Protocol for **Lipofectamine 2000** transfection of 12-20 DIV hippocampal neurons – (TWO wells; diameter - 2cm, **12-well plate**).

1. Take 2 ml aliquota with **MEM-** (MEM without serum and antibiotics) from a fridge, spray ethanol on a napkin and clean the tube with it. Put it into incubator (37 C, 0% CO_2) for 60min – **MEM-** should be at 37 C when you change solution in a well.
2. Take 2 ml aliquota with **Opti-MEM** from a fridge, spray ethanol on a napkin and clean the tube with it. Put it into incubator (37 C, 0% CO_2) for 20min – **Opti-MEM** should be at 37 C when you change solution in a well.

3. Prepare your working place in laminar – put in laminar sterile gloves, 1 ml **DNA-FREE tube A** (you need it for DNA solution),1 or 0.5 mL **DNA-FREE tube B (for DNA)**, rack for tubes, appropriate automatic pipettes – 1 ml, 200 ul and 20 ul, sterile **tube C** for Culture medium (~10-20 ml tube) , pipette tips – 1ml and 200 ul (in all procedures always use a new tip – don't use any tip twice) and a cup for used tips, expose all these to UV-light for 15-20 min. Tip boxes and tubes should be open in order for them to be directly exposed to UV light.

4. Remove the **12-well plate** and tube with **MEM-** from the incubator and place them into the laminar. Put old Culture medium from one well into **tube C and** replace it with 1 ml of **MEM-. Do the same for another well. Try to replace the medium as quickly as possible**. Then place **12-well plate** and **tube C** into the incubator. The **tube C** should be kept open in the incubator. Take aliquota with **Opti-MEM** from the incubator

5. In the laminar add appropriate volume of **Opti-MEM** (150 ul for 2 wells) into **tube A,** then add an appropriate amount of DNA into a **tube A** (1 ug of DNA for 2 wells) (**don't expose DNA to UV-light!!!!!)**. Gently vortex the **tube A** outside the laminar for about 5s.

6. Put tube E with **Lipofectamine2000** from the fridge into the laminar (**don't expose it to UV-light!!!!!) (never freeze Lipofectamine!!!!!!!).** Add an appropriate volume (150 mL for 2 wells) of **Opti-MEM** in the **tube B**, then add 7 ul of **Lipofectamine2000 from** tube E into **tube B.** Gently vortex the **tube B** outside the laminar for about 5s, then return it to the laminar.
Wait for 10 min.

7. Add all solution from **tube B** into **tube A (do all these in laminar)**. Gently vortex the **tube A** outside the laminar for about 5s.
Wait for 12 min.

8. Take the **12-well plate** with cells from the incubator and place it in the laminar. Vortex solution in **tube A** just prior to addition to the wells of interest. Be sure to add the solution dropwise to the wells (distribute it slowly and equally to wells; 150ul per well). Gently swirl the **12-well plate** 5 times to distribute the transfection solution evenly over the cells. Return the **12-well plate** to the incubator. Return **Opti-MEM** aliquota to fridge. (4 C- **never freeze Lipofectamine!!!!!!!**)
Wait for 3 hour.

9. Remove the **12-well plate** with cells and **tube C** from the incubator and place in the laminar. Replace **MEM-** in transfected wells with the original Culture medium from the **tube C**. **Try to do this as quickly as possible.** Return the **12-well plate** back to the incubator. Clear laminar, wash the **tube C** and cup for used tips.

Materials (all used materials should be specified with the current used catalogue numbers, places they are stored and their main details (e.g. volume for tubes)

1. **MEM-** – MEM without serum and antibiotics (MEM + 25mM HEPES with GlutamaxI (liquid); Invitrogen 42360-024).
2. **tube A**-1 ml **DNA-FREE tube**
3. **Tube C**- sterile **tube** for Culture medium (~10-20 ml tube)
4. **Tube B**-1 or 0.5 mL **DNA-FREE tube**
5. **Tube E – tube(aliquot) with 100-200 ul of Lipofectamine 2000,stored in freezer at 4C (never freeze Lipofectamine!!!!!!!)**
6. **Opti-MEM**