DNA con 3 Cod. 19700

Staining kit for DNA analysis in flow cytometry

50 tests

FOR RESEARCH USE ONLY

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DNA con 3

KIT INSTRUCTIONS AND PROCEDURE

INTENDED USE

The **DNAcon3** kit contains ready-to-use reagents to characterize samples in terms of cytometric ploidy and DNA content. The kit uses the intercalating properties of Propidium Iodide (PI), a DNA-binding fluorochrome, to determine the DNA content [1,2] in cells or nuclei from animal or plant tissues.

The **DNAcon3** kit is specifically designed for flow cytometry, however it is also capable of labelling the DNA of cytological or histological samples for fluorescence microscopy. The kit can be used with most commercial flow cytometers.

The test tubes in the **DNAcon3** kit contain a lyophilized buffer mixture of RNase and a chromatin stabilizer at the bottom. A Propidium Iodide solution is used to reconstitute the buffer mixture in each tube at the time of testing. Samples are processed and analyzed by flow cytometry according to the procedure described.

PRINCIPLE

Normal and neoplastic cell proliferation is characterized by a process of DNA synthesis followed by cell division (figure 1).

Non-proliferating human somatic cells contain two sets of 23 chromosomes and a normal diploid DNA content, while many neoplastic tumors show an abnormal DNA content.

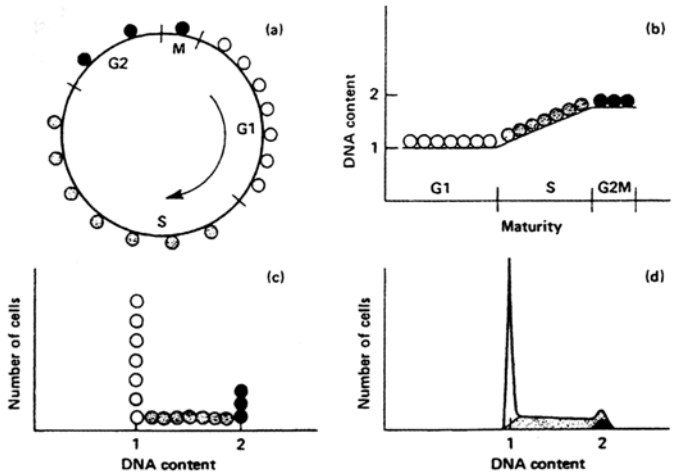

Figure 1
The cell cycle

Flow cytometric measurement of DNA content is an important diagnostic tool in clinical pathology for monitoring cell proliferation and detecting of the presence of abnormal (aneuploid) DNA content [3,4].

Aneuploid cells have a DNA content which is lower (hypo-diploid) or higher (hyper-diploid) than normal. Abnormalities detected by DNA quantitation using flow cytometry is referred to as “cytometric DNA aneuploid” [5].

THE CELL CYCLE

The repetitive growth and division of cells is called the cell cycle. The cell cycle is often divided into five phases and plotted on a DNA histogram (figure 1). The five cell cycle phases are:

1) G_1 (for “gap 1”), a period of growth before DNA replication;
2) S (for “synapses”), as DNA is replicated;
3) G_2 (for “gap 2”), a period of growth following DNA replication;
4) M (for “mitosis”), the period of cell-division;
5) G_0 represents non-dividing or quiescent cells.

(G_0) and (G_1) cells are located in the first part of the histogram, and normally represent the majority of the cell population. The second peak includes (G_2) and mitotic (M) cells. Cells in the “S phase” are located between (G_0 / G_1) and (G_2 / M) peaks. The number of S phase cells (SPC) represents the “proliferation potential” of the analyzed sample and is important in prognosis and treatment [6].

Cytometric DNA ploidy is expressed using the DNA index (DI). This index is calculated using a reference standard of normal diploid cells that are biologically equivalent to the analyzed sample. The DI is the ratio between the peak channel position of the unknown cells divided by the peak channel position of the diploid reference cells.

A normal diploid tissue should always be used as reference standard when suspected tumor samples are tested [7]. The reference normal diploid tissue samples have a DI=1. Abnormal hypo-diploid samples have a DI less than one and abnormal hyperdiploid sample have a DI greater than one. Tumor cells can also be tetraploid (DI=2) and “mixed” in the histogram with G_2+M normal diploid cells. When the DNA content value of aneuploid cells is greater than two times the reference standard cells, it is defined as hyper-tetraploid and reported as DI>2. In some tumor samples more than one cell line can be present and therefore different values of DI can be found.

Since normal diploid cells are usually included in the cell suspension obtained from disaggregated solid tumor samples, the histogram will show the diploid peak together with the aneuploid peak if the tumor is aneuploid. Conversely, if the tumor cells are “cytometrically diploid” they will be included with the normal diploid cells in the same “diploid” peak.

KIT INFORMATION

All reagents provided in this kit are intended for research use only.

a) MATERIALS / REAGENTS PROVIDED:

a) **Propidium Iodide solution** (50 ml bottle) - 50 μg/ml Propidium Iodide

Warning and precautions

Acute effects:

- Harmful if swallowed, inhaled, or absorbed throught skin.
- Causes eyes and skin irritation.
- Causes irritation to mucous membranes and upper respiratory tract.

Chronic effects:

- May alter genetic material. Toxic and may cause heritable genetic damage.
- Propidium iodide is irritating to eyes, respiratory system and skin.

Medical precautions and recommendations:

- In case of exposure, or if you feel unwell, seek immediate medical attention (bring label information if possible).
- In cases of eyes contact, rinse immediately with copious amounts of water, and seek medical attention without delay.
- Wear appropriate personal protective equipment that includes gloves and eyes/face protection.
- Do not breathe dust.
- Refer to material safety data sheet for additional information.

b) Test tubes (50 tubes / kit) capped polystyrene tubes with dehydrated buffer containing lysing agent, RNase, and chromatin stabilizer.

b) MATERIALS REQUIRED BUT NOT PROVIDED

Petri dishes
Scalpels
Pasteur pipettes
Micropipettors (50, 200, 1000 ml)
Conical centrifuge tubes 15 ml
Low speed centrifuge (150-500 g)
Fluorescence microscope
Powder-free latex gloves
Medimachine CTSV cod. 79200;
Medicons CTSV cod. 79300S;
Medicons CTSV cod. 79325N;
Filcons CTSV cod. 130-33N;

Filcons CTSV cod. 200-98N;
Filcons CTSV cod. 150-47N;
Depacons CTSV cod. 59720;
Ficoll-Hypaque (or equivalent)
Phosphate-Buffered Saline (PBS) 0.01 M pH 7.4 +-0.1.

c) KIT STORAGE

All reagents and test tubes contained in the **DNAcon3** kit are stable in the dark at room temperature (20-25°C).
Expiry: refer to the lable on the box.

Procedure

1) Pipette 1 ml of Propidium Iodide solution into each **DNAcon3** test tube containing de hydrated buffer mixture. Use one tube for each specimen and control being tested. Wait 5 minutes for the reagents to be completely dissolved.

2) Locate the sample preparation instructions for each type of specimen to be analyzed. Add the quantity of cell suspension recommended in each sample preparation procedure to the **DNAcon3** test tube prepared in step 1.

3) Vortex each test tube for few seconds following the addition of each sample.

4) Place all sample preparations in the refrigerator (2-8°C) for 60 minutes. Resuspend tubes two times during this period.

5) Filter all stained samples with a 30 μm Filcons (130-33) before running on the flow cytometer.

SAMPLE PREPARATION GENERAL REMARKS

DNAcon3 kit can be applied to any fresh (unfixed) or stored tissue samples which may be frozen (-80°C or -20°C), ethanol-fixel. Generally, fresh or frozen tissue samples give the best results. Fixed suspensions often present high rates of cell aggregates that may cause problems with histogram interpretation and quantitation. Formalin-fixed paraffin-embedded samples require experience in sample handling to obtain reliable results.

The staining procedure is dependent on the type of sample. Fresh unfixed samples can be stained for 1-2 hours and sometimes survive until the next day. Frozen samples stain rapidly (within 30 minutes) but deteriorate faster than fresh ones. Ethanol-fixed samples (as well as nuclei obtained from paraffin-embedded samples) can be stained for long periods of time, and generally survive quite well for one week.

The **DNAcon3** kit is used to estimate the proliferation potential and cytometric ploidy in

biological samples. Samples such as whole blood, bladder irrigation, body fluids, or any other samples with a natural cell suspension do not require specimen preparation for use with the **DNAcon3** kit. Solid tissue samples (both normal and abnormal) must be disaggregated prior to testing through manual or automated disaggregation procedures.

Below are working procedures for whole blood samples, isolated leukocytes, bone marrow samples, biopsies, cell cultures and solid tissue samples.

WHOLE BLOOD SAMPLES

Peripheral blood anticoagulated with sodium citrate can be directly stained:

1) Add 50 μl of whole blood to the **DNAcon3** tube staining solution tube prepared in step 1 in the main procedure.
2) Go back to the main procedure and follow steps 3 to 5.

ISOLATED LEUKOCYTES (WBC)

Leukocytes can be isolated by various methods:

a) SIMPLE GRAVIMETRIC ISOLATION OF WBC

a) Collect 5 ml of peripheral blood anticoagulated with sodium citrate and place in the refrigerator for 1 hour.
b) Gently pick-up the "buffy coat" (white blood cell layer) at the interface between red cells (bottom) and the plasma (upper) using a Pasteur pipette.
c) Place 20 μl of these recovered cells (buffy coat) in the **DNAcon3** test tube prepared in step 1 of the main procedure.
d) Go back to the main pocedure and follow steps 3 to 5.

b) LYMPHOCYTE ISOLATION BY DENSITY GRADIENT

a) Collect 2-3 ml of peripheral blood anticoagulated with sodium citrate.
b) Dilute 2 ml of blood with 2 ml of PBS or normal saline.
c) Place 2 ml of Ficoll-Hypaque in a test tube and add the diluted blood.
d) Centrifuge at 400 g for 20 minutes at room temperature.
e) Carefully remove the mononuclear cell layer with a Pasteur pipette and transfer in a new test tube.
f) Wash two times with PBS and spin at 200 g for 10 minutes at room temperature.
g) Place 20 μl of recovered lymphocyte suspension in the **DNAcon3** test tube prepared in step 1 of the main procedure.
h) Go back to the main procedure and follow steps 3 to 5.

BONE MARROW SAMPLES

Bone marrow can be analyzed either as an *aspirate* of bone *marrow* biopsie

a) Bone marrow aspirates are a "mixture" of solid tissue and marrow blood. Aspirate samples or bone marrow biopsie samples can be analyzed just as they are by disaggregating a part of the aspirate or biopsie directly in PBS by means of a glass Pasteur pipette. Aspirates can also be analyzed by separating the sample into the liquid portion (marrow blood) and solid portion (bone marrow tissue). The liquid portion of the aspirate can be treated just like a whole blood sample (see leukocytes section) listed above.

b) Bone marrow biopsie samples can be treated like a solid tumor or directly disaggregated in PBS as described below.

BIOPSIES

Endoscopical biopsies (e.g. gastric, etc) can be treated as small fragments of tissue according to the section solid tissues. Fine needle biopsies (e.g from "in vivo" breast tumors as well as from large piece of surgical tissue samples) can be treated as:

1) "True cell suspension", thus following the procedure described for *bone marrow aspirates*.
2) *"Body fluid"* whenever a few cells have been recovered.

BODY FLUIDS

In principle any body fluid "containing cells" can be stained by **DNAcon3** and analyzed by flow cytometry. The limiting factor may be the low number of cells in the suspension (e.g. cefalo-rachidian) or the "poor quality" of the cells in the suspension (e.g. urine). Cell concentration may be increased by using the following procedure:

1) Collect the suspension to be analyzed in a 15 ml conical tube and spin down at low centrifuge speed (200 g) for 10-15 minutes to preserve cell integrity.
2) Discard the supernatant
3) Vortex the cell pellet for few seconds
4) Add the Propidium Iodide solution from the **DNAcon3** test tube (prepared in step 1 of the procedure) directly into the conical tube
5) Vortex and then transfer the cell suspension back into the **DNAcon3** test tube
6) Go back to the main procedure and follow steps 3 to 5.

CELL CULTURES

Cell cultures grown in suspension are treated in the same manner as *isolated leukocytes*. For cell cultures grown in monolayer, cells must be recovered from the culture flasks by means of the combined enzymatic mechanical procedure listed below:

1) Remove flask(s) from the incubator and gently discard the culture medium.

2) Add 2 ml of a new medium containing 0.25% trypsin and 0.05% EDTA (pre-warmed at 37°C) and leave in the incubator for 15 minutes shaking the flask(s) from time to time.
3) Remove the flask(s) from the incubator and vigorously shake in order to remove cells still sticking to the surface of the flask. Recovered cells can be washed and treated according to the procedure for *isolated leukocytes*.

SOLID TISSUES (NORMAL AND ABNORMAL)

Solid tissue samples must represent the original tissue as much as possible. Remove all fat and connective tissues from the sample. In cases of large surgical samples, small pieces are collected from different areas in the tissue to guarantee a "good representative pool" of tumor cells.

Routine measurements are generally performed on the pooled samples, but more detailed studies (tumor heterogeneity for both ploidy and SPC) can be carried out on individual pieces of the same tumor. Tissue samples can be prepared using manual or automated disaggregation procedures below.

a) MANUAL DISAGGREGATION

a) Place the tissue sample on a flat plastic support (nylon or teflon are recommended).
b) Keep the tissue wet using few drops of PBS (avoid adding too much liquid to prevent tissue from floating in the liquid).
c) Carefully mince the tissue using a scalpel (or other proper tool) until complete tissue disaggregation has been achieved.
d) Recover the cell suspension by a pipette adding few drops of PBS.
e) Dilute the recovered cells with PBS (depending on the quantity of released cells up, to 2 ml).
f) Filter the suspension using a 50 μm pore size (**Filcons 150-47**).
g) Spin down, aspirate and discard the supernatant and recover the cell pellet.
h) The final cell concentration in the staining solution is important for precise DNA quantitation. Propidium iodide must be kept in large excess with respect to DNA, therefore avoid adding too many cells to the staining solution. The limit is evidenced by the change of the color of the solution from light-red to pink-violet. If this happens, immediately dilute the cell concentration by adding more Propidium Iodide solution to the tube. Check the cell concentration in the sample to be analyzed, the ideal suspension should be approximately 10^6 cells/ml.
i) Go back to the main procedure and follow steps 3 to 5.

b) AUTOMATED DISAGGREGATION

The automated **Medimachine** can be uesd to standardize processing procedures for tissues disaggregation. General instructions are listed below. For detailed information, refer to the **Medimachine** instruction manual.

a) Fill a disposable **Medicons** (one for each piece of tumor to be analyzed) with 1.0 - 1.5 ml of PBS using a 1 ml syringe prior to inserting the piece of tissue. The entire metal screen surface must be wet. In cases of very small pieces of tissue, disaggregation can be performed directly in the staining solution.

b) Place the tissue in the **Medicons**. Check the liquid level prior to replacing the cover Important: The tissue **must not** float in too much liquid over the metal surface!

c) After disaggregation recover the cell suspension using a syringe. If the liquid is very cloudy (which generally means a high cell concentration) dilute with PBS.

d) Filter the suspension using a 50 μm pore size (**Filcons 150-47**).

e) Recover the filtrate liquid and spin it down at 200 g for 10-15 min.

f) Discard the supernatant and recover the cell pellet.

g) The final cell concentration in the staining solution is important for precise DNA quantitation (See above in “Manual disaggregation”).

h) Go back to the main procedure and follow stpes 3 to 5.

Controls and standards

DNA flow cytometry measures the relative DNA content compared to a standard value. Normal diploid cells from samples are used to calculate the DNA Index (DI). Normal lymphocytes can be used as a standard for all body fluid samples, while solid tumor samples can use a piece of a similar normal tissue (generally a tissue sample from the surroundings close to the lesion area). Measurements should be performed in duplicate tubes with and without the added standard in order to better understand the diploid peak position.

Cell suspension obtained from solid tumor tissues “always” contain diploid cells that may help the DI determination but, at the same time, may cause problems in proliferation assessment (while the S phase diploid cell may overlay the distribution of the aneuploid stem line, thus interfering with the calculation of the cell cycle phases).

Instrument setup and performance

Set up the flow cytometer according to manufacturer’s instructions. Select the appropriate DNA software program. **DNAcon3** stained samples can be analyzed in either blue or green excitation according to the type of instrument (see procedure). Check instrument performance with a calibration sample (fluorescent beads or any prestained known cells, i.e., normal human lymphocytes) to verify sensitivity, linearity and coefficent of variation.

Once the instrument is aligned run the sample(s) without any variation of the instrumental

setting during the acquisition. The DNA of the nuclei in the sample intercalated by the Propidium Iodide has a red fluorescent emission that is stechiometrically function of the DNA content.

Spectrophotometric properties of PI allow excitation performed by both laser and lamp sources. The blue and green lines 488 nm and 514 nm of the argon ion laser are the most efficient. Instruments equipped with a mercury arc lamp can take the advantage of the powerful 546 nm green line as well as the other bands (UV and blue) suggested for special applications (multiparametric analyses). PI fluorescent emission, prevalently located in the red between 560-700 nm, is generally measured by means of a long-pass filter of 610 nm.

Keep the frequency of "running events" at 200-300/sec to reduce doublet counting. If higher values are noted, dilute the sample with staining solution.

Always fllter the sample (with Filcons) before placing the sample tube in the instrument for analysis.

According to the available number of channels of the memory (256, 512, 1024) adjust (with the control of PMT voltage and signal amplification) the "diploid" peak position in order to be able to see hypodiploid as well as "multiploid" cells (suggested 60, 100 and 200 respectively).

DATA ANALYSIS

Results obtained using **DNAcon3** staining procedure and flow cytometric analyses are shown in figures 2 and 3.

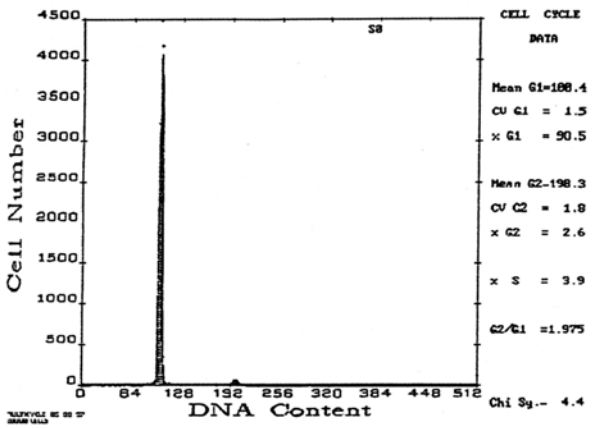

Figure 2
Normal diploid sample. Data handled by Multicycle*

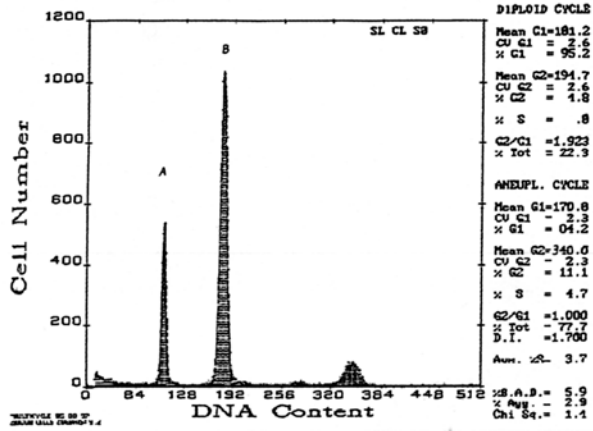

Figure 3
Tumor sample showing together with a diploid peak (A) an aneuploid sub-population B of DI=1.70 (hyperdiploid). Date handled by "Multicycle*.

Multicycle is a Phoenix Flow Systems program.

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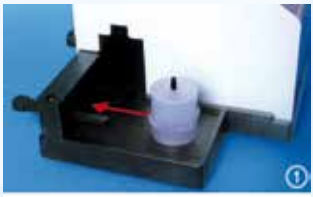
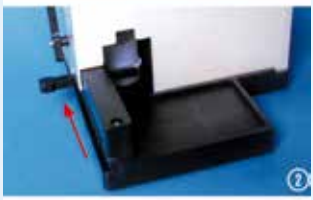
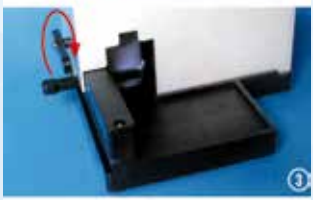
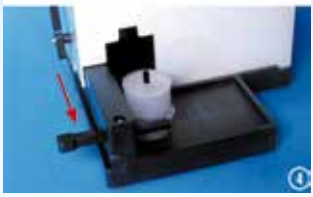
Medimax system









Filcons

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