Leibniz-Institut
DSMZ-Deutsche Sammlung von
Mikroorganismen und Zellkulturen GmbH

Handling of retinoblastoma cell lines

This protocol describes the standard procedure to cultivate retinoblastoma cell lines provided by DSMZ.

Cell line origin

Retinoblastoma cell lines were derived from tumor material extracted of enucleated eyes of patients with retinoblastoma. Retinoblastoma is a tumor of the retina occuring in children under the age of five. The molecular cause is, in most cases, biallelic inactivation or the tumor suppressor gene *RB1*. In rare cases, retinoblastoma arises due to amplification of *MYCN*. Inactivation of *RB1* can be caused by promoter hypermethylation, different mutations in the *RB1* gene and deletion of the gene. Often tumor material shows loss of heterozygosity (LOH), meaning loss of the second allele of *RB1* or the entire chromosome 13.

Molecular characterization

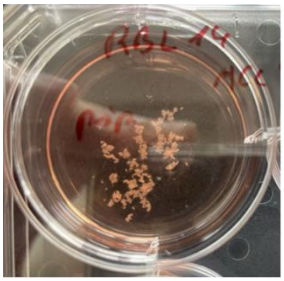
Described *RB1* mutation can be determined by Sanger sequencing of the respective exon
Hypermethylation of the RB1 promoter can be tested by bisulfite treatment of genomic DNA followed by sequencing (Sanger = non-quantitative; deep amplicon sequencing = quantitative), or by MLPA
Analysis of larger exon deletions and promoter methylation status can be done by MLPA using the SALSA MLPA Probemix P047 RB1 of MRC Holland
Zygosity and number of chromosome 13 can be identified by microsatellite analysis of chromosome 13 or STR, MLPA or analysis of chromosome spreads

Material

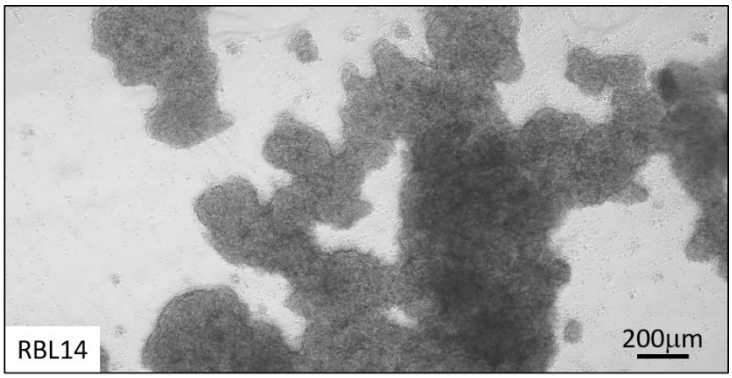
- Medium: DMEM high glucose (Gibco #11965065), 15 % FBS, 1 mM sodium pyruvate (Gibco #11360039), 1 x Glutamax (Gibco #35050037), 50 µM ß-mercaptoethanol (Gibco #31350010), 10 µg/ml human insulin (Gibco #12484014)
- D-PBS without Magnesium and Calcium (Gibco #14190144)
- Accutase (Merck #A6964-100ml)
- Trypan blue (Sigma #T8154)
- DMSO (Honeywell #D5879)
- Serological pipettes, 5 ml and 10 ml
- 6-well plates, TC-treated (Nunc #140675)
- Wide-bore tips – or 1000 µl tips cut with scissors – opening of tip should be around 3 mm
- Hemocytometer

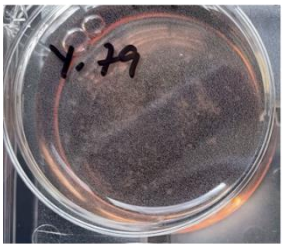
Sample images

Images of each cell line can be found in the online catalogue under: (https://www.dsmz.de/collection/catalogue/human-and-animal-cell-lines/catalogue)

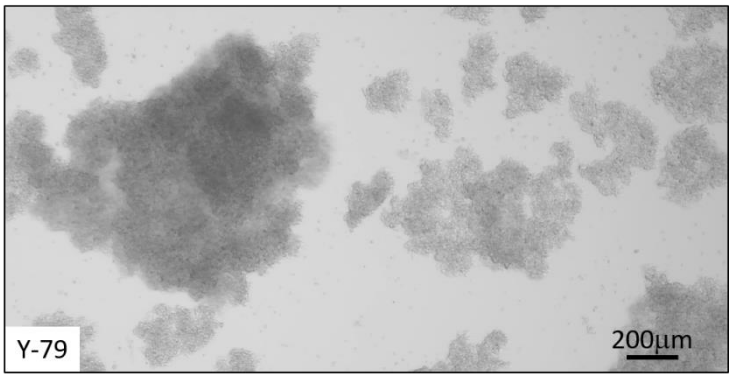


RBL14





Y-79



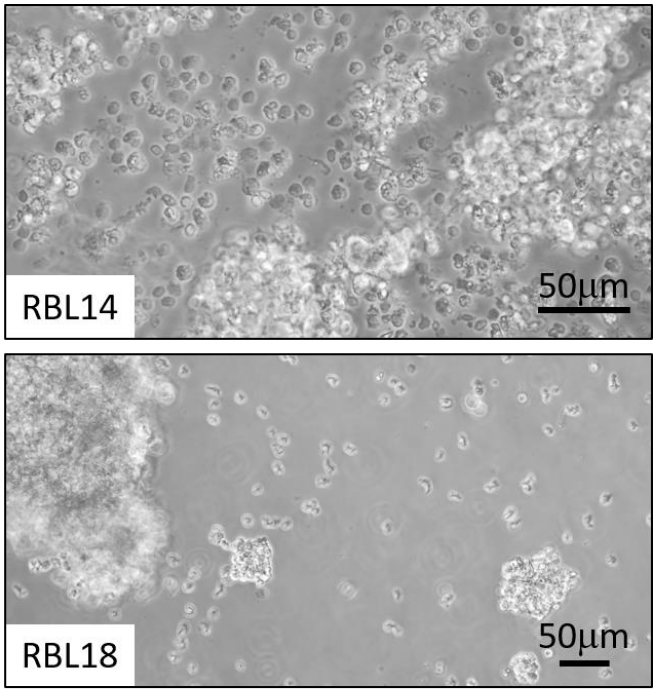
General remarks to cell culture

- Follow your own institutional guidelines concerning tissue culture and aseptic technique
- Tissue culture processes must be performed in a biosafety cabinet with laminar airflow
- All solutions and equipment having contact with cells must be sterile
- Personnel needs to be trained in tissue culture and sterile techniques
- Tissue culture waste should be autoclaved

- Retinoblastoma cell lines usually grow in suspension, forming spheroids that from larger, loose and soft aggregates
- Cave: Aggregates are sensitive to destruction by pipetting!
- Cultivate in standard, TC-treated 6-well plates
- Use serological pipettes or wide-bore / cut pipette tips
- we define “split-ready” as wells that contain sufficient cell material: many aggregates with a size of 1 to 1.5 mm and/or pH of medium changes to orange in 2-3 days using 3 ml of medium

Thawing of cells

- Cells are frozen as aggregates!
- Prepare 15 ml tube with 10 ml pre-warmed medium
- Transfer cryo vial to 37 °C water bath, incubate until small ice clumps are still visible
- Carfully wipe tube with ethanol-drained cloth
- Transfer 1ml volume of cryo vial into prepared 10 ml of medium, use wide-bore or cut tip, do not resuspend
- (Alternative: pour content of cryo vial into the prepared 10 ml of medium)
- (Optional: take out 500ul aliquot, resuspend and dilute 50 µl of cell suspension 1:1 with trypan blue to assess viability and rough cell number in hemocytometer)
- Centrifuge 300g, 5 min, RT
- Discard supernatant
- Carfully resuspend in 3 ml medium using 5 ml serological pipette – aggregates should remain intact
- (Alternative: add medium and tap or swirl tube to loosen pellet, pour into well)
- Transfer to 1 well of 6-well plate
- Add another 3 ml of medium to well
- Incubate at 37 °C, 10 % CO2



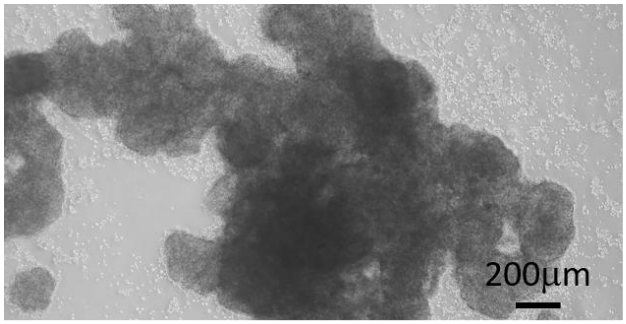
About 60 min after thawing

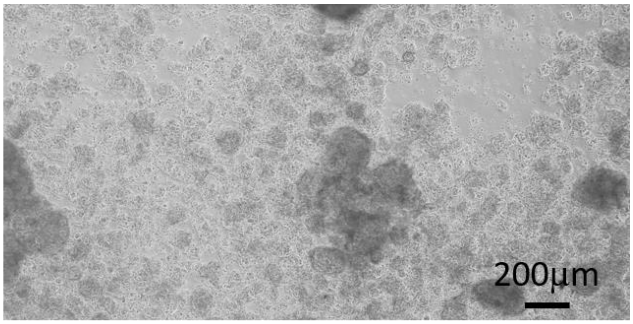
Maintenance of cells (media change)

- Do not perform complete medium changes, some of spent medium should remain
- In good conditions, cells are fed every 3-5 days (pH change of medium should be visible)
- Remove medium from well, be careful not to aspirate cell aggregates
- Carefully add fresh medium (position pipette at rim of well, let medium run slowly into the well)

Propagation and Passaging of cells (break up of aggregates)

- Passaging ratio is usually 1:2 to 1:3 every 5-8 days (roughly once per week)
- Rule of thumb: wells are split-ready (contain sufficient cell material) when aggregates have a size of 1 to 1.5 mm and/or pH of medium changes to orange in 2-3 days using 3 ml of medium;
- Transfer about one half of cell aggregates to new well, add new medium to both wells
- Pipette up and down slowly and carefully 2-3 times to break up aggregates
- Large aggregates need to be broken up to ensure further proliferation





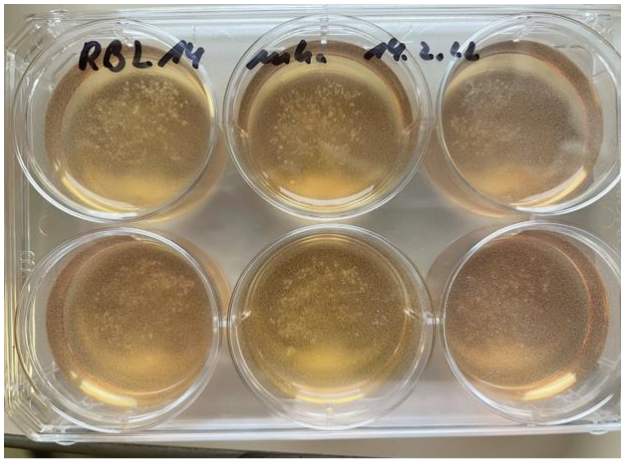
Cell line RBL14 before and after splitting with 2-3 times pipetting with cut tip

Preparation of cell pellets

- Cell pellets can be frozen at -80 °C for DNA, RNA or protein isolation
- Use content of of one split-ready well (see page 2 for definition) per pellet
- Transfer cells to 15 ml tube
- Centrifuge 300 g, 5 min, RT
- Discard supernatant
- Resuspend cells in PBS, transfer to Eppendorf tube
- Short spin at max speed to pellet cells
- Discard PBS
- Freeze at -80 °C immediately

Cryopreservation

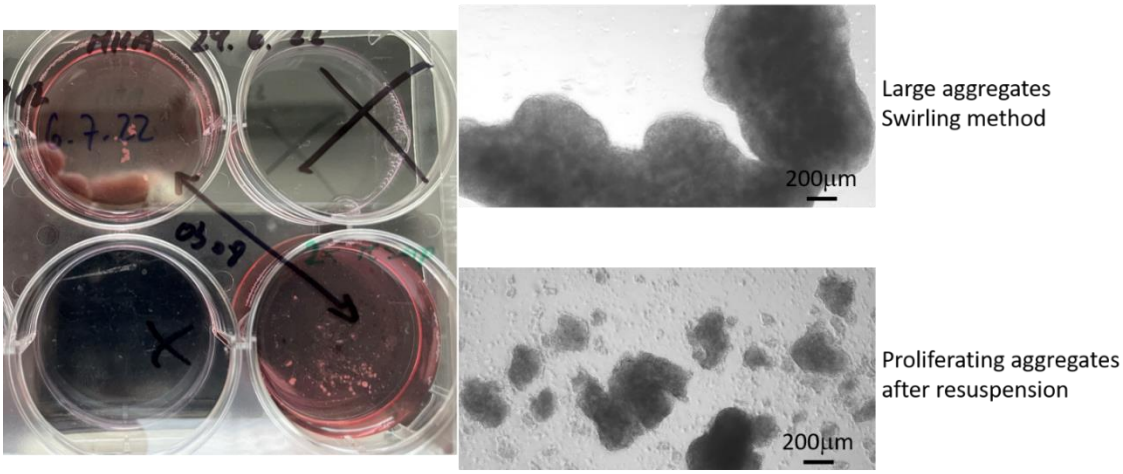
- Freeze content of one split-ready well (see page 2 for definition) into one cryo vial
- Prepare cryo vials, label with cell line name, date, comment on amount of cells (1x 6-well), your name
- Collect cells in 15 ml tube
- Centrifuge at 300 g, 5 min, room temperature
- Resuspend pellet in 70 % DMEM high glucose, 20 % FBS, 10 % DSMO



Cell line RBL-14 before freezing

Preparation of large aggregates for cryosectioning

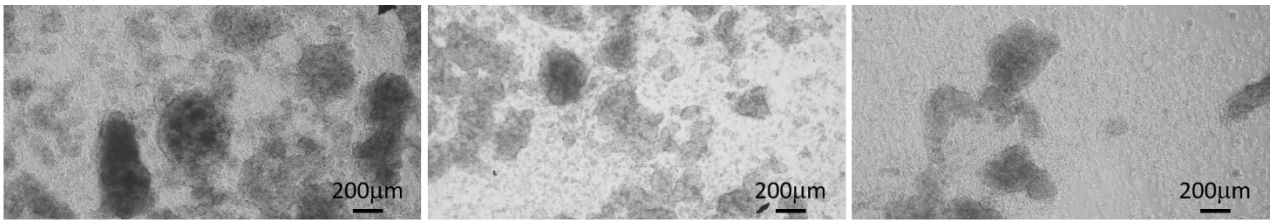
- In case you need large aggregates (more than 2 mm) for your analysis (e.g. cryosectioning), we recommend the following procedure:
- Use one split-ready well, swirl plate in a circle to collect aggregates in the middle of the plate
- Aspirate off medium
- Add new medium until aggregates reach desired size
- Caution: cell aggregates will become large, but stop proliferating at some point! Do not use this procedure for regular propagation and expansion of cells



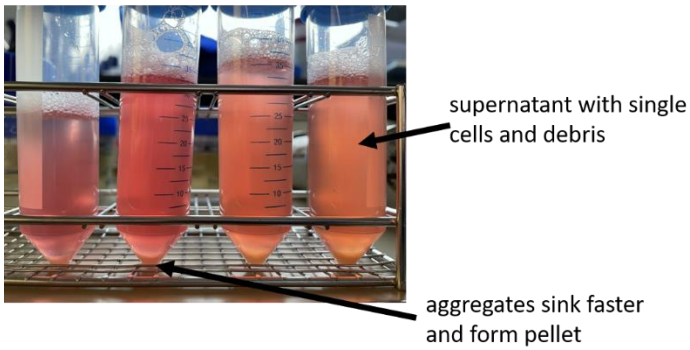
Cell line RBL14

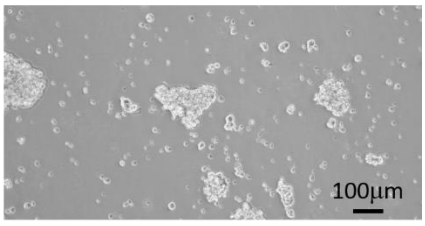
Measures to reduce cell debris

- Aggregate cultures can accumulate cell debris over time. To reduce this cell debris, the following procedure can be tested:
- Transfer cells of 1 well of 6-well plate into 15 ml tube
- Let stand for about 10 min – large aggregates will sink, smaller cell debris will stay in solution
- Discard supernatant
- Add 3 ml of medium, transfer cells to 1 well of 6-well plate with serological pipette, no pipetting up and down!



Cell line RBL13 with cellular debris in culture

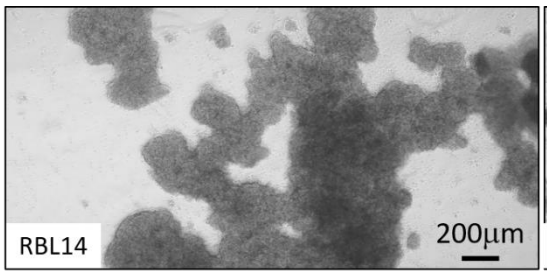




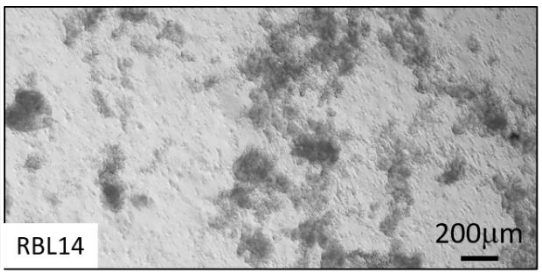
Cell line RBL13 after incubation

Estimation of cell number

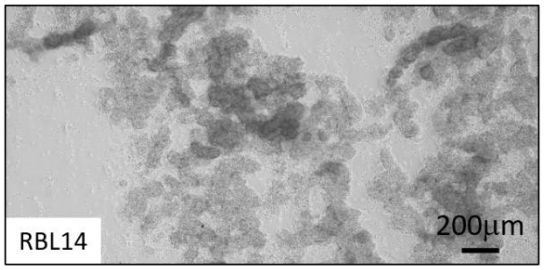
- For estimation of a cell number, aggregates need to be disintegrated with Accutase.
- Use one split-ready well (see page 2 for definition) for counting
- Transfer cells to 15 ml tube
- Centrifuge 300 g, 5 min, room temperature
- Discard supernatant
- Resuspend cells in PBS (careful pipetting, 2-3 times)
- Centrifuge 300 g, 5 min, room temperature
- Discard supernatant
- Resuspend pellet in 3ml Accutase, transfer to 1 well of 6-well plate, incubate 7 min in incubator at 37 °C
- Add 3 ml of medium, resuspend by careful pipetting, 2-3 times, aggregate size will be 100 – 200 µm
- Dilute 50 µl of cell suspension 1:1 with trypan blue
- Count and assess viability in hemocytometer
- Usual numbers are 4-8 x 10^6 cells per split-ready well, with a viability between 60-90% (depends on aggregate size at Accutase treatment – larger aggregates will result in more dead cells)
- Centrifuge 300 g, 5 min, room temperature
- Discard supernatant
- Resuspend in 3 ml fresh medium, careful pipetting, 2-3 times
- Transfer to 1 well of 6-well plate
- Cells should re-aggregate in 24 hours and continue to proliferate



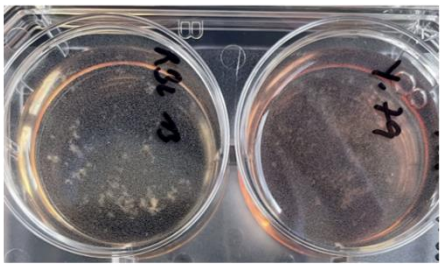
Before Accutase treatment



6 min Accutase treatment



24 h after Accutase treatment


Cell lines Y-79 and RBL13
5 days after Accutase treatment