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Cytogenetic analysis of telomere dysfunction

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Abstract

Dysfunctional telomeres arising either through natural attrition due to telomerase deficiency or by the removal of telomere binding proteins are recognized as double stranded breaks (DSBs). Repair of DSBs is crucial for the maintenance of genome stability. In mammals, DSBs are repaired by either error prone nonhomologous end joining (NHEJ) or error free homologous recombination (HR) and can be visualized as chromosomal fusions.

2. Materials

2.1 Cell culture and harvesting

1. Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone SH30022.01), supplemented with 10% Fetal Bovine Serum (FBS, Sigma F-6178).
2. Penicillin-streptomycin (100 U/ml Penicillin G and 0.1mg/ml streptomycin – Gemini Bio-products Cat. no. 400-109).
3. Mouse Embryonic Fibroblasts (MEFs)
4. Trypsin (0.25%) and ethylenediamine tetra acetic acid (EDTA) (1mM) solution (Gibco 25200).
5. Colcemid (0.05µg/ml) from Gibco (Cat. No. 15212-012).
6. A 0.075M solution of KCl in water.
7. Freshly prepared solution of Methanol : acetic acid (3:1)(v/v).
8. 15 ml conical centrifuge tubes
9. Glass Pasteur pipettes
10. Glass slides with frosted ends.

2.2 Giemsa staining

1. 4% Giemsa stain diluted in 0.01M phosphate buffer (0.469g NaH₂PO₄ + 0.937g Na₂HPO₄/L ddH₂O) (Gibco Cat. No. 10092-013)

2.3 PNA Telomere FISH (Fluorescence in situ hybridization)

1. PNA telomere probe (Tam-OO-(CCCTAA)₄) Panagene Inc. (Cat. No. F2002)
2. DAPI (Vectashield H-1200)
3. Triton-X 100 (Sigma-BP337 500)
4. Formaldehyde (Sigma Cat. No. F7508-500G)
5. 2% Bovine Serum Albumin

6. tRNA (100µg/ml)

Preparation of PNA probe in hybridization buffer:

ddH ₂ O	2.85µl
tRNA(100µg/ml)	0.15µl
2% Bovine Serum Albumin	1.5µl
Formamide	21µl
0.6XSSC	3µl
PNA probe (10ng/µl)	1.5–3.0µl

3. Methods

To get a good mitotic yield, cells should be growing in exponential condition and the cells should be freshly fed with the media a day before the harvest. Colcemid treatment should be monitored to obtain an optimum length of the chromosomes.

3.1 Harvesting the cultures for chromosomal preparations

1. Plate about 1 million Mouse Embryonic Fibroblast (MEF) cells in 100 mm petridishes and add 10 ml culture media supplemented with 10% FBS and Penicillin-streptomycin. Incubate the cultures at 37° C overnight.
2. Next day examine the cultures for the presence of doublets indicating presence of dividing cells and at the time such doublets are present add colcemid (0.05µg/ml) to the cultures and incubate them at 37° C for 45 minutes to 1 hour.
3. Aspirate the medium and rinse the cells with PBS.
4. Detach the cells using 0.025% trypsin and transfer them to 15 ml conical centrifuge tubes.
5. Spin at 1500 rpm for 6–8 minutes.
6. Remove the supernatant leaving about 150–200ul of media in the tube. Gently pipette up and down to break the clumps.
7. Add 5–7 ml of 0.075M KCl solution and gently mix with a Pasteur pipette.
8. Incubate at room temperature for 15 minutes.
9. Add about 1 ml of methanol: acetic acid (3:1) solution to the tube and mix slowly.
10. Centrifuge at 1500 rpm for 6–8 minutes and remove the supernatant.
11. Break the pellet by tapping the bottom of the tube and slowly add about 1 ml of fixative. Mix with a Pasteur pipette to break any clumps and add 5–6 ml more fixative.
12. Spin at 1500 rpm for 6 minutes and remove the supernatant.
13. Repeat steps 11 and 12 thrice.
14. Resuspend the final pellet in small volume of fixative so that the cell suspension looks slightly milky.
15. Take clean slide and pour distilled water on it. Drain the slide, hold it at an angle and drop 2–3 drops of cell suspension on the slide.
16. Either air dry the slide or dry it on a hot plate heated to about 60° C.

17. The slides can be stored at room temperature for about 2 weeks or for longer period at -80°C .

3.2 Giemsa staining

Giemsa staining of the slides prepared from cell culture harvest is a useful tool in the study of chromosomes. Slides are stained in a 4% Giemsa stain diluted in 0.01M phosphate buffer (0.469g NaH_2PO_4 + 0.937g $\text{Na}_2\text{HPO}_4/\text{L}$ dd H_2O) for 5 minutes, washed in double distilled water and air dried. Observe the stained slide under a bright field microscope using 60X oil immersion objective.

3.3 PNA Telomere FISH (Fluorescence in situ hybridization)

1. Immerse the slide in Phosphate Buffered Saline (PBS) for 2 minutes.
2. Fix the slide in 4% formaldehyde in PBS for 2 minutes
3. Wash in PBS three times (5 minutes each)
4. Treat slides with pepsin (1 mg/ml in 10mM HCl) for 10 minutes at 37°C , wash with PBS.
5. Fix the slide in 4% formaldehyde in PBS for 2 minutes.
6. Dehydrate the slide in 70%, 85% and 100% ethanol 2 minutes each and air dry.
7. Apply 30ul PNA (Peptide Nucleic Acid) telomere probe in hybridization buffer to each slide.
8. Cover slide with coverslip and seal with rubber cement.
9. Denature the slide for 3 minutes at 80°C on a heat block and then incubate for 2–3 hours in dark at room temperature.
10. Remove the coverslip gently with forceps and wash the slide in 70% formamide/ 0.06XSSC (Saline-Sodium Citrate) (pH 7.2) 2X 15 minutes.
11. Wash with PBS containing 0.05% triton X100 3 times, 5 minutes each.
12. Apply a drop of mounting media with DAPI to the target area of the slide.
13. Cover with a coverslip and allow the solution to spread evenly under the coverslip.
14. Observe the stained slide under a fluorescence microscope using 60X oil immersion objective and appropriate filters.

4.0 Analysis of chromosomal fusions

Analyse Giemsa stained slides under Bright field microscope using 60X oil immersion lens.

In laboratory mouse, *Mus musculus*, the diploid chromosome number is $2n=40$. All mouse chromosomes, including the sex chromosomes, are acrocentric in morphology.

Translocations involving total chromosomes or whole chromosome arms can be classified into three basic types, centromere-centromere (C-C), centromere-telomere (C-T) and telomere-telomere (T-T) fusions. Depending on the way the fusion has occurred, the resulting translocated chromosome may exhibit different morphology.

1. In Centromere –Centromere type fusion, one chromosome has a break in the long arm immediately below the centromere and the other has a break in the telomeric region above the centromere. This results in an asymmetrical bivalent chromosome and a “bare” centromere which is subsequently lost. If the fusion occurs between

the two mouse chromosomes retaining both centromeres, then one centromere may be rendered inactive.

2. In the Centromere-Telomere type fusion, two chromosomes fuse, one at the centromeric end and other at the telomeric end. This type of translocation can result in either a long acrocentric chromosome or a biarmed chromosome depending on which centromere is lost or inactivated. It is also called tandem translocation.
3. In Telomere-Telomere translocations two chromosomes fuse at the telomeric ends. Here again, the morphology of the resulting chromosome could either be dicentric with one centromere at each end or a long acrocentric chromosome if one of the centromere is lost or inactivated.
4. Ring chromosomes may be formed by:
 - a. Telomere-Telomere fusion between telomeres present at both ends of a single chromosome.
 - b. Sister chromatid fusion at one end of the same chromosome leading to the formation of a ring like structure.
 - c. Fusion at the telomeric regions of the long arms of two acrocentric chromosomes.

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