K562 cell culture and formaldehyde cross-linking

1. Take out the K562 vial (1 million cells in 1 ml) from liquid nitrogen and thaw it in 37 degree waterbath. Suspend the washed cells in 10 ml RPMI with 10% FBS and GIBCO Antibiotic-antimycotic (Cat. No. 15240-062 5ml per 500 ml of culture.
2. Centrifuge at 700 rpm for 5 min.
3. Suspend the cells in 10 ml RPMI with 10%FBS and transfer them into a small cell culture flask (not a spinner flask) to be incubated in CO2 incubator.
4. These cells get into log phase in 5 to 7 days. Start counting at day 3. When the cell density reaches 0.7 to 0.8(x 10?)/ml. split the culture to about 0.4 million cells per ml with fresh RPMI with 10% FBS (this is the growth medium for K562). From this point on the cells should double every 24 hours,
5. From now on expect the cell density to double every 24 hours. (When the total cell number reaches 2X10>7, they can be stored as stock in liquid nitrogen at 1 million cells/ml (a total of 20 vials) in straight serum (FBS) containing 10% DMSO)
6. Split the cells when the density reaches around 0.75 million/ml.
7. Grow the cells to required numbers.

Note: For our labs purposes, we calculate the number of cells we will need for an experimental group, open a fresh vial of frozen cells, grow up the desired number of cells and then stop the culture-opening a fresh vial of cells for next experimental group. Therefore the cells are rarely passaged for more than 4 weeks.

Formaldehyde cross linking (all the solutions should be at room temp).

1. Centrifuge the required number of freshly growing K562 cells and suspend them in RPMI (without serum and antibiotics) to 2X10>7 cells /ml density. Add equal volume of freshly made 2% formaldehyde in RPMI to the cells. Rotate on end to end shaker for 10 minutes. Add 2M glycine stock to the final concentration of 125 mM and rotate on a shaker at room temp for 5 minutes followed by centrifuging at 300 rpm on a table top CS-6R centrifuge.
2. Discard the sup and process the pellet as per the ChIP protocol.

Inducible shRNA cell lines in K562 (shX_K562)

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Description of the cell line

K562, a non-adherent, human erythromyeloblastoid leukemia cell line was used as the parent cell line for making inducible shRNA cell lines. K562 cells were transduced with lentiviral vectors carrying an inducible short hairpin RNA (shRNA) to a specific transcription factor gene targeted for reduced expression. Cells with stable integration of shRNA constructs were selected with puromycin (2 μg/ml) for approximately three weeks. Doxycycline (3 μg/ml) was used to induce shRNA to reduce the expression of the targeted gene. A successful shRNA cell line showed at least a 70% reduction in expression of the target transcription factor as measured by qPCR.

For simplicity and ease of identification, we named these cell lines shX_K562, where X is the transcription factor targeted by shRNA and K562 denotes the parent cell line. For example, shATF3_K562 cells are K562 derived cells selected for stable integration of shRNA targeting the transcription factor ATF3 gene and showed at least a 70% reduction in the expression of ATF3 gene when measured by qPCR.

Description of the shRNA plasmids

We purchased the short hairpin RNA constructs (shRNAmir) from Thermo Fisher Scientific either in TRIPZ or GIPZ vectors as glycerol stocks. Short hairpin constructs express human microRNA-30 (miR30) as primary transcripts. The hairpin stem consists of twenty-two nucleotides of dsRNA and the nineteen nucleotides from human miR30 forms the loop. For more detailed description of the shRNA vector design refer to the technical bulletin from Thermo Fisher Scientific (http://www.openbiosystems.com/collateral/rnai/pi/pTRIPZ_Technical_Manual.pdf).

The addition of doxycycline (tetracycline derivative) induces expression of shRNA and turboRFP in TRIPZ vectors with a Tet-on configuration. While, GIPZ vectors lack the Tet-regulatory element and express turboGFP as the visible marker. We subcloned GIPZ constructs into TRIPZ plasmids according to the manufacturer's protocol, making all shRNA constructs have Tet-on configuration. To summarize, all shRNA constructs have a puromycin selection marker to select stable cells and a doxycycline inducible promoter regulating the expression of shRNA and red fluorescent protein.

Myers lab protocol for making and maintaining inducible stable shRNA cell lines

Part I of the following protocol elucidates the steps involved in making a stable shRNA cell line. Part II delineates the methods for storing, thawing, propagating, and harvesting of the shX_K562 cells.

Culture media composition:

- **Growth media:** RPMI-1640 with glutamine (VWR 16777-146), 10% Fetal Bovine Serum (FBS, Hyclone SH30071.03) and 1% Pen-Strep (Invitrogen 15140-163).
- **Selection media:** RPMI-1640 with glutamine (VWR 16777-146), 10% Fetal Bovine Serum (FBS), 1% Pen-Strep (Invitrogen 15140-163) and 2 μg/ml puromycin (Invitrogen A11138-03).
- **Induction media:** RPMI-1640 with glutamine (VWR 16777-146), 10% Fetal Bovine Serum (FBS), 1% Pen-Strep (Invitrogen 15140-163), 2 μg/ml puromycin (Invitrogen A11138-03) and 3 μg/ml doxycycline (Clontech 631311).

General protocol notes:

- Incubate all cultured cells at 37°C in a humidified incubator with 5% CO_2.
- Do not grow cells to maximum density for any of the experiments.
- Replace selection media and induction media every 24 hours, unless specified otherwise in the protocol.

I. Detailed protocol for making shX_K562 cells

The following 6 steps to make stable knockdown (shX_K562) cells are described below:

1. Package shRNA constructs into lentivirus vector and concentrate the virus
2. Determine the effective multiplicity of infection
3. Identify puromycin concentration for selection of the stable cells
4. Transduction and selection of the stable cells
5. Induction of shRNA with doxycycline
6. Calculate the percentage knockdown in expression using qPCR

1. Package shRNA constructs into lentivirus vector and concentrate the virus

Materials required:

- 100 mm cell culture dish (Corning 3296)
- Arrest-In (Thermo Fisher ATR1743)
- Lenti-X 293T (Clontech 632180)
- Lenti-X concentrator (Clontech 631232)
- Midi-prep kit (Qiagen 12145)
- shRNA plasmid constructs, glycerol stocks (Thermo Fisher, TRIPZ or GIPZ)
- Trans-lentiviral packaging kit (Thermo Fisher TLP4692)
- Non-silencing TRIPZ construct (Thermo Fisher RHS4743)

Steps to complete before starting with packaging of shRNA constructs:

- Subclone GIPZ plasmid constructs into TRIPZ vector constructs according to manufacturer's recommendations (Thermo Fisher Scientific).
- Isolate plasmid DNA from the glycerol stocks according to manufacturer's (Thermo Fisher) protocol and using a Qiagen Midi-prep kit as recommended (Qiagen).
- Quantify the plasmid DNA and check quality (recommended $A_{260/280}$ =1.8-2).

Procedure:

1. Plate Lenti-X 293T cells at a density of 5.5 x 10^6 cells per 100 mm plate in growth media and incubate at 37°C, 5% CO2 incubator overnight.

Note: Use LentiX-HEK293T cells with less than 5 passages for transfections. Lenti-X 293T cells (100 mm plate) transfected with 80% - 90% efficiency generate approximately 1 x 10^6 - 1 x 10^7 virus particles.

2. Dilute 9.0 µg plasmid DNA (pTRIPZ) and 28.5 µg Trans-lentiviral packaging mix (28 µL) into 1 ml (total volume) of growth media without FBS in a microfuge tube for each plate. Mix well using a vortex.
3. Dilute 187.5 µl of Arrest-In into 1 ml (total volume) of serum free medium (growth media without FBS) in a separate microfuge tube.
4. Combine DNA from step 2 with the diluted Arrest-In transfection reagent (step 3), in a 5 ml tube, vortex to mix. Incubate for 20 minutes at room temperature.
5. Add 3 ml of serum free medium to each tube.
6. Aspirate media from the cells (step 1) and add mixture from step 5 to the plate. Incubate transfected cells at 37°C in CO_2 incubator for 6 hours.
7. Aspirate the transfection mixture and add 12 ml growth medium to transfected cells. Return cells to the CO_2 incubator at 37°C for 48 hours.

Note: From this point forward cells and the supernatant will have live lenti-virus particles. Follow BSL 2+ guidelines while working with cells and disposing used plasticware.

8. Collect the virus-containing supernatant in a 50 ml sterile, capped, conical tube and store the supernatant at 4°C. Do not throw away the cell culture plate.
9. Add 12 ml of growth media to the culture plate, from which virus supernatant was collected in step 8, and incubate for 24 hours in the CO_2 incubator at 37°C.
10. Collect the supernatant from the cells and combine with the previously collected supernatant.

Note: Always keep viral supernatant and virus pellets on ice.

11. Centrifuge tube with collected virus supernatant at 500 g for 10 minutes at 4°C.
12. Transfer the clear supernatant (approximately 24 ml) to a fresh 50 ml tube and add 8 ml Lenti-X concentrator solution to it.
13. Incubate the solution at 4°C overnight.
14. Centrifuge at 1500 g for 45 minutes at 4°C to collect lenti-virus pellet.
15. Resuspend the pellet (shRNA lenti-virus pellet) in 325 µl growth media to make virus stock solution.
16. Store the virus stock solution at -80°C in single use aliquots (~100 µl each). Save ~25 µl of the virus stock to determine virus titer or transduction units per ml.

Note: Store virus stock solutions as single use aliquots as repeated freezing and thawing results in reduced virus titer.

2. Determine effective multiplicity of infection (MOI)

MOI is defined as the ratio of virus particles (transduction units) to the number of target cells.

MOI = Transduction units / No. Of cells to transduce

The goal of determining effective MOI is to estimate the amount of virus particles required to obtain similar copies of virus in most of the transduced cells.

Materials required:

- Non-silencing GIPZ construct (Thermo Fisher RHS4346)
- 96-well culture plate (Corning 3340)
- 24-well culture plate (Corning 3337)
- Doxycycline (Clontech 631311)

General protocol notes:

- To determine effective MOI we used a control GIPZ construct expressing non-silencing shRNA and truboGFP.
- The percentages of transduced K562 cells are approximations based on turboGFP expression.
- shRNA lentivirus constructs at an MOI of 0.5 transduced 60% of the target K562 cells and most of the transduced cells expressed similar fluorescence levels when observed under the microscope.

Procedure:

1. Calculating Transduction Units per ml of virus stock (Virus titer)

    a. Seed a 24 well tissue culture plate with Lenti-X 293T cells at a density of 5 x 10^4 cells per well in growth media and grow overnight.

    b. Make 8 five-fold dilutions of the virus stock in a round bottom 96-well plate using growth media without FBS (80 µl growth medium with no FBS + 20 µl of viral stock).

Note: Use wells numbered A1 to A8 to make viral dilutions, where A1 is a five fold dilution and A2 is 25 fold dilution and so on.

    c. Remove growth media from the cells in 24-well plate.

    d. Add 225 µl of serum-free media to each well of 24-well plate and transduce the cells by adding 25 µl of diluted virus from the 96-well late (step b) to first three rows of 24-well plate (triplicates). Add growth media with no virus to the fourth row.

Note: Add diluted virus from wells A2 to A7 of the 96-well plate (step b) to well A1 through A6 of the 24-well plate (step d). Repeat the same for Row B and Row C of the 24-well plate. Wells A1, B1 and C1 of the 24-well plate are treated as replicates.

    e. Incubate transduced cells at 37°C in CO_2 incubator for 4 hours.

    f. Remove transduction solution from cells and add 1 ml of growth media. Incubate the cells for 48 hours.

    g. Remove growth media from cells using aspiration and add 1 ml of induction media (growth media with 2 µg/ml doxycycline) to each well and incubate for 48 hours.

    h. Count the red fluorescent (RFP expressing) cells or colonies of cells in triplicates and take an average.

    i. Calculate the transduction units per ml (TU/ml) or virus titer using the following formula

No. Of RFP positive colonies x dilution factor x 40 = TU/ml

Note: Start with the step 2 only after calculating TU/ml.

2. Determine effective MOI for K562
    a. Add 5 x 10^4 K562 cells per well in a 24-well plate and incubate overnight.
    b. Centrifuge the plate and remove as much media as possible by aspiration.
    c. Add virus stock at an MOI of 0, 0.3, 0.5, 1.0, 2.0 and 5.0 to first three rows of the 24-well plate having K562 cells. Add one MOI per well in triplicates. Add growth media only to one row (i.e., six wells) of the plate.

Note:

- *Add one MOI per well. For example, add MOI of 0 to A1, 0.3 to A2 and so on. Repeat the same for Rows B and C of the 24-well plate. A1, B1 and C1 are treated as technical replicates.*
- *Add growth media only to Row D.*
- *Calculate volume of virus required to get the desired MOI e.g., 0, 0.3 as following*

Volume of virus = (5 x 10^4 x MOI) / TU/ml

    d. Add growth media without FBS to the cells making a final volume to 250 µl (volume of virus + growth media without FBS = 250 µl).

Note: If volume of virus added to the cells exceeds 250 µl (indicating low virus titer), it is recommended to harvest more viruses or concentrate virus in lesser volume as suggested in Step 1 (see above Package shRNA constructs into lentivirus vector and concentrate the virus).

    e. Incubate transduced cells at 37°C for 4 hours. Add 1.0 ml of growth media. Incubate cells for 48 hours.

Note: In our experiments, transduction mixture did not any adverse effects on K562 cells. Therefore, we suggest not removing the transduction mixture from these cells to avoid loss of cells in the process.

    f. Observe cells in all three replicates for turboGFP (green florescence) expression under the microscope.
    g. Count the green florescent cells or colonies of cells in triplicates and take an average.
    h. Record the MOI showing transduction of approximately 60% cells and more importantly exhibit that most of the transduced cells express similar green florescence levels.

3. Identify puromycin concentration for selection of stable cells

Materials required:

- 24-well culture plate (Corning 3337)
- Puromycin (Invitrogen A11138-03)
- Trypan blue (Lonza 17-942E)

We determined purmomycin concentration for K562 cells using the standard kill curve protocol recommended by Thermo Fisher Scientific. For K562 cells, 2 µg/ml puromycin in growth media is optimum for selection for stable shRNA K562 cells. Growth media with 2 µg/ml puromycin killed almost 100% of K562 cells with no shRNA integration within 96 hours to 120 hours. We counted cells every 24 hours using Trypan blue to assess cell death after puromycin treatment.

4. Transduction and selection of stable shRNA cells

Materials required:

- 24-well culture plate (Corning 3337)
- Puromycin (Invitrogen A11138-03)

Procedure:

1. Plate 5 - 8 x 10^4 cells per well in a 24-well plate in growth media. Incubate overnight at 37°C.
2. Gently mix virus stock at an MOI of 0.5 with growth media without FBS for a total volume of 100 µl.

Note: Thaw virus stocks on ice and return any unused viruses immediately to -80°C. We recommend determining titer of virus stock after every three freeze thaw cycles.

3. Remove the growth medium from the cells and add virus solution from step 3.
4. Add additional growth media without FBS to the cells to make a total volume of 200 µl.

Note: The volume in step 4 can be increased up to 250 µl, if virus titer is low. If more than 150 µl of virus stock is required, we recommend harvesting more viruses to get higher titer as described in Step 1 (see above Package shRNA constructs into lentivirus vector and concentrate the virus).

5. Approximately 6-8 hours post-transduction add 1ml of growth media to the cells in step 2 and incubate for 48 hours at 37°C.
6. Replace culture media with 1 ml of growth media in the cells and incubate for 48 hours.
7. Remove growth media by aspiration and add 1 ml of selection to cells. Return cells to 37°C incubator.

8. Replace media with freshly prepared selection media every 48 hours. After the first week of selection replace media every 24 hours.
9. Monitor the cells daily and observe the percentage of surviving cells. At some time point almost all of the cells surviving selection will be harboring the shRNA.

General protocol notes:

- We typically grow cells for 3-4 weeks to ascertain that all surviving cells harbor shRNA and acquire required number of cells for the downstream experiments.
- To check if all the surviving cells have shRNA constructs, induce a portion of cells with induction media (see Step 5) and observe them under a microscope for expression of red fluorescence. All cells should express turboRFP.
- Split cells when cell density reaches 5 - 8 x 10^5 per ml.

5. Induction with Doxycycline

General protocol notes:

- The induction time required for maximum knockdown of the targeted transcription factor should be determined empirically. We recommend collecting doxycycline induced cells every 24 hours for four days to determine the optimum induction time.
- Time required for observed knockdown in expression varies with the candidate shRNA. We have observed knockdown in expression of some targeted gene within 6 hours of induction while in some other cases induction.
- Count doxycycline induced cells every 24 hours to assess affect of shRNA on cell growth.

Procedure:

1. Transfer puromycin selected cells (approximately 4x10^7) to 50 ml tube and centrifuge at 1200 rpm for 5 minutes.
2. Remove the supernatant and wash cells with PBS. Divide cells into two 50 ml tubes. Centrifuge at 1200 rpm for 5 minutes. Collect the cell pellets.
3. Resuspend cells in one tube in 100 ml of selection media and resuspend second cell pellet in 100 ml induction media.

Notes:

- *Prepare fresh induction media.*
- *Maintain cells on induction media for the duration of the experiment. Change induction media every 24 hours.*
- *Split cells if density reaches 5 - 8 x 10^5 per ml.*

- *Harvest uninduced cells (cells with selection media) to use as reference for qPCR measurements. A one time harvest of uninduced cells is sufficient for each time series of doxycycline induced cells.*

4. Plate the cells in T-225 flask and incubate at 37°C for varying amounts of time (from 6 hours to 144 hours).
5. Observe the cells under the microscope to see the expression of turboRFP.

Note: TurboRFP will become visible (under a fluorescent microscope) within 24 hours and will be at full intensity by 72 hours.

6. To collect cell pellets for qPCR at each time point of doxycycline induced cells and uninduced cells, centrifuge 1 x 10^7 cells at 1200 rpm for 5 minutes at 4°C. Remove the media and wash the cell pellet with cold PBS. Centrifuge at 2000 rpm for 5 minutes at 4°C. Snap freeze the cell pellet in dry ice and then store at -80°C.

Note:

- *To turn off the expression of shRNA:*
    - *Collect doxycycline induced cells in a 50 ml tube and centrifuge at 1200 rpm for 5 minutes.*
    - *Remove the supernatant using aspiration and wash cells twice with PBS. Centrifuge cells at 1200 rpm for 5 minutes to collect cell pellet.*
    - *Resuspend cells in selection media and incubate at 37°C. The turboRFP protein will be turned over in approximately 72 hours and will no longer be visible under the microscope, indicating that shRNA is no longer expressed in the cells.*

6. Calculate percentage knockdown in expression using qPCR

Materials required:

- 96-well or 384 well qPCR plates
- Dynabeads mRNA Direct Kit (Invitrogen, 610.12)
- DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher F415L)
- cDNA synthesis kit (Thermo Fisher F470L)

Note:

- *Collect following samples to evaluate the percentage knock down of targeted factor*
    - *Uninduced and induced shX_K562 cells.*
    - *Uninduced and induced control K562 cells (shNS_K562, a Non-Silencing pTRIPZ construct (Thermo Fisher RHS4743)).*

- *For normalizing the qPCR data, we use average expression of 10 endogenous control genes: GAPDH, ACTB, GUSB, UBC, G6PD, POL2RA, TUBB, TBP, SDHA and B2M.*

Procedure:

1. Prepare mRNA from the collected cell pellets with the Dynabeads mRNA Direct Kit as instructed by the manufacturer.
2. Synthesize cDNA from mRNA using cDNA synthesis kit.
3. Use DyNAmo Flash SYBR Green qPCR Kit for qPCR, as per the recommended protocol on ABI 7900.
4. To calculate percent knockdown of the target transcription factor, record the expression of endogenous control genes and candidate transcription factor in:
5. Uninduced (cells with no doxycycline) and induced shRNA cells (shRNA cell line for candidate transcription factor).
6. Uninduced and induced vector only (non-silencing control, shRNA plasmid with no short hairpin) control.
7. Use ΔΔct method to calculate percent knockdown in expression of target factor in induced shX_K562 cells using uninduced shX_K562 cells as reference.
8. Use ΔΔct method to calculate percent knockdown in expression of target factor in induced shNS_K562 cells using uninduced shNS_K562 cells as reference to evaluate off target effects.

Note:

- *A stable inducible shX_K562 cell lines show at least 70% reduction in the expression of targeted transcription factor gene.*
- *We prefer using uninduced shX_K562 cells as the reference for calculating percentage knockdown.*
    - *Uninduced shX_K562 cells serve as controls for off targets resulting from the integration of candidate shRNA in each shX_K562 cell line.*
    - *Induced shNS_K562 cells control for general off target effects because of the experimental procedure in making the shX_K562 cells and doxycycline induction.*

II. Detailed protocol for storing, sub-culturing and harvesting shX_K562 cells

1. Liquid Nitrogen Storage

1. Take 2 x 10^7 cells in 50 ml conical tube and centrifuge at 1200 rpm for 5 minutes to collect the cell pellet.
2. Wash the cell pellet with PBS and centrifuge at 1200 rpm for 5 minutes to collect the cell pellet.
3. Resuspend the cell pellet in 1 ml of growth medium supplemented with 5% (v/v) DMSO.
4. Aliquot approximately 5 x 10^6 cells per cryogenic vial,
5. Freeze in an isopropanol jar at -80C overnight, and then transfer to liquid nitrogen storage.

2. shX_K562 Cell Culture Protocol (Thawing and propagating)

1. Thaw a vial of shX_K562 cells as quickly as possible in a water bath at 37°C.
2. Add 1 ml thawed cells to 9 ml of pre-warmed growth media in a 15 ml tube.

Note: Do not use selection media at the time of thawing the cells.

3. Spin cell suspension at 1000 rpm for 5 minutes. Discard media and resuspend cells in 10 ml of growth media. Transfer cells to a T-25 flask and incubate for 48 hours.
4. Collect cells in 50 ml tube and centrifuge at 1200 rpm, 5 minutes. Discard the supernatant and suspend cells in 20 ml of selection media.
5. Divide cell suspension in two T-25 flasks and incubate at 37°C.
6. Split cells when cell density reaches approximately 5 - 8 x 10^5 cells per ml.
7. Transfer puromycin selected cells (approximately 4x10^7) to 50 ml tube and centrifuge at 1200 rpm for 5 minutes.
8. Remove the supernatant and wash cells with PBS. Divide cells into two 50 ml tubes. Centrifuge at 1200 rpm for 5 minutes. Collect the cell pellets.
9. Resuspend cells in one tube in 100 ml of selection media and resuspend second cell pellet in 100 ml induction media.
10. Grow induced and uninduced cells for desired amount of time.

Note:

- *Keep cell density below 2 x 10^5 cells per ml when inducing with doxycycline.*
- *Split doxycycline induced cells as regular K562 or uninduced shX_K562 cells i.e., split cells when cell density is 5 - 8 x 10^5 cells per ml.*

3. Crosslinking and Harvesting for crosslinking, RNA and Protein analysis

1. Count uinduced and induced cells.

Note:

- *Collect uninduced shX_K562 cells at the maximum time point used for doxycycline-induced cells.*
- *Collect cells for doxycycline induced shX_K562 cell pellets at each time point of interest after induction.*

2. Collect cell pellets for RNA (qPCR, RNA-seq) or protein analysis (WB, IP-WB)
   a. Take 8x 10^7 cells in 50 ml tubes and centrifuge cells at 1200 rpm for 5 minutes.
   b. Resuspend cells in 32 ml growth media and centrifuge at 1200 rpm for 5 minutes.
   c. Keep cells on ice and discard the supernatant.
   d. Resuspend cells in equal volume of cold PBS and aliquot equally in to eight 5 ml tubes.
   e. Centrifuge again at 2000 rpm for 5 minutes at 4°C to collect the cell pellets.
   f. Snap freeze the cell pellet in dry ice and store at -80°C.
3. Crosslinking shX_K562 cells for ChIP (uninduced and induced cells)
   g. Collect 2 x 10^7 in a 50 ml tube and centrifuge cells at 1200 rpm for 5 minutes. Discard the supernatant.
   h. Resuspend cells in 35 ml growth media.
   i. Add 1% formaldehyde (final concentration), mix gently and incubate at room temperature for 10 minutes.
   j. Add glycine to a final concentration of 0.125 M and mix gently.
   k. Centrifuge cells at 2000 rpm for 5 minutes at 4°C and discard the supernatant.
   l. Keep cell pellet on ice and resuspend cells in equal volume of cold PBS.
   m. Centrifuge cells at 2000 rpm for 5 minutes at 4°C to collect the cell pellet.
   n. Snap freeze the cell pellet in dry ice and store at -80°C.