Lymphocyte Preparation for FISH analysis:

Test Material

- Specimen preparation of metaphase spreads from whole peripheral blood

Additional Material:

- Hybridization oven (heating oven; 37°C)
- Centrifuge appropriate for 15ml tubes (1000rpm; room temperature (RT))
- Water bath (37°C, 67°C)
- Refrigerator (2-8°C)
- Filter flask connected to water-jet aspirator pump
- Cell culture flasks (without surface treatment; 25 cm^2)
- Blood collection system (e.g. Monovetta) incl. sodium heparin
- Centrifuge tubes, cone-shaped 15ml
- Lymphocyte separation medium incl. phytohemagglutinin (PHA)
- Demecolcine [10 µg/ml] (100 µl per culture)
- 0.075 M KCl (14ml per culture)
- Carnoy's fixative
- Pasteur pipettes
- Rack

Preparatory Steps

Day 1:

- *Pre-warm hybridization oven to 37°C*
- *Blood collection (incl. heparin)*
- *Defat uncoated glass slides (e.g. Superfrost)*: Incubation in 100% ethanol at least o/n and rinse with deionised water afterwards. Store the slides in deionised water at 2-8°C until use.

Day 4:

- *Pre-warm water bath to 67°C*
- *Prepare ethanol series: 70%, 90%, 100%*
- *Prepare 70% ethanol at -20°C for storage of samples*
- *Prepare Carnoy's fixative*: Mixing of methanol and glacial acetic acid 3:1. A volume of 30 ml per culture is needed.
- *90 min before starting with sample preparation, add 100 µl Demecolcine per culture*

  Please note, the shorter the incubation time of the Demecolcine the longer the chromosomes BUT at the same time the amount of metaphases will be reduced.

- *30 min before starting with the sample preparation, pre-warm KCl solution at 37°C*
- *During KCl incubation, prepare Carnoy's fixative and cool down to 2-8°C*

Please note: Solution should be stored at 2-8°C until use.

1.2 Protocol [Day 1]

- Fill 10 ml lymphocyte separation medium into cell culture flask
- Add 1 ml blood (incl. heparin) into 10 ml lymphocyte separation medium
- Incubate cell culture flask in upright position for approx. 72h (3 days) at 37°C

1.3 Lymphocyte Preparation [Day 4]

- Decant culture into 15 ml centrifuge tube
- Centrifuge for 5 min, 1000 rpm
- Discard all but 0.2 - 0.5 ml of the supernatant and resuspend the pellet
- Add 7 ml warm hypotonic KCl solution and resuspend the pellet by inverting the culture flask
- Incubate suspension in the water bath for 10 min at 37°C
- Centrifuge for 10 min, 1000 rpm
- Discard all but 0.2 - 0.5 ml of the supernatant and resuspend the pellet
- Repeat hypotonic treatment with warm KCl (steps 4-7)
- Add dropwise (!) the cold Carnoy's fixative while agitating constantly

  Please note, the first ml should be added over a period of 2 min.
- Incubate 10 min at RT
- Centrifuge for 10 min, 1000 rpm
- Discard all but 0.2 - 0.5 ml of the supernatant and resuspend the pellet
- Wash 3x with 7 ml cold Carnoy's fixative

  Please note, addition can be done more rapidly at this step.
- Discard all but 1 ml of the supernatant and resuspend the pellet

  Please note, residual volume determines the concentration of the suspension.

- Test slides can be prepared by dropping suspension onto uncoated glass slides.

  Please note, suspension can be diluted by addition of Carnoy's fixative or can be concentrated by an additional centrifugation step with subsequent discarding of the supernatant.

- Suspension needs to be fixed at least o/n. Preparation of metaphase spreads can be done the next day.

  Please note, alternatively, the suspension can be immediately dropped onto uncoated, defatted glass slides. Place glass slides into staining jars including Carnoy's fixative and store o/n at -20°C.

1.4 Preparation of metaphase spreads [Day 4]

- Put defatted glass slides (which have been stored in deionised water) directly horizontal onto a rack which is placed in a 67°C pre-warmed water bath.
- Take a small amount of the lymphocyte suspension by using the pasteur pipette and drop from a height of 5-10 cm 1-2 drops onto the glass slide.
- Incubate for 10 min in the water bath and dry slides afterwards at RT. *Please note, marking the area of interest with a diamond pen on the backside of the slide may facilitate further processing.*
- After drying, dehydrate glass slides in 70%, 90%, and 100% each for 1 min.
- Specimens can be stored short term at RT. Long term storage can be done in 70% ethanol at -20°C. Before performing the FISH experiment, specimens need to be incubated at least o/n in 70% ethanol at -20°C.

Proceed using the Zyto*Light* FISH Cytology Implementation Kit [Z-2099-20]