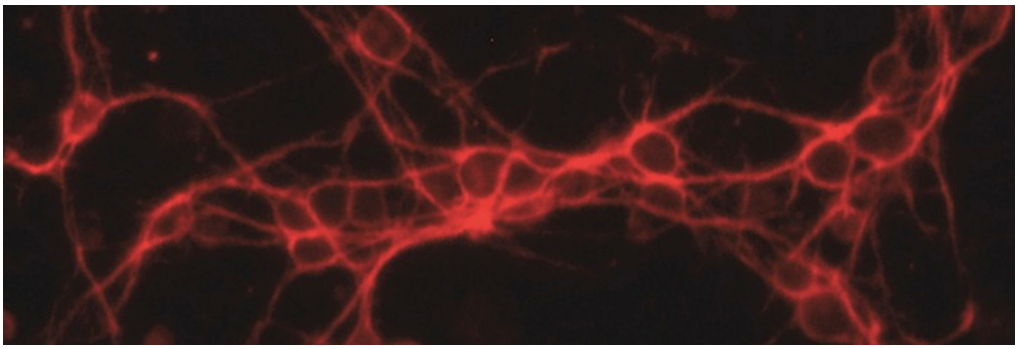
Cortical Neuron Isolation Kit

User Manual

Cat. No. CN-K



The ultimate procedure for cortical neuron isolation within a day!

Check all kit components immediately upon arrival for completeness and storage conditions

FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC AND THERAPEUTIC USE.

Table of Contents

Abbreviations

BSM	basal support medium
CDS-1	cell dissociation solution 1
CDS-2	cell dissociation solution 2
CDS-3	cell dissociation solution 3
CNS	central nervous system
L1 CAM	L1 cell adhesion molecule
MAD-N	multiple adhesion dish (for cortical neurons)
MDS	mild dissociation solution
NGM	neuronal growth medium
OSDM	oligodendrocyte survival & differentiation medium
P.Neural-plus	P.Glia defined neural medium plus
RT	room temperature
SDM	separation density medium

Kit Components and Storage

Please examine all kit components immediately upon arrival for leakage or breakage. Notify P.Glia or your distributor immediately if any problems occur.

Please note that if kit components thaw during shipment, their activity remains unaffected if they are stored at the adequate temperature (see Table 1: notes 1, 2)

All kit components must be transferred immediately into a freezer and stored at -20°C until use. Once thawed, cell culture media must be stored at 2-8°C as indicated in Table 1. Do not refreeze.

Table 1. Cortical Neuron Kit components and storage conditions.

Cat. No.	Description	Amount	Storage / Stability	Note
CDS-1	Cell dissociation solution 1	1 x 1 ml	-20°C for 12 months	**1**
CDS-2	Cell dissociation solution 2	1 x 1 ml	-20°C for 12 months	**1**
CDS-3	Cell dissociation solution 3	1 x 100 µl	-20°C for 12 months	**1**
MDS	Mild dissociation solution	1 x 5 ml	-20°C for 12 months	**1**
BSM	Basal support medium	1 x 30 ml	-20°C for 12 months 2-8°C for 1 month	**2**
NGM	Neuronal growth medium	1 x 12 ml	-20°C for 12 months 2-8°C for 2 weeks	**2**
MAD-N	Multiple adhesion dish (for neurons)	1 x 95 mm ∅	-20°C for 6 months	**1**
-	Cell strainer, 40 µm	1 x 40 µm	RT or 2-8°C	**-**
Manual	Cortical Neuron Isolation	1 x manual	1 x check list	

1	If thawed during shipment, freeze again at -20°C until use. No loss of activity!
2	If thawed during shipment and not going to be used within one week, freeze again at -20°C in working aliquots until use. Always thaw overnight at 2-8°C. Avoid repeated freezing and thawing which will impair the activity of culture media!

CN-K is a basic kit for cortical neuron isolation from early postnatal mouse or rat brain (for overview, see Table 2 on page 9).

Additional P.Glia media & reagents needed would depend on your specific applications and can be purchased separately (see page 4 and page 12).

Additional Materials Required

- HBSS (Hanks´ balanced salt solution), without calcium chloride and magnesium sulfate, sterile-filtered
- PBS, pH 7.4 (10 mM phosphate buffer pH 7.4, 140 mM NaCl, 3 mM KCl), sterile-filtered
- **P.Neural-plus** (P.Glia defined neural medium plus): can be used for in vitro myelination assays / neuron-oligodendrocyte co-cultures without further additives (optional)
- sterile instruments for tissue preparation (fine scissors, tweezers, forceps)
- sterile tubes (15 ml) and culture dishes (60 mm ∅)
- sterile serological pipettes, 1 ml-micropipette and pipette tips
- sterile pasteur/fire-polished pasteur pipettes (optional)
- standard cell culture equipment: inverse microscope, centrifuge (optimally with a swing-out rotor), laminar flow, humidified 37°C, 5% CO_2 incubator
- **Attention!** Adjust **speed (390 ± 1 x g)** and **time (5 min)** of your lab centrifuge and keep these parameters throughout the whole isolation procedure.

Additional P.Glia media and reagents which can be purchased separately if needed:

Cat. No.	Description	Size	Storage
NGM *	**Neuronal growth medium** is a serum-free chemically defined medium specially formulated to support the growth of cortical neurons/progenitors from neonatal mouse or rat brain in culture.	50 ml	-20°C
P.Neural-plus *	**P.Glia defined neural medium plus** is a chemically defined medium best suited for co-cultures of CNS neurons and oligodendrocytes under serum-free conditions.	50 ml	-20°C
OSDM	**Oligodendrocyte survival and differentiation medium** is a unique serum-free medium specially formulated to support the survival and terminal differentiation of brain-derived OPCs into mature oligodendrocytes (OLs).	30 ml	-20°C
PL-D *	**Poly-lysine containing dish:** allows the rapid expansion of cortical neurons within 2 days of culture in neuronal growth medium (NGM).	8 x 60 mm	2-8°C
CDS-3	**Cell dissociation solution 3** is a ballanced DNase-containing medium allowing the gentle dissociation of cellular aggregates into single cells.	2 x 1 ml	-20°C

* see also under „Cortical neuron expansion and further applications“ on page 12
See also **P.Glia** website for related products and applications.

Introduction

The cerebral cortex of the mammalian brain is the most complex and fascinating structure created during nervous system evolution. With its billions of neurons belonging to hundreds of different subtypes, the cortex is the brain region where „higher" cognitive functions take place. Understanding the cellular and molecular processes involved in neuronal specification and function could have important implications for a number of neurological disorders associated with cognitive impairment and neuronal dysfunction/damage. Cellular models based on primary neurons have, therefore, become an invaluable tool for the analysis of neuronal function and the development of new diagnostic and therapeutic agents for brain repair following neurotrauma or degeneration.

In all mammalian species, the cerebral cortex is organized into six cell layers of different cell densities and neuronal morphologies which appear in a strict temporal and spatial order to create different types of neurons. All these neurons belong to two major classes of excitatory **pyramidal (projection) neurons** and inhibitory **interneurons** differing in their origin, morphology, electrophysiology and connectivity[1,2]. While cortical interneurons originate from neural progenitors in the ganglionic eminence which migrate tangentially into the cortex, all pyramidal neurons arise from cortical progenitor cells in the dorsolateral wall of the telencephalon and populate the cortex in a characteristic radial migration pattern. Cortical progenitors (mainly radial glial cells with stem cell-like properties) undergo multiple steps of proliferation, neuronal commitment, cell migration, subtype specification and terminal differentiation[1].

The chart bellow gives a short summary of the present knowledge about cortical neuron origin and migration and the specification of cortical projections/ cortical connectivity (Figure 1).

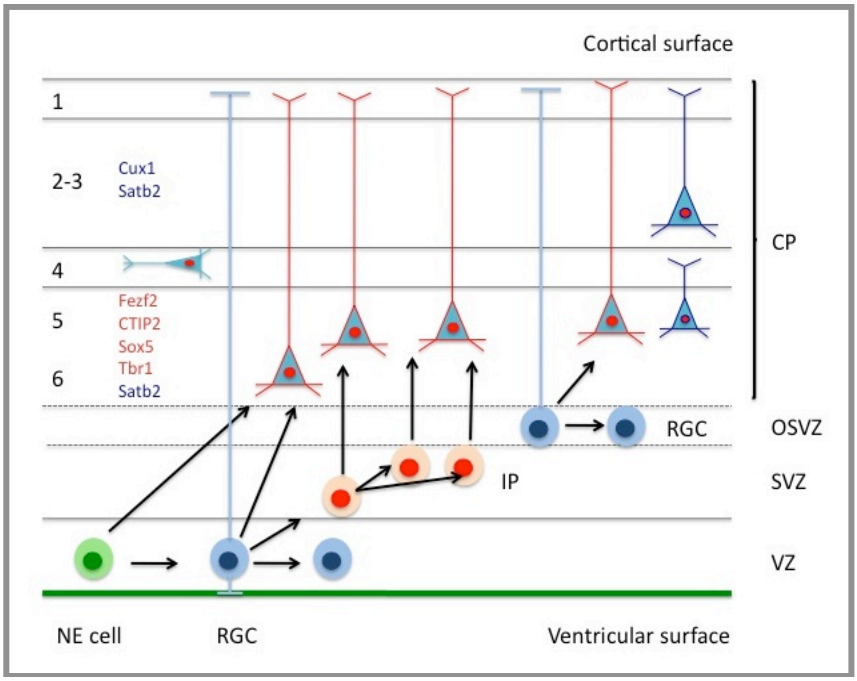


Figure 1. Cortical progenitors and laminar specification of cortical projections.

Cortical progenitors (lower part of the scheme): During embryonic development (E9-10 in the mouse), cerebrocortical progenitors are generated from neuroepithelial (**NE, green**) cells derived from the anterior part of the neural tube which can give rise to both cortical neurons and **radial glial cells** (**RGC, blue**). RGC in the ventricular zone (**VZ**) constitute the major progenitor pool in the early postnatal and adult mammalian brain. They undergo

symmetric and asymmetric cell divisions and thereby generate different neuronal cell types while maintaining a pool of progenitors in the ventricular zone. A second, RGC-derived, pool of **intermediate (or basal) progenitors** (**IP, red**) with stem cell properties is present in the subventricular zone (**SVZ**) of the cortex. Another major progenitor type was observed within the outer subventricular zone (**OSVZ**) of the developing human cortex. These **outer RGC** (**blue**) share common features with the regular RGC, including the potential for self-renewal and cortical neuron generation. Committed neurons migrate radially in an inside-out order to form the laminar structure of the cortical plate (**CP**), with early-generated neurons residing in deep layers and late-generated neurons in the upper layers.

Laminar organization of cortical projections (upper part of the scheme): The cerebral cortex is organized into six different layers (**1-6**) containing defined subtypes of neurons with a specific pattern of connectivity. **Corticofugal neurons** (**red**) reside in the deep cortical layers (5 and 6) and mainly send their axons to subcortical structures (i.e. thalamus, basal ganglia, brainstem, spinal cord). **Corticocortical-projecting neurons** send projections within the cortex (**blue**), including callosal projections, and reside primarily in the upper layers, with a small contingent present in layer 5. Each neuronal subtype is characterized by a specific gene expression (transcription factors in **red** and **blue**). Among these genes, *Fezf2* is one of the master genes for corticofugal neuron specification. **Layer 4 neurons** mainly receive inputs from other brain regions.

At the molecular level, the highly regulated processes of cell cycle exit, cell migration and neuron specification are governed by various extrinsic (*i.e.* ***neurotrophic factors, Reelin***[3] *and associated cell signaling pathways,* ***Sema3A***[4] *and its coreceptor Neuropilin1*) and intrinsic factors (*mainly cytoskeletal proteins and cytoskeleton-associated factors:* ***TUBA1, Dcx, Filamin A, GTP-binding proteins***)[5] and by differential gene regulation (***Neurog2, Rnd2, Nap1, Fezf2, Dlx****, others*[2,6-9]). During pattern formation, cortical cell migration and axon pathfinding are controlled by the regulated expression of neural recognition molecules (*i.e.* ***L1, NCAM, contactins***) and extracellular matrix receptors (***integrins, proteoglycans***) on neuronal cell bodies and axons and their associated signaling pathways[10]. In the course of maturation and synaptogenesis, cortical neurons are characterized by the stable expression of neuron-specific proteins and enzymes, such as ***tyrosine hydroxylase, glycolytic enzymes, synaptophysin, synapsin IIa, α and ß synucleins, glutamate*** (***NMDA, AMPA***) and ***GABA receptors*** (inhibitory neurons).

The neuronal phenotype selected with this kit corresponds to immature cortical neurons.

References

1. Merot J, Retaux S, Heng JI. Molecular mechanisms of projection neuron production and maturation in the developing cerebral cortex. Semin Cell Devel Biol. (2009) 20:726–734.
2. Gaspard N, Vanderhaeghen P. Laminar fate specification in the cerebral cortex. F1000 Biol Rep. (2011) 3:6.
3. Gupta A, Tsai LH, Wynshaw-Boris A. Life is a journey: a genetic look at neocortical development. Nat Rev Genet (2002) 3:342–55.
4. Polleux F, Morrow T, Ghosh A. Semaphorin 3A is a chemoattractant for cortical apical dendrites. Nature (2000) 404:567–73.
5. Hammond V, Tsai LH, Tan SS. Control of cortical neuron migration and layering: cell and non cell-autonomous effects of p35. J Neurosci. (2004) 24:576-87.
6. Heng JI, Nguyen L, Castro DS, Zimmer C, Wildner H, Armant O, et al. Neurogenin 2 controls cortical neuron migration through regulation of Rnd2. Nature (2008) 455:114–8.
7. Yokota Y, Ring C, Cheung R, Pevny L, Anton ES. Nap1-regulated neuronal cytoskeletal dynamics is essential for the final differentiation of neurons in cerebral cortex. Neuron (2007) 54:429–45.
8. Chen B, Wang SS, Hattox AM, Rayburn H, Nelson SB, McConnell SK. The Fezf2–Ctip2 genetic pathway regulates the fate choice of subcortical projection neurons in the developing cerebral cortex. Proc Natl Acad Sci USA (2008) 105:11382–7.
9. Koizumi H, Tanaka T, Gleeson JG. Doublecortin-like kinase functions with doublecortin to mediate fiber tract decussation and neuronal migration. Neuron (2006) 49:55–66.
10. Maness PF, Schachner M. Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration. Nat Neurosci. (2007) 10:19-26.

Principle and Benefits

The **MAD** (multiple adhesion dish) principle of neural cell isolation relies on basic knowledge in cell biology that the strength of cell adhesion to the surrounding matrix or to other cells strongly depends on the affinity of different cellular receptors to their extracellular ligands and the respective activation of intracellular signaling. Ligand-receptor interactions may thus result into diverse adhesive forces varying from very stable to weak or even repellent. The unique properties of **MAD** are based on accordingly selected multiple cell binding sites supporting the adhesion of different cell types and of different adhesive strength. Taking advantage of such molecular mechanisms, MAD allows the isolation a single cell type out of a complex mixture due to its weak adhesiveness. Moreover, this takes place under defined culture conditions favoring the growth of the desired cell type (Figure 2).

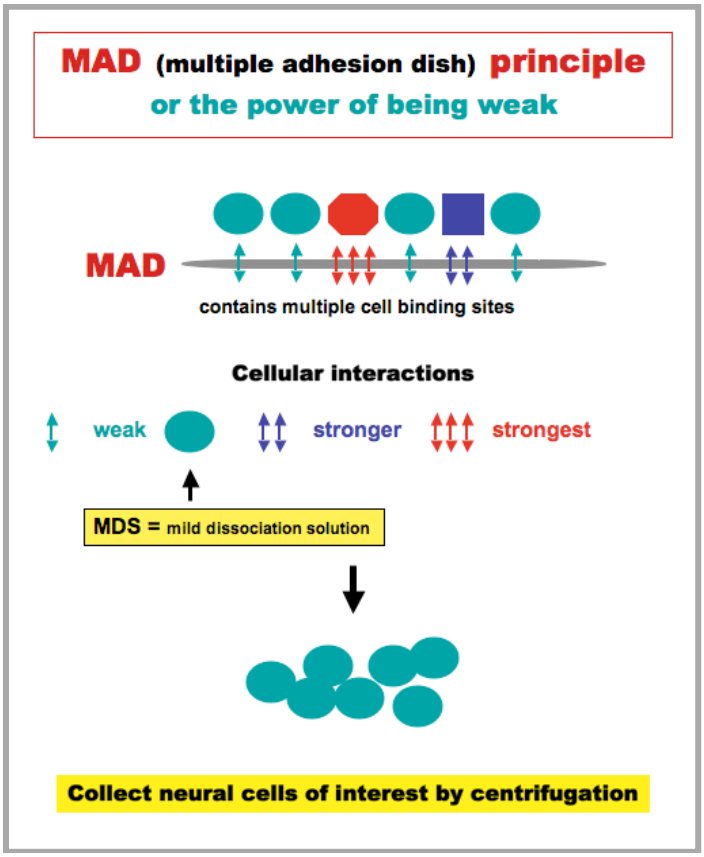


Figure 2. The MAD principle.

The unique properties of **MAD** are based on multiple cell binding sites supporting the adhesion of different cell types and of different adhesive strength, from weak to very strong. Weak cellular interactions with the MAD surface can be disrupted by treatment with a mild dissociation solution (MDS) and target cells collected by centrifugation.

How it works

For cortical neuron isolation, all you need to do is to dissociate neonatal (postnatal day 0-1) brain tissue and plate single cell suspensions into **MAD-N** in a specialized neuronal growth medium (**NGM**). Then just let brain cells undisturbed for one day in culture. The concept of the cell binding sites is such that cellular interactions of neural progenitors with the MAD surface are the weakest ones. Upon short treatment with mild dissociation solution (**MDS**), cortical neurons can easily be detached from the MAD surface and

collected by centrifugation. The procedure allows an easy and reliable isolation of cortical neurons (within one day) and further expansion (within 1-2 days) before use. It has been optimized for neonatal (P0-1) mouse or rat brain tissue.

The flow chart below (Figure 3) gives a short cut of the bench protocol.

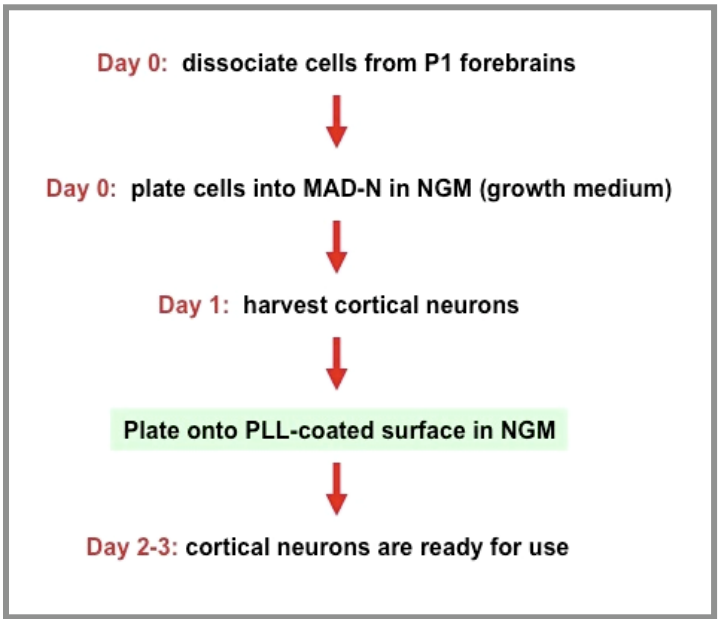


Figure 3. Bench protocol (short cut).

When plated into the desired well or dish format (coated with poly-L-lysine, PLL), MAD-derived neurons can be further expanded in growth medium (**NGM**) or directly used for the experimental set-up of interest. MAD-derived neurons can be further cultured in **P.Neural-plus** (P.Glia defined neural medium plus) with or without further additives.

What you get

After one day of culture in NGM, many single or groups of proliferating neurons, but almost no astrocytes, are present in the MAD-N culture dish. The following figure (Figure 4) shows cortical neurons immunostained for L1 CAM after 3 days of culture in NGM.

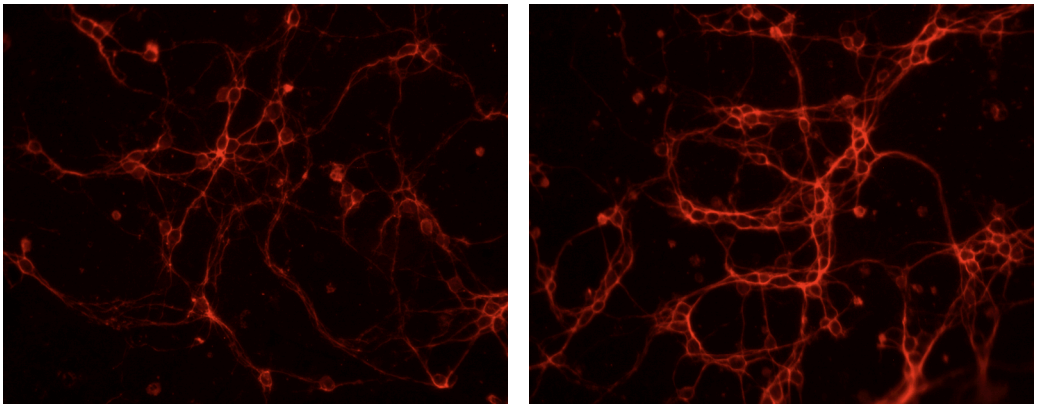


Figure 4. MAD for cortical neuron isolation.

P1 mouse forebrain-derived cells were grown for one day on **MAD-N** in neuronal growth medium (**NGM**) and

cortical neurons were collected from the dishes after MDS (mild dissociatiation solution) treatment. MAD-derived cortical neurons were plated into 8-chamber slides (PLL-coated) in **NGM** and immunostained for L1 CAM after 2 days in culture. Cellular phenotype: **L1+ TUJ-1- A2B5-**

The cell isolation and culture conditions promote a rapid increase in the number of cortical neurons from P0-1 mouse or rat brain within one day and do not require embryonic brain tissue and long culture periods.

Purity and cell yields. Since the control of neuronal cell detachment from MAD-N under a microscope can be very individual-dependent, the purity of isolated cells after one day may slightly vary.

In order to obtain high cell yields, it is strongly recommended to exactly stick to the parameters (i.e. enzymatic treatment, number of culture dishes, etc.) worked out for the number of animals given in Table 2.

Table 2. Number of postnatal (P0-P1) animals and amounts of reagents required.

Treatment / Reagent	**3 x P1 mouse forebrains**	**2 x P1 rat forebrains**
CDS-1 (1 x 1 ml)	8 min, RT	10 min, RT
CDS-2 (1 x 1 ml)	5 min, RT	5 min, RT
CDS-3 (1 x 100 µl)	10 min, RT	10 min, RT
MAD-N culture	1 x 95 mm dish: 1 day	1 x 95 mm dish: 1 day
MDS (1 x 5 ml) *4-5 ml/dish (95 mm)*	1-2 min agitation, RT *(detachment control!)*	1-2 min agitation, RT *(detachment control!)*
BSM	1 x 50 ml	1 x 50 ml
NGM (for cortical neurons)	1 x 12 ml	1 x 12 ml
P.Neural-plus	(optional)	(optional)
Cell strainer, 40 µm	1 x 40 µm	1 x 40 µm

Benefits at a glance:

- cost-effective and time-saving (at least fourfold)
- animal-saving (at least 50%)
- easy handling (no longer than 2 hours work on the bench at Day 0)
- no dedicated equipment required
- from postnatal mouse or brain
- high cell yields (>2 x 10^6 cells within 2-3 days) ●
- unique **MAD-N** properties: selective support of neuronal cell adhesion and growth
- unique properties of **NGM** (neuronal growth medium)
- unique differentiation medium **P.Neural plus**

● Please note that in primary cell cultures, the final cell yields may vary dependent on animal species, number and age of animals used, between individual animals, operator´s skills and/or additional materials used.

Protocol

PLEASE READ THIS PROTOCOL CAREFULLY BEFORE STARTING WITH CELL ISOLATION

A. General considerations

You should be aware of the following ***safety rules*** concerning aseptic handling of primary animal tissue and cell culture materials:

1. Decontaminate all surfaces of workbenches, microscope(s), laminar flow, etc. going to be used for tissue preparation and cell culture with 70% alcohol to ensure they are sterile.
2. Decontaminate external surfaces of all vials, bottles and micropipettes with 70% alcohol to ensure they are sterile.
3. Make sure all solutions, cell culture materials and instruments for tissue preparation are sterile.
4. Always thaw cell culture media overnight at 2-8^{o}C (see Table 1 for further details).
5. Perform cell culture work only in a sterile hood or laminar flow.
6. Do not pipette with mouth.
7. Always wear gloves and a clean lab coat when working with primary animal tissue.
8. After thawing, always keep enzyme solutions on ice until use.

The following protocol is recommended for the isolation of cortical neurons using **forebrains** from **P0-1 mice** or **rats**. Amounts of solutions / incubation times indicated at different steps of this protocol have been optimized exactly for the number of postnatal animals used (see Table 2, page 9). Change of any parameters is not recommended and no responsibility will be taken accordingly.

To facilitate the step-by-step performance avoiding unprepared handling, some ***preparatory steps*** (***in italic bold***) have also been included. The whole procedure takes about 1.5-2 hours (from tissue preparation to culturing of brain cells).

Attention! All P.Glia dishes should be washed 2 x 10 min with PBS before use.
Before starting with cell isolation, place MAD-N dishes at RT and rehydrate for 10 min in PBS. Wash once with PBS and keep in Neuronal Growth Medium (6 ml/95 mm dish) ***in a humidified CO_2 incubator until use.***

B. Tissue dissociation and single cell isolation

Day 0

1. Decapitate P1 mice and collect heads in a sterile dish. Decontaminate the entire surface of the skin with 70% alcohol and wait for 3-5 min to ensure it is sterile.
2. ***In the meantime, prepare 2 x culture dishes (60 mm ∅) with HBSS (2 ml/dish).***
3. Carefully remove the skin from the cranial region of each head.
4. Cut out the dorsal part of each skull very carefully with sterile scissors. Avoid disruption of the underlying brain tissue.
5. Dissect out brains with sterile tweezers and collect them into a culture dish containing 2 ml HBSS.
6. Separate forebrains from the rest brain regions and transfer into another HBSS-containing culture dish. **No need to remove meninges!**
7. ***All the following steps are performed in a sterile laminar flow!*** Aspirate HBSS and add 1 ml **CDS-1** to the dish at room temperature (RT).

8. Quickly disaggregate the tissue with fine tweezers (into pieces of about 2-3 mm) and transfer tissue pieces with the enzyme solution into a 15 ml tube using a sterile pasteur pipette.
9. Incubate for 8-10 min (see Table 2) at RT and gently rock the tube from time to time to provide an even enzyme access to the tissue.
10. Immediately add 6 ml ice-cold HBSS, close the tube and mix the content. Collect cells and tissue pieces by centrifugation (5 min, 390 x g, 4°C).
11. ***In the meantime, prepare a fire-polished pasteur pipette (with an aperture of 0.5-1 mm in diameter) using a gasburner (optional).***
12. Aspirate supernatant very carefully (the tissue pellet is usually not very compact!) and add 1 ml **CDS-2**. Incubate for 5 min at RT and rock the tube from time to time.
13. Dissociate tissue pieces with a fire-polished pasteur pipette by gently pipetting 6 times up and down to obtain single cell suspensions (*If no gasburner is available to prepare a fire-polished pasteur pipette, use a 1ml-micropipette instead*). Avoid foaming of cell suspensions which may disrupt living cells.
14. Add 9 ml of ice-cold **Basal Support Medium**, mix the tube content and carefully pass the cell suspension through a **40 µm cell strainer** placed onto a sterile 50 ml tube. Collect cells by centrifugation (5 min, 390 x g, 4°C).
15. ***Get sure that MAD-N was rehydrated and placed into a humidified CO_2 incubator (see instructions on page 10).***

C. Cortical neuron propagation and isolation

1. Aspirate the supernatant from the tube and resuspend the cell pellet in 1 ml ice-cold **Neuronal Growth Medium** with a 1 ml micropipette and add the cell suspension to the **MAD-N** dish containing 6 ml **Neuronal Growth Medium**.
2. Mix the dish content to allow an even distribution of brain cells over the substrate surface and leave cells undisturbed for 1 day in vitro (1 div) in a humidified 37°C, 5% CO_2 incubator.

Day 1

3. Examine cell culture under a microscope by gently agitating the **MAD-N**. After 1 div in NGM, many proliferating neurons (single cells or cell groups) and few astrocytes are present in the MAD-N culture dish.
4. ***In the meantime, equilibrate appropriate amounts of mild dissociation solution and Basal Support Medium (5 ml of each solution/dish) to RT or pre-incubate in CO_2 incubator.***
5. Carefully remove the medium from **MAD-N** and apply immediately PBS (5 ml/dish) equilibrated to RT by gently pipetting at the inner dish wall. Agitate the dish to remove floating cellular material and carefully aspirate the solution. Wash with PBS once more if necessary. Apply immediately **mild dissociation solution** (4-5 ml **MDS**/dish) by gently pipetting at the inner dish wall. Avoid dislodging cells!
6. Agitate the dish for 1-2 min by controlling neuronal cell detachment from MAD-N under a microscope. Do not pipette! **Neuronal cells would readily detach from the substrate and occasionally present astroglial cells should remain in the dish!** Since MDS treatment (by agitation) can be very individual-dependent, the purity of isolated cells may vary.
7. Transfer cell suspension into a 15 ml tube and carefully add 5 ml Basal Support Medium at the inner dish wall to collect residual cells. Collect cells by centrifugation (5 min, 390 x g, 4°C).
8. Since isolated cells tend to aggregate, resuspend cell pellet in 100 µl **CDS-3** and leave for 10 min at RT. Disaggregate cells by slowly pipetting 6-7 times up and down.

9. Resuspend cell pellet in the appropriate amount of **Neuronal Growth Medium** and directly plate cells into your final dish or slide format (coated with poly-L-lysine, PLL), dependent on your specific application. ***Please note that cortical neurons might be poorly adhesive on poly-D-lysine coated surfaces!***

D. Cortical neuron expansion and further applications

When plated into the desired well or dish format (PLL-coated) or into **PL dishes** (from P.Glia), MAD-derived neurons can be expanded in Neuronal Growth Medium (**NGM**) for further two days or directly used for the experimental set-up of interest.

For in vitro myelination assays, MAD-derived neurons can be co-cultured with oligodendrocytes in **P.Neural-plus*** (P.Glia Defined Neural Medium plus; from P.Glia) or **OSDM*** (Oligodendrocyte survival & differentiation medium**;** from P.Glia) without further additives.

After cortical neuron expansion in NGM, change half of the culture medium with **P.Neural-plus** or **OSDM** and then change half of the culture medium every 2-3 days.

* **P.Neural-plus** and **OSDM** are not included with this kit and can be purchased separately if needed.

For further details and treatments, see also **CN-Kp** Manual (pages 12-13).