**Standard Operating Procedure, SOP**

**Titel:**

**Cell Seeding and maintenance**

**for combinatorial RNAi screening**

**Valid: ERC Project**

**Author(s):** Laufer, Christina

*(Surname, Name)*

**Approved by:**

*(Surname, Name)*

**Staff Signature:**

*(signing below indicates that you have read this SOP and understand the material contained in it)*

|  |  |  |
| --- | --- | --- |
| **Date** | **Name** | **Signature** |
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |

**Revision History**

|  |  |  |  |
| --- | --- | --- | --- |
| **Version** | **Reason for changes** | **Changed at** | **Changed from** |
| 01 | New document | 25.10.2016 | Christina Laufer |
| 02 | Adapted after first rounds of screening | 06.12.2016 | Christina Laufer |
|  |  |  |  |
|  |  |  |  |
|  |  |  |  |
|  |  |  |  |
|  |  |  |  |
|  |  |  |  |
|  |  |  |  |
|  |  |  |  |
|  |  |  |  |

**Table of contents**

1 Purpose 4

2 Description 4

2.1 General information 4

2.2 Process 4

2.3 Locations 4

3 Materials & Reagents 4

4 Procedures 5

4.1 Preparations 5

4.2 Plate & query preparation 6

*4.2.1 Query preparation* 6

*4.2.2 Plate preparation* 7

4.3 Cell preparation 7

4.4 Cell seeding 8

4.5 Cleaning 9

5 Safety information 9

6 Other applicable documents 9

7 Attachments 9

7.1 Media, buffers and solutions 9

# 1 Purpose

This standard operation procedure explains the cell seeding and maintenance procedure used in SYNGENE combinatorial RNAi screening in *Drosophila* D.mel-2 cells. It might need to be adapted for other cell lines or screening purposes.

# 2 Description

## 2.1 General information

For library design and set composition, please see file‚ SYNGENE library.pptx’ in *PowerFolders/ERC/D1086-ERC-Project-SYNGENE-library/SYNGENE\_Library/General\_Information.* The assay plates and query dsRNAs are prepared in Mannheim and transferred to Heidelberg. They are stored frozen in room 3.120 (HTS2).

## 2.2 Process

Each Monday and Thursday 2 sets of SYNGENE library assay plates (40 plates per set) are screened, each in combination with one query dsRNA design.

This SOP describes the procedure for 1 set of 40 plates, including preparation of assay plates and cells, cell seeding and transferring the plates to incubation, as well as preparing cells for the subsequent screen. The procedures for two subsequently performed sets take about three hours in total.

SOPs for previous and subsequent screening steps can be found under *PowerFolders/ERC/SOPs.*

## 2.3 Locations

Room 3.111 (HTS1) Screening lab

Room 3.121 Cold room

Room 3.120 (HTS3) Location of ERC freezers (F17, F22)

# 3 Materials & Reagents

* Water bath TW12 Julabo
* Centrifuge with plate holder 5810 Eppendorf
* Multidrop Combi (B110 Nr.3)

with standard cassette (ERC1 or ERC2) Thermo Fisher

* Cellometer Auto 100 PeqLab
* Cell Counting Chambers SD100 Nexcelom

Bioscience

* PlateLoc heat sealer
* PlateLoc seal
* Wheaton Spinner flasks 500 ml

With Corning stirrer

* Measuring cylinder 500 ml
* Serological Pipets, 5 ml, 10 ml, 25 ml, 50 ml
* 50 ml Tubes Falcon
* T175 cell culture flasks with ventilated caps
* Wipes
* 384 well microtiter plates, black, clear bottom 781091 Greiner bio-one
* 384 well microtiter plates, clear 781182 Greiner bio-one
* Express Five serum-free cell culture medium 10486-025 Gibco

+ 10% GlutaMax (100x) 35035-038 Gibco

* 1 x PBS 10010-015 Gibco
* 2 x Trypan Blue (0.4%) T8151 Sigma
* 70 % EtOH

# 4 Procedures

**Note:** For more information on the instruments, please see the *Instruments SOP* or ask the *responsible persons* (see black board (Labjobs sheet) or WIKI)

## 4.1 Preparations

*1 day in advance:*

* Print **tracer sheet** (*PowerFolders/ERC/SOPs*) and fill in starting date (date of cell seeding!) and screen ID (all pages!) in the headline
* Find sets for upcoming screens in freezer F17, room 3.120 (HTS3) (2 consecutive sets per drawer, check the barcodes!)
* Transfer the whole drawer with the two sets to the cold room (room 3.121) and place the rack in the metal shelf to the left for thawing
* Find the needed query dsRNA in freezer F22, drawer A1, blue-capped vials in box. Put them into the cold room for thawing as well
* Sign in **tracer sheet**

*1 hour in advance:*

* Start with preparations, check if everything listed is ready
* Turn on and open sterile cell culture hood and let run for 15 min
* Put medium in water bath on 25°C (2 bottles)
* Clean workspace under hood with 70% EtOH
* Check if barcodes of plates in coldroom match the screen ID on the tracer sheet
* Transfer assay plates of both sets to room temperature ≥ 30 min before screening
* Centrifuge the plates at 1300 rpm for 1 min (accel: 4, brake: 4, rad: 16.1, g=304)
* Wipe plates with 70 % EtOH, place on table right to the hood (sorted downward)
* Place Falcon tube with 50 ml of 70% EtOH for cleaning Multidrop and Falcon tube with 50 ml of medium for flushing Multidrop under hood
* Note Multidrop cassette # on **tracer sheet**
* Rinse cassette with 70% EtOH from outside, put under hood and let dry, then mount and set up Multidrop (see Intruments SOP)
* Wipe medium bottle with 70% EtOH and place unter hood. When opening new bottle, add 1 bottle (100 ml) of Glutamax.
* Clean and equilibrate Multidrop:
* Prime with ~20 ml 70% EtOH
* Let sit for 5 min with relaxed tubing
* Prime with remaining 70% EtOH
* Prime with ~25 ml medium/10% Glutamax

**Notes:**

* Change Casette every two weeks and give the used one to calibration
* Always empty cassette tubing before switching from EtOH to medium.
* Relax tubing in breaks between dispensing steps.

## 4.2 Plate & query preparation

### *4.2.1 Query preparation*

* Total volume of medium/query to be prepared (including excess volume):

30µl x 384 wells x 45 Plates = 518,4 ml

* Stock solution of query dsRNA: 1µg/µl
* Total volume of medium: 514 ml (29,75 µl x 384 wells x 45 plates)
* Total volume of query dsRNA: 4,4 ml (0.25 µl x 384 wells x 45 plates)

### *4.2.2 Plate preparation*

* **Control plates 1 and 2 + seeding control/view plate 1:**

Dispense **30 µl/well** of Express Five medium + 10% Glutamax per well with Multidrop

Mode: **high-speed**

Multidrop program: **Erc\_add\_query**

* **SYNGENE assay plates + seeding control/view plate 2:**

Dispense **30 µl/well** of medium/10% Glutamax **with query dsRNA**

Mode: **high-speed**

Multidrop program: **Erc\_add\_query**

* Sort plates for screening, numeric ascending, control plate 1 before plate \_1001, control plate 2 after plate \_2019, seeding control plates 1 + 2 after ctrl plate 2

## 4.3 Cell preparation

**Notes:**

* For each set of 40 plates, fresh cell suspension is prepared, even though the sets are screened consecutively.
* 500 ml cell suspension is prepared for 40 plates. The excess volume is to ensure enough volume in spinner flask (too little volume while spinning can cause trouble with cells).

1. Detach cells by pipetting
2. Take an aliquot of 100 µl cell suspension for cell counting
3. Dilute PBS/Dye with cell suspension in ratio 1:2 (50µl PBS + 50 µl Trypan blue + 100 µl cell suspension) and use 20 µl to count the cells
4. Count cells with Cellometer, program *Dmel2.viability trypanblue 0.1%* (see also Instruments SOP)
5. *Count the following flasks:*
   1. Flask 1 for maintenance
   2. Flask 2 and 3, pooled for first query/design
   3. Flask 4 and 5, pooled for second query/design
6. *Count each by the same procedure*
   1. Take 1 ml from culture into fresh tube
   2. Prepare fresh tube with 50 µl PBS + 50 µl 2x TrypanBlue
   3. Transfer 100 µl of culture i. to Eppi ii. and mixe by pipetting 5 times up and down
   4. Transfer 20 µl of stained cells c) into Nexcelom counting slide

(**Note:** remove both foils (upper and lower) from counting slide)

* 1. Set slide in Cellometer auto
  2. Press “preview image” and “count current image”

1. Take notes and switch off the device by pressing the “ON” Button once
2. Put down the following numbers in the **tracer sheet**

* Total number of cells
* Number of live cells
* Number of dead cells
* Viability (%)

1. Prepare 5 flasks for next cell seeding (1 for maintenance, 2 for each set):

* On Mondays, prepare 60 mio cells in 25 ml Express V /10% Glutamax in T175 cell culture flask, incubate at 25°C, in Brunswick incubator, no CO2 Adjustment
* On Thursdays, prepare 35 mio cells in 25 ml Express V /10% Glutamax in T175 cell culture flask, incubate at 25°C, in Brunswick incubator, no CO2 Adjustment

Set concentration of cell solution to **0.9 mio cells/ml** by diluting cell suspension in Express Five medium/10% Glutamax in sterile measuring cylinder.

1. Note down the calculation in the **tracer sheet**.

## 4.4 Cell seeding

**Notes:**

* First, dispense 3x into a dummy plates (empty assay plate, autoclave after dispensing)

1. Transfer cell solution in corning Spinner Flask
2. Stirrer setting: ca. 50% max speed
3. Dispense **10 µl/well** of prepared cell suspension

Mode: **slow**

Multidrop program: **Erc\_cell\_seeding**

1. Hot seal plates with PlateLoc (see Intruments SOP)
   * + Settings: 2.5 sec, 178°C
     + Let plates cool-down before stacking
2. Spin microplates at 140 g (882 rpm) for 1 min (accel: 4, brake: 4, rad: 16.1)
3. Incubate plates for 96 hours (4 days) @ 25°C, no CO2 adjustment, upper binder incubator
4. Sign in **tracer sheet**

## 4.5 Cleaning

1. Clean Multidrop by priming with

* ca. 20 ml express Five medium (before performing a second cell seeding)
* 70% EtOH and ddH2O when the cell seeding is finished

1. Clean bench space with 70% Ethanol

# 5 Safety information

**70% Ethanol:**

Ethanol is flammable (liquid and vapor) and can have severe health effects upon inhalation and contact with skin/eyes. Take the appropriate health precautions.

# 6 Other applicable documents

* **Tracer sheet** (PowerFolder -> ERC -> SOPs)
* Instruments SOP (for Multidrop dispenser, Cellometer cell counter and PlateLoc heat sealer)

# 7 Attachments

## 7.1 Media, buffers and solutions

***Express Five serum-free cell culture medium + 10% GlutaMax***

To a freshly opened bottle of Express Five Medium, add 100 ml of 100x Glutamax.

***Disinfection reagent*, *70% Ethanol, 1l***

Dilute 700ml Ethanol absolut with 300ml ddH2O