals according to genotype (or other experimental intervention), to avoid coprophagic sharing of gut microbiota between different interventions.

Note: The coprophagic nature of mice means that, by eating the fecal pellets of other mice, they can share their gut microbial community and influence microbiota composition (Boga-tyrev et al., 2020).

3. Modulate the number of mice in each home cage depending on the desired level of social enrichment required.



Note: Mice are social animals so single housing (social isolation) is a stressor (particularly in female mice) and should be avoided unless social isolation is part of the experimental design. Whilst we would suggest that 2–4 mice per standard mouse cage is a practical number, some experimenters use larger numbers of mice per cage in their environmental enrichment conditions, to increase social enrichment. Apart from this exception, the mouse numbers per cage should be matched between the housing conditions. Furthermore, maximal numbers of animals per cage (depending on cage sizes) may be recommended by local animal ethics committees. [Troubleshooting 2](#).

- All mice should be housed in the same experimental room with the appropriate light/dark cycles and controlled for temperature and humidity.

Note: Keep in mind that open-top cages located at the top of cage racks may receive more light via illumination directly above the lids of the cages.

Note: This protocol below describes the specific steps for assembling standard cages, environmental enrichment cages, exercise (running wheel) cages and super-enrichment arenas ([Mazarakis et al., 2014](#)) for laboratory mice. However, this protocol can also be used for laboratory rats, allowing for the increased cage sizes (and running-wheel sizes) needed for rats ([Matsumoto et al., 2008](#); [Simpson and Kelly, 2011](#); [Birch et al., 2013](#); [Mika et al., 2015](#)). Furthermore, laboratory mice have a greater tendency to use running wheels (for voluntary exercise) than laboratory rats, and therefore some investigators use treadmills (usually non-voluntary and thus stress must be considered) for exercise studies in rats (and mice).

Institutional permissions

Experiments were all approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee, AEC project number 19-012-FINMH and conducted per the National Health and Medical Research Council animal research guidelines. Every effort was made to minimize the number of animals used and ensure ethical treatment. All experiments conducted using animals require permissions and approval from the relevant institutions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Male & female mice, age 6 weeks, strain R6/1 B6.Cg-Tg(HDexon1)61Gpb/J	The Jackson Laboratory	JAX: 0006471
Deposited data		
Datasets and metadata	This study	NCBI Sequence Read Archive under BioProject: PRJNA770470
Other		
Standard mouse cage base and wire lid (34 cm × 16 cm × 16 cm)	Wiretainers	MB1
Environmental enrichment & exercise home-cage base and wire lid (40 cm × 28 cm × 18 cm)	Wiretainers	RB2
Super-enrichment arena base and lid (80 cm × 43 cm × 51 cm)	Kmart	120 L Plastic Storage Tub On Wheels
Sterilized wood chips	Able Scientific	ASTAEB-FA
Sterilized tissues	CareYou	Soft White Tissue Box 2 ply – Polar Serene
Standard chow	Ridley	Barastoc Rat & Mouse Pellet
Water bottles and cap (250 mL)	Techniplast	ACBT0262 & ACCP2521
Running wheel & stand - 12 cm diameter	Kellyville Pets	46025

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Running wheel lubricating oil (non-toxic)	Local hardware store	Non-toxic oil
<u>Housing</u> : plastic pot, upside down plastic basket, plastic mouse house.	Able Scientific	ASNKPB-PC
<u>Tunneling</u> : plastic open tunneling, plastic closed tunneling, tubes or piping	Able Scientific	ASMFTR-PC
<u>Climbing</u> : plastic or metal ladders, plastic or thick metal wire, rope.	Local hardware store	Polyester climbing rope
<u>Metal items</u> : ladders, coat hangers.	Able Scientific	ASKEC-SS
<u>Ceramic or glass items</u> : cups, bowls, plates.	Local department store	Small glass cup, small ceramic bowl
<u>Floor texture items</u> : plastic grids, metal floor gratings.	Able Scientific	ASIFTB-PC
<u>Shapes and textures</u> : Cones or triangular items, brushes or bristled items.	Local department store	Plastic funnel, nail brush
<u>Movement</u> : plastic or metal balls, ping-pong (table-tennis) balls, balls with bells, tumbler balls.	Able Scientific	ASK3290
Cable ties	Kmart	500 Pack Cable Ties

MATERIALS AND EQUIPMENT

- Size of cages, environmental enrichment objects (Figure 1) and running wheels (Figure 2) may vary depending on the animal model, and the use of larger appropriate toys is common for super-enrichment (where the housing chamber is larger; Figure 2).
- The use of suitable nesting and bedding materials is an essential husbandry practice that should be considered, depending on specific study aims.
- Make sure that environmental items are open and big enough so that animals will not get stuck (e.g., tubes/tunnels must be of wider diameter than the largest animal's widest diameter), which could temporarily prevent them from accessing food and water (however all animals should be monitored daily regardless).
- Non-toxic lubricating oil for running wheels is used sparingly to avoid squeaking noises, which can be a stressor for rodents. Only a small amount of non-toxic lubricant should be used and any excess lubricant carefully wiped off, to avoid ingestion.
- Due to this protocol being used in a gut microbiome-related project, paper, wood and cardboard items were excluded from enrichment housing and the super-enrichment arena as they can carry bacteria, can be ingested by the animals (thus modifying gut microbiota) and are difficult to sterilize effectively. However, if experimenters are not specifically investigating gut microbiota, then paper and cardboard enrichment items can be used, and encourage naturalistic tearing and shredding behaviors (relevant to the ethology of nest building).
- Mice should have *ad libitum* access to food and sterilized water. Access to food or water may be restricted (to temporarily increase hunger or thirst) depending on the specific study aims (e.g., behavioral tasks requiring specific food or water rewards), providing local animal ethics approvals are obtained.

STEP-BY-STEP METHOD DETAILS

Assemble standard cages

⌚ Timing: 5 min

This step aims to create standard housing cages, where no additional items are added.

1. Fill the base of the 34 cm × 16 cm × 16 cm cage with sterilized wood shavings, enough to cover the base to approximately 1 cm of depth and four tissues.



Figure 1. Assembled environmental enrichment home cage (40 cm × 28 cm × 18 cm) demonstrating examples of each of the enrichment items

2. Add the lid to the cage.
3. Add the appropriate amount of food to each cage and water bottles in the designated area for mice to have *ad libitum* access.
4. Place mice into the cage, secure the lid and make sure to attach relevant identification tags or cards to the outside of the cage.

Assemble environmental enrichment cages

⌚ Timing: 10 min

This step aims to create an environmental enrichment cage through the addition of tunneling, climbing, housing and novel objects into the home cage, thus enhancing sensory, motor and cognitive stimulation in comparison to standard housing. At least one tunneling, housing, climbing and somatosensory item needs to be placed into each home cage.

5. Fill the base of the larger 40 cm × 28 cm × 18 cm cage with sterilized wood shavings, enough to cover the base to approximately 1 cm of depth and four tissues.
6. Add eight enrichment items listed in the materials section above into the cage including:
 - a. One housing item.
 - b. One tunneling item.
 - c. One climbing item.
 - d. One somatosensory item from each subcategory.

Note: You can use cable ties to attach climbing objects to the wire frame of the cage. Please see [troubleshooting 1](#) for considerations when choosing enrichment items. We suggest a standard number (eight in this case) of enrichment items per cage, to ensure at least a minimal level of environmental novelty and complexity, however this number can be adjusted according to specific conditions (e.g., cage sizes and enrichment-object sizes).

7. Assemble the lid and cage:
 - a. Once the lid has been assembled with the addition of any climbing object place it on top of the base of the cage.
8. Add the appropriate amount of food to each cage and water bottles in the designated area for mice to have *ad libitum* access.

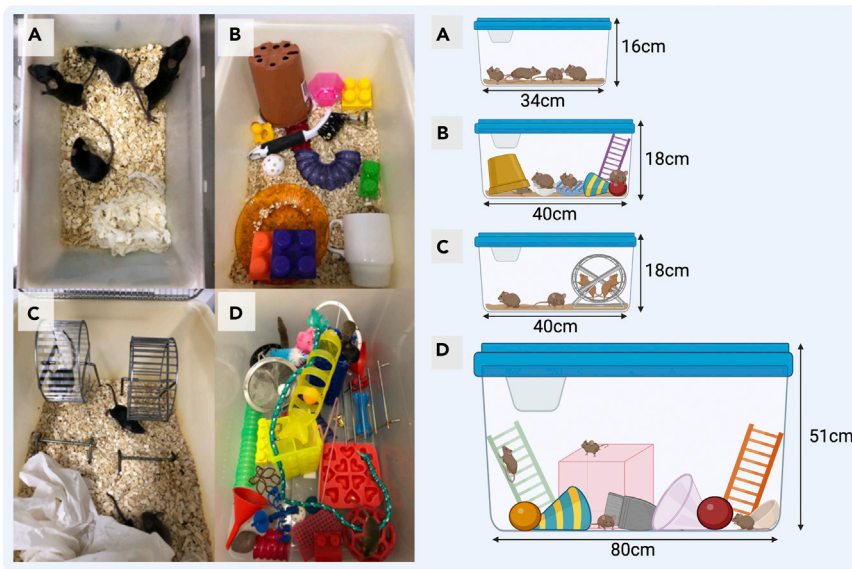


Figure 2. Photographs of assembled housing conditions and diagrammatic representation of housing sizes (A–D) (A) 34 cm × 16 cm × 16 cm standard home cage, (B) a 40 cm × 28 cm × 18 cm environmental enrichment home cage, (C) a 40 cm × 28 cm × 18 cm exercise home cage, (D) 80 cm × 43 cm × 51 cm super enrichment arena.

9. Place mice into the cage, secure the lid and make sure to attach relevant identification tags or cards to the outside of the cage.

⚠ **CRITICAL:** Environmental enrichment cages should not contain running wheels, unless your goal is to investigate the cumulative effects of both interventions (environmental enrichment and running exercise). If environmental enrichment does include running wheels, then it should be assumed that rodents will choose to use the running wheels (due to the hedonic, and possibly addictive, effects of such physical exercise) more than most other enrichment objects. It must be ensured that all running wheels have free and unrestricted spinning capacity (under voluntary ad-libitum exercise conditions), and thus enrichment objects (or bedding materials) cannot be allowed to impede the spinning of any running wheels, and this must be checked (along with the animals themselves) daily.

Assemble exercise cages

⌚ **Timing:** 5 min

This step aims to create an exercise cage through the addition of running wheels to enhance motor stimulation in comparison to standard housing.

10. Fill the base of the larger 40 cm × 28 cm × 18 cm cage with sterilized wood shavings, enough to cover the base to approximately 1 cm of depth and four tissues.
11. Assemble running wheels:
 - a. Place the running wheel stand into the sides of the running wheel.
 - b. Use oil to lubricate the join between the running wheel and the stand to make sure the wheel moves smoothly and without any noise, making sure to remove any excess lubricant (so that the rodents cannot ingest it).
12. Add running wheels into the cage:

Note: The number of running wheels will be dependent on the number of mice in the cage, with one running wheel per two mice. Due to the size of the cages used in this protocol we recommend

a maximum number of two wheels per cage, noting that two mice can run in parallel on a single wheel (although such behavior may be affected by dominance hierarchies). [Troubleshooting 3](#).

13. Add the lid to the cage.
14. Add the appropriate amount of food to each cage and water bottles in the designated area for mice to have *ad libitum* access.
15. Place mice into the cage, secure the lid and make sure to attach relevant identification tags or cards to the outside of the cage.

Note: Running trackers can also be added to the running wheels to track physical activity (i.e., total distance run on each wheel per day). Ideally, in group-housed rodents, this would be combined with a technology such as RFID tracking via injectable microchips, to establish which animal is on which wheel at any point in time. However, in the absence of such tracking technology, if all animals within a cage are of the same experimental group (e.g., same genotype), then it is possible to measure the average total distance run per animal, per day (most running will occur during their active dark cycle). Another surrogate measure of exercise is to measure the amount of food consumed (by weighing the chow left in the food hopper daily) and water consumed, and regularly weighing the animals. This provides a rough estimate of energy intake, and energy expenditure, allowing for averaging of food/water intake in group-housed cages.

Assemble super-enrichment arena

⌚ Timing: 10 min

This step is optional and aims to create a super-enrichment playground arena to further enhance sensory, motor and cognitive stimulation in addition to enrichment housing. Due to the larger size of the arena, at least two tunneling, climbing and somatosensory items from each category need to be placed into each arena. This arena can be used to ensure that environmentally enriched mice receive substantial environmental enrichment.

16. Locate the previously purchased base of an arena.

Note: No sterilized wood shavings or tissues are added to the playground arena.

17. Add enrichment items listed above into the arena including a minimum of:
 - a. Two tunneling items.
 - b. Two climbing items.
 - c. Two somatosensory items from each subcategory.

Note: No housing items or food and water are added to the playground arena, to encourage exploration and play rather than nesting. Enrichment items can be stacked on top of one another if they are stable, to encourage climbing and exploration.

18. Place mice into the arena and make sure to attach relevant identification tags or cards to the outside of the arena and add the lid to the arena to ensure mice are contained for the exposure period.

⚠ **CRITICAL:** Super-enrichment arenas must not contain running wheels, unless your goal is the cumulative effect of both interventions.

Housing maintenance

⌚ Timing: weekly, timing depends on total number of cages

Once a week all cages from all housing conditions should be changed for cleanliness purposes, with replacement of bedding materials including wood chips and tissues, removing accumulated feces and urine from the cage floor. All new houses should be assembled before changing cages so that mice can be moved directly from the old cage into the new one. Do not change cages more than weekly to avoid increased handling as a potential confounder.

Standard cage weekly maintenance

19. Following steps 1–4 to assemble a new standard cage.
20. Remove the entire old standard cage including the cage, lid, old wood chips and tissues.
21. Transfer mice directly into newly sterilized and assembled standard cage with wood chips and tissues.

Enrichment cage weekly maintenance

22. Following steps 5–9 to assemble a new environmental enrichment cage.
23. Remove the entire old environmental enrichment cage including the cage, lid, old wood chips, tissues and all enrichment items for sterilization.
24. Transfer mice directly into newly sterilized and assembled environmental enrichment cage with wood chips and tissues and a specified number (in our case eight) of new enrichment items should be added into the cage in a novel configuration.

Exercise cage weekly maintenance

25. Following steps 10–15 to assemble a new exercise cage.
26. Remove the entire old exercise cage including the cage, lid, old wood chips, tissues, running wheels and stands for sterilization.
27. Transfer mice directly into newly sterilized and assembled exercise cage with wood chips and tissues and running wheels on stands.

Super-enrichment arena maintenance

Due to the study aims, mice were placed in the super-enrichment area three times a week for one hour. After each one-hour session, mice are returned to the home cage and the following steps are to be completed:

28. Remove all enrichment toys from the super-enrichment arena.
29. Clean the arena using paper towel and ethanol.
30. Add new enrichment items in novel configuration to ensure maximal environmental novelty, complexity, and exploration for each one-hour session.

After three weekly sessions:

31. Remove the entire old super enrichment arena including the cage, lid and all enrichment items for sterilization.

Housing exposure duration

© Timing: Study dependent

For the aims of the [Gubert et al. \(2021\)](#), study animals were randomly assigned to either standard housed, environmentally enriched or exercise housing conditions for six weeks of exposure (six weeks of age to 12 weeks of age) due to the neurodegenerative nature of this mouse model of Huntington's disease (relative to the randomized littermate wild-type control mice) and previous studies (reviewed by

[Nithianantharajah and Hannan, 2006](#)). Enrichment-housed mice were exposed to the super-enrichment playground three times a week for one hour for two weeks (10 weeks of age to 12 weeks of age).

Note: This timing was specific to the aims and mouse model used in our study. Furthermore, all mice were housed in a room with a 12-hour light/dark cycle (lights on at 7.00 am), controlled for temperature (22°C) and humidity (45%). A lux meter should be used to measure luminance in housing rooms (and other laboratory rooms where live animals spend time) and, in housing rooms, cage positions should be randomized (animals in open-top cages on the top row of cage racks may be exposed to more light than those positioned in middle or lower shelves of these racks).

EXPECTED OUTCOMES

Mice should be actively exploring the super-enrichment arena during the one-hour exposure time. This can be heard as they move around the arena, or by briefly and carefully lifting the arena lid for visual inspection of movement. If the lid of super-enrichment arena is transparent, then video recording (via a tripod or other fixed-mounted camera) of animals can be performed.

LIMITATIONS

This protocol does not account for variation in exposure time to each of these housing conditions, nor does it track the physical activity of the mice in environmentally enriched conditions or the amount of running in the exercise conditions (however, approaches to measuring running distances are discussed in this protocol). Furthermore, as mentioned above, video recording of animals can be performed while animals are exploring enriched environments. Standard-housed cages in this protocol are smaller than the environmental enrichment and exercise cages, however contained the same number of mice, leading to different mice-per-area densities.

TROUBLESHOOTING

Problem 1

Animals chewing on environmental enrichment toys (step 6).

Potential solution

Consider the materials used in your home-cage enrichment and super-enrichment housing and only include hard plastic or metal items if necessary. This is an important factor to consider for microbiome-related studies, where ingested non-chow materials (that are not present in standard housing) could modulate gut microbiota.

Problem 2

Animals fighting in grouped housing conditions (step 3 in [before you begin](#)).

Potential solution

This can be seen in both males and females and is a natural social behavior for murine models. Depending on your experiment, consider whether group housing is best. Animal ethics rules should be carefully followed, and in cases of injury such as bite marks and/or bleeding (occasionally observed in males of particular genetic strains) the dominant animal (aggressor) should be identified and removed. However, it should be noted that single housing is a form of social isolation (a stressor in social rodents such as laboratory mice, particularly females), so that any rodents maintained under single housing should never be analyzed as part of an experimental group where the data is pooled with other rodents that have been group housed. Furthermore, it is always best to keep littermate mice together (with sexes separated to different cages) after weaning, to reduce the potential for fighting. And after sexual maturation (approximately 6–8 weeks) it is best not to combine non-littermate male mice from different cages, as the shift in dominance hierarchies may increase the likelihood of fighting (however this is dependent on genetic background strain and environmental conditions, as are all behaviors).

Problem 3

One animal monopolizing the running wheel (step 12).

Potential solution

You can include enough running wheels to account for the number of animals per cage, to ensure at least one wheel per two mice. Two mice can run together on the same wheel, depending on the size of the wheel and other factors, such as social dominance hierarchies.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anthony J. Hannan (anthony.hannan@florey.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets and metadata related to this study have been deposited in the NCBI Sequence Read Archive. The accession number for the raw sequence reads reported in this paper is BioProject: PRJNA770470.

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AUTHOR CONTRIBUTIONS

Conceptualization and methodology, C.G., T.R., and A.J.H.; investigation, C.G. and C.J.L.; resources, T.R. and A.J.H.; writing – original draft, C.J.L.; writing – review & editing, C.G., C.J.L., T.R., and A.J.H.; funding acquisition, A.J.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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