1. About the kit

Xpert™ Karyotyping Teaching Kit has been developed for teaching short term in vitro culture of peripheral blood lymphocytes for cytogenetic studies in educational organizations. Cytogenetic studies include metaphase and prometaphase studies carried out on lymphocytes to detect chromosomal aberrations associated with structural and numerical abnormalities.

CCK012, Xpert™ Karyotyping Teaching Kit is sufficient for preparation of 10 mitotic spreads (Cell suspension obtained from 1 flask is sufficient for dropping 2 slides).

Significance of reagents and materials provided in the kit:

a. HiKaryoXL™ RPMI-1640 Medium:
HiKaryoXL™ RPMI Medium is a karyotyping medium composed of a basal medium RPMI 1640 and supplemented with L-Glutamine, FBS, phytohemagglutinin (PHA-M), Penicillin, Streptomycin and Sodium bicarbonate. AL165A is a complete medium and does not require supplementation with any additional components.

b. HiKaryoXL™ Colchicine Solution:
Colchicine is an alkaloid that is isolated from the plant Colchicum autumnale. Colchicine is used for arresting the cells in metaphase. Arresting of cells in metaphase allows an increased yield of mitotic cells for analysis. Colchicine inhibits microtubule polymerization by binding to tubulin. Availability of tubulin is essential for mitosis and therefore colchicine effectively arrest cells in metaphase. TCL062 is a sterile filtered solution of 10µg per ml Colchicine in phosphate buffered saline.

c. Potassium chloride solution 0.075M:
Potassium chloride solution is a hypotonic solution widely used in cytogenetics. It aids in the spread of metaphase chromosomes by causing swelling and enlargement of the cells, facilitating their better analysis. TCL040 is a sterile filtered 0.075M potassium chloride solution.

d. Giemsa stain solution:
Giemsa stain is a mixture of methylene blue, eosin, and azure B. It is specific for the phosphate groups of DNA and attaches itself to where there are high amounts of adenine-thymine bonding. Giemsa stain is mainly used for staining of peripheral blood smears and specimens obtained from the bone marrow. It is used to obtain differential white blood cell counts. Giemsa stain is also used in cytogenetics to stain the chromosomes and identify chromosomal aberrations. It is commonly used for G-Banding (Giemsa-Banding) studies. TCL083 is a 20X sterile filtered concentrated solution of Giemsa stain.

e. Tissue culture flasks:
Tissue culture flasks provided in the kit have a vented cap and surface area of 25cm². These are surface non-treated flasks used for culturing non-adherent (suspension) cells.
f. **Glass slides:**
Glass slides provided in the kit have fine polished edges (45°) and are frosted at one end on both the sides. Frosting aids in labeling of slides.

g. **Centrifuge tubes:**
Centrifuge tubes are sterile, individually packed and have a capacity of 15ml.

2. **Kit contents and storage:**

On receipt, remove the contents of the kit and place them in appropriate storage locations as per recommended storage temperature.

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
<th>Quantity</th>
<th>Store at</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL165A</td>
<td>HiKaryoXL™ RPMI Medium With L-Glutamine, FBS, PHA-M, Penicillin, Streptomycin and Sodium bicarbonate 1X Liquid Karyotyping Medium</td>
<td>5 X10ml</td>
<td>-20°C</td>
</tr>
<tr>
<td>TCL062</td>
<td>HiKaryoXL™ Colchicine Solution With 10µg/ml Colchicine in Phosphate Buffered Saline</td>
<td>1ml</td>
<td>2 - 8°C</td>
</tr>
<tr>
<td>TCL040</td>
<td>Potassium chloride solution 0.075M</td>
<td>30ml</td>
<td>15-30°C</td>
</tr>
<tr>
<td>TCL083</td>
<td>Giemsa Stain Solution</td>
<td>5ml</td>
<td>15-30°C</td>
</tr>
<tr>
<td>TCS4</td>
<td>Tissue Culture Flask 25cm², Vented cap, Volume 50ml Non-treated</td>
<td>5Nos</td>
<td>15-30°C</td>
</tr>
<tr>
<td>CG17</td>
<td>Glass slides Fine polished edges, Frosted One End Both Sides</td>
<td>10Nos</td>
<td>15-30°C</td>
</tr>
<tr>
<td>CG274</td>
<td>Centrifuge tubes, 15ml Sterile, Conical bottom</td>
<td>5Nos</td>
<td>15-30°C</td>
</tr>
<tr>
<td></td>
<td>Heparinized Blood Collection Tube</td>
<td>1No.</td>
<td>2 - 8°C</td>
</tr>
</tbody>
</table>

* Quantities supplied in excess to compensate operational losses

3. **Materials required but not provided in the kit**

3.1 Blood collection accessories
- Needle
- Syringe
- Tourniquet
- Cotton
- Spirit solution

3.2 Equipments
- Laminar air flow hood
- CO₂ Incubator at 37°C
- Compound light microscope with 100X oil immersion objective
- Centrifuge
- Water bath at 37°C

3.3 Consumables
- Micropipettes
- Serological pipettes
- Pipette aid (LA692)
- Disposable gloves
- Lab coat
- Isopropanol spray
- Tissue paper
- Coplin jar for staining (PW354)

3.4 Chemicals (for preparation of Carnoy's fixative)
- Methanol
- Glacial acetic acid
4. Aseptic techniques and good cell culture practices

a. Use Personal Protective Equipment (PPE), (laboratory coat, gloves and eye protection) at all times while working in a cell culture lab. Use head caps to cover hair.
b. PPE for tissue culture facility should be kept separate from PPE worn in general laboratory environment.
c. Before starting tissue culture work switch on the UV light in the biosafety cabinet for 15-20 minutes.
d. Keep all the work surfaces free from clutter.
e. All reagent and media bottles should be labeled correctly with name and date of preparation and should be kept at recommended storage temperatures.
f. Clean the working area of the laminar air flow hood with 70% isopropanol.
g. Prior to starting work all reagent and media bottles, pipettes, tip boxes should be sprayed with 70% isopropanol.
h. Arrange the work station in such a way that you have an easy access to all the items and a wide clear space in the centre of the bench.
i. Keep all the reagents and media bottles to the left hand side of work station and the consumables and discard beaker to the right hand side of work station for efficient working.
j. While working do not contaminate the gloves by touching anything outside the cabinet (especially face and hair). In case they become contaminated then, respray with 70% isopropanol before proceeding.
k. In case of any spillage while working, mop up immediately and swab the area with 70% isopropanol.
l. Avoid rapid movement within and immediately outside the cabinet. Slow movement will allow the air within the cabinet to circulate properly.
m. Avoid speaking, sneezing and coughing while working in the cabinet to prevent the contamination.
n. Pipette tips, waste reagents and waste medium should be discarded carefully into a separate discard beaker.
o. Once the work is finished, clear the working area and clean with 70% isopropanol.

5. Directions for use

Step 1: Blood lymphocyte culture

1. Thaw HiKaryoXL™ RPMI Medium (AL165A) overnight at 2 - 8°C. Before adding to the flask, ensure that the medium is brought to room temperature.
2. Draw 8-10ml peripheral blood from a healthy volunteer, in heparinized blood collection tube. Cap the blood collection tube tightly and mix gently by inverting 2-3 times to avoid coagulation.
   **Note**: Do not shake vigorously.
3. Arrange all the required accessories in laminar air flow hood as mentioned in section 4.
4. Mix the thawed medium by gentle swirling and aseptically add 10ml in each flask with the help of sterile pipette and pipette aid.
5. Add 800µl freshly drawn heparinized blood in each flask.
   **Note**: Volume of blood to be added in the medium depends on age and physical condition of an individual.
   *Normal healthy person* - 800µl
   *Children and infants* - 600µl
   *Women (during pregnancy / postpartum)* - 1ml
6. Mix the blood thoroughly in medium by gentle swirling and shaking the flask.
7. Incubate the flasks in an upright (vertical) position at 37°C and 5% CO₂ for 72 hours.
   **Note**: Refer figure A for position of the flasks.

Fig A: Upright position of flask during incubation

Step 2: Lymphocyte harvesting:

1. After 72 hours of incubation, take the flasks out from an incubator and aseptically add
100µl of colchicine solution (TCL062) in each of them. Shake well to mix.

2. Incubate the flasks in upright position at 37°C and 5% CO₂ for additional 2 hours.

*Note: Refer figure A for position of flasks. Incubation time of less than 1 hour might result in reduced mitotic index*

*During incubation period of 2 hours, prepare the grease-free slides (refer point 1 & 2 in step 3).*

3. After 2 hours, take out the flasks from the incubator and transfer the entire contents of the flask in the individual sterile 15ml centrifuge tube.

*Note: Further steps can be performed outside the laminar air flow hood, on a clean bench*

4. Centrifuge the tubes at 1000rpm for 10 minutes.

5. Discard the supernatant.

6. Warm the KCl solution (TCL040) by placing the bottle in a hot water bath for 10 minutes.

7. Resuspend the pellet in 5ml of warm hypotonic KCl solution by adding it drop wise while agitating the cells.

8. Mix by inverting the tubes 2 - 3 times.

9. Incubate in a water bath at 37°C for 35 minutes.

10. During this incubation period, prepare Carnoy's fixative by mixing methanol and glacial acetic acid in 3: 1 ratio. Mix 75ml methanol and 25ml glacial acetic acid to make 100ml fixative. Store it at -20°C until use.

*Note: For processing of 5 tubes, approximately 75ml fixative is required (15ml per tube). It is recommended to prepare extra amount of fixative to compensate for operational losses.*

11. After incubation with KCl, add 5ml of ice-cold fixative to each tube. Mix thoroughly by gently inverting the tubes 2 - 3 times.

12. Centrifuge at 1000rpm for 10min.

*Note: For periods between centrifugation, keep the fixative at -20°C. Fixative should be ice-cold for getting proper results.*

13. Discard the supernatant and again add 5ml of freshly prepared ice-cold fixative with constant mixing. Leave the cells at 4°C for 10-15 min.

14. Centrifuge at 1000rpm for 10 minutes.

15. Repeat the step no. 13 and 14.

16. Discard the supernatant and resuspend the pellet in 0.5ml of fresh cold fixative.

17. Keep the tubes in refrigerator until use.

*Note: If sufficient time is not available for preparation of slides on the same day, store the tubes containing cell suspension overnight in refrigerator at 2 - 8°C. On next day, mix the cell suspension by pipetting, add 2 - 3ml of freshly prepared Carnoy's fixative. Centrifuge at 1000rpm for 10 minutes. Discard the supernatant, add 0.5ml of fresh cold fixative and proceed for slide preparation.*

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**Step 3: Slide preparation and staining:**

1. Clean the slides with mild detergent and wash thoroughly under running tap water to make them grease-free.

2. Place the clean slides in a beaker containing water in such a way that the slides are completely immersed in water. Keep the beaker in a refrigerator at 2 - 8°C and allow the slides to cool until use.

*Note: Above 2 steps can be performed during incubation period of 2 hours with colchicine solution. This will save time.*

3. Once the cell suspension is ready, take out the ice-cold slides from the refrigerator.

4. Tilt the ice cold wet slide at 45° angle. Drop 50µl of cell suspension at the bottom of the slide with the help of micropipette in such a way that the suspension hits hard on the slide and then runs down the surface.

5. Similarly drop 50µl in the center and 50µl at the top of the slide.

*Note: Ensure that the direction of dropping is from bottom to the top. Refer Fig B.*

6. Allow the slides to air dry.

7. Fix the smear over a hot plate or a boiling water bath.

8. Prepare Giemsa stain solution by diluting concentrated (20X) Giemsa stain in distilled water in 1: 20 ratio just prior to staining.
9. Stain the fixed slides in Giemsa stain for 20 minutes.  
   *Note: For uniform staining, completely cover the slides with stain solution.*
10. Gently wash the stained slides with distilled water to remove excess of stain.
11. Allow the slides to air dry.

6. Observations:

![Fig C: Lymphocytes and chromosomes stained with Giemsa (40X).](image)

Mitotic chromosomes

Stimulated lymphocytes

![Fig D: Mitotic chromosomes stained with Giemsa. Short and long arms visible (100X oil Immersion).](image)

7. Troubleshooting:

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cell growth or very slow growth</td>
<td>Incubation temperature or CO₂ percentage in the incubator too high or too low</td>
<td>Check incubator temperature. It should be at 37°C ± 0.5°C. Check percentage of CO₂ inside the incubator. It should be 5 ± 0.5%</td>
</tr>
<tr>
<td></td>
<td>Blood used for culture is not fresh</td>
<td>Always use freshly drawn blood</td>
</tr>
<tr>
<td>No chromosomes or scattered chromosomes</td>
<td>Cells burst during harvest procedure</td>
<td>Ensure gentle addition of fixative and hypotonic solution</td>
</tr>
<tr>
<td>No metaphases</td>
<td>Harvesting not performed in exponential phase</td>
<td>Harvesting should be done between 70 - 72 hours</td>
</tr>
<tr>
<td>Chromosomes not well spread</td>
<td>Presence of cell aggregates</td>
<td>Disperse cell clumps before adding colchicine</td>
</tr>
<tr>
<td>Chromosomes not uniform</td>
<td>Non uniform drying of slide</td>
<td>Avoid blowing. Drop the cell suspension from a height</td>
</tr>
<tr>
<td></td>
<td>Debris on slide affecting drying in some spots</td>
<td>Ensure that the slides are clean and grease-free</td>
</tr>
</tbody>
</table>

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Email: info@himedialabs.com