Metaphase Chromosome Spread Preparation Protocol
10/5/2007

Materials and Reagents:

Item	Vendor	Catalog #
KaryoMax	Gibco	15210-040
PBS (1X without Ca or Mg)	Gibco	14190-144
ES cell medium (see specific ES cell culture protocol for either KOMP-CSD or KOMP-Regeneron)		
Trypsin (see specific ES cell culture protocol for either KOMP-CSD or KOMP-Regeneron)		
KCl (10x, 5.6% KCl: 5.6g KCL in 100ml Sterile Distilled Water. Mix until completely dissolved. Store at room temperature for up to 12 months.)	EM Science	PX1405-1
Methanol	EMD	MX0485 P-4
Glacial acetic acid	Fisher	A38-212
Vectashield mounting medium with DAPI	Vector Lab	H1200

Preparation of Metaphase Spreads:

1. Thaw or passage ES cells 2 to 3 days prior to performing chromosome spreads onto a 35mm gelatinized dish.

2. Feed 3 hours prior to harvesting with 2ml ES cell media.

3. Add 200 ul of Colcemid to a final concentration of 1 ug/ml, incubate at 37^{o}C for a 1 hour.

4. Aspirate medium and rinse with PBS. Trypsinize cells with PBS.

5. Spin at 900 rpm for 5 min.

6. Aspirate supernatant, leave about 200ul of media in the tube. Pipette up and down to break any clumps.

7. Add 5ml 0.56% KCl solution and invert once. Stand at RT for 5 min. Spin at 900rpm for 5 min.

8. Aspirate supernatant, resuspend pellet in remaining drop and add 5ml of methanol:glacial acetic acid (3:1) solution. This is the most critical stage of the preparation. It is very important to disrupt the pellet before addition of the fixative and to add the fixative only one or two drops at a time for the first 2.5ml, tapping bottom of tube to mix cells all the time. The remaining 2.5ml can be added at only twice the speed. Spin at 900 rpm for 5 min.

9. Repeat step 8 once.

10. Aspirate supernatant. Resuspend pellet in small volume of fixative, probable less than 200ul, until cell suspension slightly milky.

11. Take a small quantity of cell suspension in 200ul pipettte. Hold the pipette vertically with the end about 10cm above the slide. Release a single drop form this height onto the center of the slide. Allow the slides to air-dry thoroughly for minimum of 1 hour. Prepare about 6 slides for each cell line.

Photographing and counting chromosomes:

1. Add a drop of mounting solution with DAPI to each slide, over the dried cell prep. Place a coverslip and seal around sides with clear nail polish.

2. Examine under oil immersion at 10x100 magnification, using an UV light source. Scan each slide and photograph the first 24 good spreads you find for each clone. (The "good" spreads are those in which the chromosomes are clearly form one cell only. Sometimes two cells spread their chromosomes in the same place. Also, odd small number of chromosomes can sometimes be seen, that are clearly less than a complete representation of one cell. Don't count those.)

3. Count the number of chromosomes in each spread. Chromosomes can be most accurately counted by identifying the centromeres, which light up a little more than the rest of the chromosome. Mark each centromere out with a pen as it is counted. Most commonly we find cells prove to be carrying between 38 to 42 chromosomes. We inject the clones are identified as being >70% euploid.

Quick Reference:

0.56% KCL (1x Working Solution) – 5 ml per sample

Chill on ice before using

Number of Samples	Volume of 5.6% Stock	Volume of Sterile Distilled Water
1	0.5 ml	4.5 ml
2	1 ml	9 ml
3	1.5 ml	13.5 ml
4	2 ml	18 ml
5	2.5 ml	22.5 ml
6	3ml	27ml

Fixative (3 parts Methanol + 1 part GAA) – 10 ml per sample

Chill on ice before using

Number of Samples	Volume of Methanol	Volume of Glacial Acetic Acid
1	7.5 ml	2.5 ml
2	15 ml	5 ml
3	22.5 ml	7.5 ml
4	30 ml	10 ml
5	37.5 ml	12.5 ml
6	45ml	15ml