cytognos

LEUCOFINDER™
Ref: CYT-LF-50

CE

For In Vitro Diagnostic use

INTENDED USE

LeucoFinder™ kit is designed for enumerating residual leucocytes in leucoreduced blood products for transfusion by flow cytometry.

SUMMARY AND EXPLANATION

In recent years, the enumeration of absolute cell counts has been shown to be relevant in different research and clinical settings. One of these applications is the enumeration of residual leukocytes when evaluating leucoreduced blood products for transfusion since an accurate count is critical to prevent febrile reactions, micro organism transfer, alloimmunization, platelet refractoriness and immunosuppression (1). Leucoreduction is performed by combined depletion techniques or by double filtering the product and the final quality control assay must indicate the detection of less than $1x10^6$ residual white blood cells (rWBC) per unit to confirm that the leucoreduction process is adequate (1, 2). In this situation, flow cytometry constitutes a more sensitive, efficient and rapid alternative to haematology analysers and other traditional counting techniques of enumerating rWBC in leucoreduced blood products due to its ability to process different volumes of sample and a large number of samples in a short time (3, 4).

PRINCIPLES OF THE PROCEDURE

LeucoFinder™ kit is an efficient single-platform method for the enumeration of rWBC by flow cytometry which combines the detection of the fluorescence signal from a DNA marker incorporated into the nucleus of rWBC allowing their discrimination, with the use of Perfect-Count Microspheres™ for their absolute count.

- Labelling of residual leucocytes with the DNA marker enables them to be identified and discriminated from some non-nucleated populations, such us erythrocytes and platelets.
- The Perfect-Count™ Microspheres is a microbead-based single platform system, which assures the accuracy of absolute count results. Its unique internal quality control system contains two types of beads (defined as bead A and bead B) with densities around the upper and lower densities of peripheral blood cells (5). Variations of the ratio between beads type A and B warns about problems during sample preparation and/or acquisition which could invalidate final results. This system may be used as a double reference standard which firstly assures the accuracy of the assay and secondly ensures accurate calculation of the number of residual leucocytes per µL.

- The proportion and range that is considered acceptable for this proportion of type A beads and type B beads present in the vial, is specified at the end of this technical data sheet. Once the sample is acquired in the Flow Cytometer, the user should check that the proportion between the two reference beads subpopulations with different densities (A and B) are the same or fall into the acceptable range of variability within the proportion existing in the original mixture. This way, the user can check that the distribution of the two reference beads in the vial is homogenous and that the acquisition of both cells and beads has been randomly selected.
- The total number of beads per microliter is specified in the technical data sheet appearing with this product. The calculation of the absolute number of residual leucocytes in the leucoreduced bags can be made based on the following formula:

$$\text{Absolute Count (rWBC/µl)} = \frac{\text{Nº of rWBC counted}}{\text{Total Nº of microspheres counted (A+B)}} \times \text{Nº of Perfect–Count Microspheres / µl (known concentration)}$$

- Multiplying rWBC/µL by the volume of the pack (in µL) results in the total number of residual leucocytes in the entire pack

REAGENTS

Leuco-Finder kit includes two components:

DNA labelling solution: It contains a specially formulated Propidium Iodide solution with the capacity to rapidly enter the nucleus of rWBC while minimizing the presence of residue that may interfere in further studies with the cytometer. The fluorescent signal emitted by this DNA marker may be detected in channels FL2 and/or FL3 of the Flow Cytometer.

Composition of the DNA labelling solution:

- Propidium Iodide.
- RNAse.
- 0,1% Sodium azide (NaN3).
- Detergent for the permeabilization of the cell membrane.
- Stabilizing buffer.

Perfect-Count Microspheres™: It is an efficient microbead-based single platform method for absolute counts, which contains two different beads types (beads type A and beads type B) with different light scatter, fluorescence and floatation characteristics. Both types of microspheres remain stable for a long time, are easily detectable and differentiated by the flow cytometer because of their different fluorescence intensities. Bead A having low FSC, lower SSC, dimmer FL1, FL2 and FL3 expression and is FL4 negative, compared to Bead B which has low FSC, slightly higher SSC, brighter FL1, FL2 and FL3 expression and is FL4 positive.

- Microspheres of type A are fluorescent beads of 6,4 µm excitable at 506 nm.
- Microspheres of type B are fluorescent beads of 6,36 µm excitable at wavelengths from 365 to 650 nm

The microspheres suspension contains protein supplements to prevent beads adhesion to the tube walls.

WARNINGS AND RECOMMENDATIONS

1. For In Vitro Diagnostic Use.
2. This product is supplied ready for use. If it is altered by dilution or addition of other components, it will be invalidated for in vitro diagnostic use.
3. The reagents are stable until the expiration date shown on the label if it is properly stored. Do not use the product after the expiration date shown on the label. If the reagents are stored in conditions different from those recommended, such conditions must be validated by the user.
4. Alteration in the appearance of the reagent, such us precipitation or discolouration, indicates instability or deterioration. In such cases, the reagent should not be used.
5. The two components of LeucoFinder™ kit contain sodium azide (CAS-Nr. 26628-22-8) as a preservative, however, care should be taken to avoid microbial contamination of reagent or incorrect results may occur.
   - Sodium azide (NaN3) is harmful if swallowed (R22). If swallowed, seek medical advice immediately and show this container or label (S46).
   - Wear suitable protecting clothing (S36).
   - In case of contact with eyes, rinse immediately with plenty of water and seek medical advice (S26).
   - Contact with acids liberates very toxic gas (R32)
   - Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in metal drains where explosive conditions may develop.
6. DNA labelling buffer contains propidium iodide (CAS-Nr: 25535-16-4) a potential carcinogen, which is harmful if swallowed (R22). If swallowed, seek medical advice immediately and show this container or label (S46). Propidium iodide irritates eyes, mucous membranes and skin (R36/37/38). If contacts occur, flush immediately with plenty of water. Wear suitable protecting clothing (S36).
7. All patient specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection (6), and disposed according to the legal precautions established for this type of product. Also recommended is handling of the product with appropriate protective gloves and clothing, and its use by personnel sufficiently qualified for the procedures described. Avoid contact of samples with skin and mucous membranes. After contact with skin, wash immediately with plenty of water.

8. The primary sample pipetting step plays the major role in influencing measurement precision and accuracy, therefore reverse pipetting must be used for both the sample and the counting microspheres. The pipette plunger is pressed to the second stop, the fluid is aspirated in slight excess, and the aspirated sample is dispensed against the dry round bottom of the test tube to the first pipette stop, leaving some residual sample in the pipette tip. It is imperative that the sample is dispensed to the bottom of the dry tube and does not adhere to the side.
9. It is not recommended to use the first sample taken for dispensing (dry tip dispensing). In order to perform a wet tip dispensing draw the sample to the second pipette stop, make two or three gentle dispense cycles at the first stop, keeping the pipette tip within the sample and finally dispense at the first stop against the lower end of the wall of the tube.
10. It is recommended to verify the accuracy of the pipette for optimal results. Pipette calibration can be performed using distilled water (1 µl distilled water = 1 mg) and a precision weighing scale.
    - Place a test tube on a precision balance.
    - Tare the balance to read zero.
    - Pipette 100 µL of distilled water into the test tube.
    - Record the obtained weight.
    - Repeat this process at least 10 times
    - Calculate the mean, standard deviation (SD) and percent coefficient of variation (% CV) of the weightings.
    - The % CV should be <2,0%.
11. It is recommended that a fresh positive control, prepared adding 25 µL of plasma from any spontaneously sedimented anticoagulated fresh blood sample, was acquired following the procedure indicated in point 8.3 of this insert.
12. The bead count of Perfect-Count MicrospheresTM varies by lot. It is critical to use the bead count and the proportion of beads type A and beads type B of the lot which is being used.
13. To ensure reliable statistics for the rWBC events acquisition should be stopped when 10.000 to 20.000 events have been collected in the Perfect-Count microspheres region (R2)(1, 7).
14. Avoid evaporation and leakage of LeucoFinderTM kit and samples to prevent erroneous results.
15. Preparation methods requiring a wash step should not be used due to an unknown loss of cells.
16. Use of the reagent with incubation times or temperatures different from those recommended may cause erroneous results. Any such changes must be validated by the user.

STORAGE CONDITIONS:

Store at 2-8 ºC. DO NOT FREEZE.
This product is photosensitive and should be protected from light during storage or during incubation with cells.
Once opened, LeucoFinder™ kit must be stored in an upright position to prevent the possibility of leakage.

SPECIMEN COLLECTION AND PREPARATION

Process samples of leucoreduced blood products (red blood cells, platelets and plasma samples) within 48 hours following leucoreduction. Stored samples should be kept at 2-8 ºC (7).

PROCEDURE

Reagents Provided

LeucoFinder™ kit (Ref: CYT-LF-50), sufficient for 50 tests.

Reagents and Materials Required but Not Provided

- Flow cytometer. LeucoFinderTM kit is designed for use on a flow cytometer equipped with a 488 nm Argon ion laser for fluorescence excitation, and appropriate computer hardware and software. The flow cytometer must have a minimum of two fluorescence channel detectors and threshold on FL2.
- Disposable round bottom 6 mL tubes of 12x 75 mm.
- Precision micropipettes (100µL) with tips.
- Timer
- Vortex mixer

- Parafilm
- 25 µL of plasma from any spontaneously sedimented anticoagulated fresh blood sample.

Sample Preparation

1. Verify the accuracy of the pipette. Pipette calibration can be performed using distilled water (1µL of distilled water = 1mg) and a precision weighing scale. In point 5.10 (Warnings and Recommendations) of this insert a detailed pipetting verification procedure can be found.
2. Homogenize the sample by thorough manual mixing (no vortex).
3. Pipette by reverse pipetting technique 100 µL of the leucoreduced product into each tube. Points 8 and 9 of Warnings and Recommendations make reference to this reverse pipetting technique.
   For the preparation of the fresh positive control tube, add 25 µL of plasma from any spontaneously sedimented anticoagulated fresh blood sample.
4. Stain cells adding 100 µL of the DNA labelling solution to each tube. Mix gently in the Vortex and incubate during 5 minutes at room temperature in the dark.
5. Immediately prior to using the Perfect-Count MicrospheresTM, mix the vial manually for 30 to 40 seconds (do not use Vortex). With the same pipette used for the sample dispensing, add 100 µL (the same volume as the one used previously for the sample addition) of Perfect-Count MicrospheresTM to each tube using again the reverse pipetting technique.
6. Cover the sample tube with Parafilm and homogenize manually for a few seconds before acquisition on the flow cytometer.

Flow Cytometric Analysis

A. Cytometer Setup

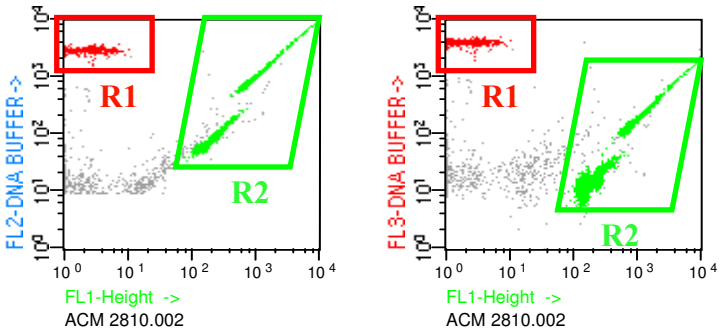
Follow the manufacturer's instrument set up procedure and run the protocol compatible with the colour combination. The procedure for adjustment of the Instrument settings is as follows:

- Check amplification modes: FSC and SSC parameters must be set on linear amplification and FL1, FL2 FL3 and FL4 parameters must be set on logarithmic amplification.
- Set the Threshold or Discriminator in parameter FL2 at a sufficient value to exclude all events other than those from leucocytes or microspheres. To this end, in BD Biosciences cytometers the threshold value in FL2 is usually set at around 250, while in Coulter cytometers the discriminator value in FL2 is usually correct when set around 2.
- Set all compensation settings to zero except for the FL1-%FL2 setting, where the same value featured in the instrument setting commonly used for surface antigens will be kept.

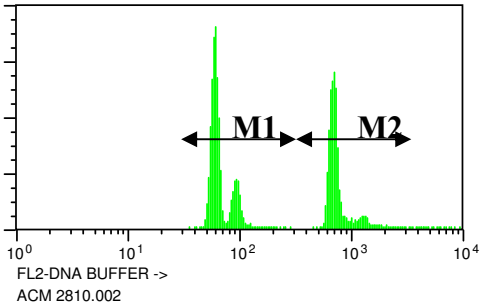
DNA labelling may be detected in parameters FL2 and/or FL3 while Perfect CountTM Microspheres are detected with different intensities in FL1, FL2 and FL3.

In the data acquisition template the following figures should be used

- FL1 vs. FL2 (FL1/FL2) dot plot and/or FL1 vs. FL3 (FL1/FL3) dot plot with two regions to select residual leucocytes (R1) and total microspheres (R2)



- Gating the residual leucocytes (R2) on a FL2 histogram, identify and select the two types of microspheres with two lineal regions (M1 and M2).



It is suggested that an enriched control sample, prepared with 25 µL of plasma from any spontaneously sedimented anticoagulated fresh blood sample, is acquired to check the correct configuration of the instrument.

B. Sample Acquisition

Gently mix the samples manually immediately prior to running on the flow cytometer to ensure thorough resuspension of cells and microspheres.

Acquire and store all events. To ensure reliable statistics for the rWBC events acquisition should be stopped when 10.000 to 20.000 events have been collected in R2 (the Perfect-Count MicrospheresTM region)(1, 7).

It is recommended to acquire at a low or medium speed to avoid cell aggregates.

C. Data Analysis

Cytognos S.L. recommends that the software INFINICYT is used for data analysis. Contact Cytognos S.L. or its authorized distributor about the advantages and ways to get this analysis software.

Manual data analysis may be done by using:

- A FL1/FL2 dot plot or a FL1/FL3 dot plot with two regions to select residual leucocytes (R1) and total microspheres (R2) and the *region statistic* box related to this dot plot. The data concerning the number of total microspheres (A+B) acquired and the number of residual leucocytes acquired will be used in the calculation of the absolute number of residual leucocytes present in the sample (Nº rWBC/µL).
- A FL2 histogram gating the residual leucocytes (R2) where the two types of microspheres are identified and selected with two lineal regions (M1 and M2) and the *region statistic* box related to this histogram. The percentage of type A and type B beads detected will be used to verify that the acquisition of the sample has been made in a random manner and that the calculation of the absolute number of residual leucocytes is reliable.

Calculation of Absolute Counts

- Verify on the FL2 histogram statistics table that the proportion between the two reference beads subpopulations with different densities (A and B) are the same or fall into the acceptable range of variability within the proportion existing in the original mixture indicated by the manufacturers at the end of this technical data sheet in point 14 about lot specific data.

- The absolute number of residual leucocytes in the sample is determined by dividing the number of leucocyte events acquired (R1) by the number of Perfect-Count microspheres acquired (R2), and multiplying this result by the microsphere concentration (microsphere concentration is indicated in point 14 about lot specific data).

$$\text{Absolute Count (rWBC/µl)} = \frac{\text{Nº of rWBC counted}}{\text{Total Nº of microspheres counted (A+B)}} \times \text{Nº of Perfect–Count Microspheres / µl (known concentration)}$$

- Multiplying rWBC/µL by the volume of the pack (in µL) results in the total number of residual leucocytes in the entire pack.

Quality Control

LeucoFinderTM kit is the only available single platform method for the enumeration of rWBC with a double internal standard represented by two different types of beads (type A and type B), which determines if

preparation and acquisition of the sample by the flow cytometer is performed homogeneously. LeucoFinderTM kit contains two types of beads which float at different levels in the tube and the accuracy of the assay is checked by verifying that the proportion of both types of beads after acquisition of the sample agrees with the manufacturers indicated proportion at the end of this technical data sheet in point 14 about lot specific data.
For optimal results, ensure that the pipettes and the cytometer are calibrated according to the frequency recommended by the manufacturers.

LIMITATIONS

- The primary sample pipetting step plays the major role in influencing measurement precision and accuracy, therefore a calibrated pipette and reverse pipetting technique must be used for both the sample and Perfect-Count MicrospheresTM. Find detailed information in points 8-10 of Warnings and Recommendations.
- Process samples of leucoreduced blood within 48 hours following leucoreduction.
- Gently mix samples manually immediately prior to running on the flow cytometer to ensure thorough resuspension of cells and microspheres.
- Stained leucoreduced samples must be analyzed on the flow cytometer within 60 minutes of adding Perfect-Count MicrospheresTM.
- The numbers of Perfect-Count MicrospheresTM included in the LeucoFinderTM kit varies by lot. It is critical to use the specified numbers of microspheres and the proportion between the different types of microspheres (beads) shown on the Lot Specific Data for the particular lot being used.
- Nucleated erythrocytes contain nucleic acid and could be detected as residual leucocytes in this assay. However, nucleated erythrocytes are not present in detectable quantities in blood from normal individuals (8).

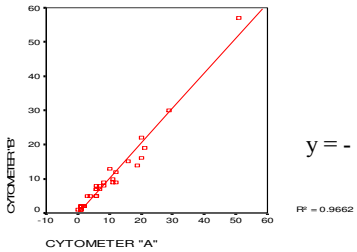
RANGES OF REFERENCE

European Community guidelines and International Society of Blood Transfusion indicate that less than 1x106 rWBC should be present in each leucoreduced blood component bag. This value is currently 5x106 in the UK. Outside the UK, in an ordinary leucoreduced blood component of about 250-300 mL, the measured rWBC level is expected to be approximately <3 cells/µL to ensure validation (7, 9).

PERFORMANCE CHARACTERISTICS

Precision

A study was performed with 32 samples in two different flow cytometers to assess stain-to-stain precision. The degree of correlation obtained was r2= 0,966. Results are shown in the following figure:

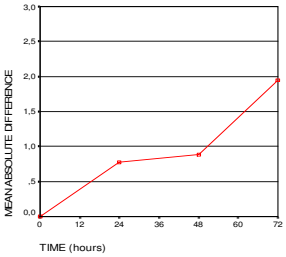


y = - 0,57+1,025X

Stability

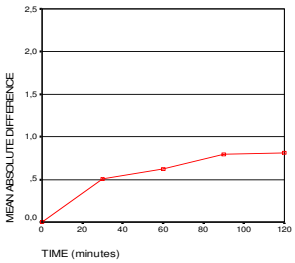
Sample stability was evaluated by comparing the results obtained with 10 samples stained and acquired within 1 hour after leucoreduction, to the same 10 samples aliquots held for 24 hours, 48 hours or 72 hours after leucoreduction. The results of the absolute differences between samples prepared immediately and samples processed later are shown in the following table and figure:

TIME	MEAN OF THE DIFFERENCES
24 HOURS	0,773
48 HOURS	0,885
72 HOURS	1,943



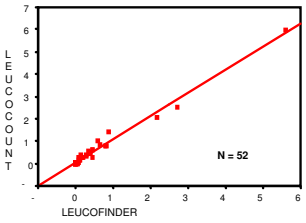
To check the stability of processed samples, 10 samples results were compared when acquired in the Flow Cytometer immediately after adding Perfect-Count MicrospheresTM, at 30 minutes, 60 minutes, 90 minutes and 120 minutes from its preparation. The results of the absolute differences between samples acquired immediately and samples acquired later are shown in the following table and figure:

TIME	MEAN OF THE DIFFERENCES
30 MIN	0,501
60 MIN	0,623
90 MIN	0,795
120 MIN	0,815



Accuracy

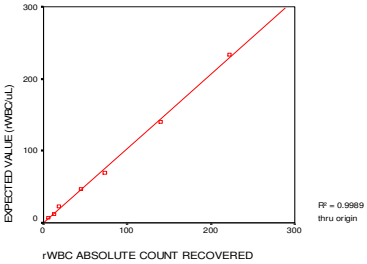
Results obtained with LeucoFinder™ were compared with the ones obtained by a similar method for residual Leucocytes counts commercially available: LeucoCount™ (BD Bioscience). The degree of correlation between both products was r^2= 0,991. Results of 52 samples are shown in the following figure:



$$Y = - 0,347 + 0,952\ X$$

Linearity

Measurement of 7 serial dilutions was made of a concentrated normal whole blood sample to achieve a range of rWBC concentrations. The following figure shows excellent correlation (r^2= 0,999) obtained. The limit of detection of this method is 1 rWBC / 10 µL of sample, being the maximum concentration analyzed with this method of 500 rWBC / µL.



REFERENCES

1. Mandy F, Brando B. In Current Protocols in Cytometry 6.8.1-6.8.26 (2000) John Wiley&Sons, Inc.
2. European Working Group on Clinical Analysis (EWGCCA) Cytofluorometric methods for assessing absolute number of cell subsets in blood. Cytometry (Comm. Clin. Cytometry) 42: 327-346 (2000)
3. Center for Biologics Evaluation and Research. Recommendations and licensure requirements for Leucocyte-reduced blood products. Rockville, MD: Food and Drug Administration, 1996

4. Dzik S, Moroff G, Dummont L. A multicenter study evaluating three methods for counting residual WBCs in WBC-reduced blood components: Nageotte hemocytometry, flow cytometry, and microfluorometry. Transfusion 40: 513-520 (2000).
5. Storie I, Sawle A, Goodfellow K, Whitby L, Granger V, Ward RY, Peel J, Smart T, Reilly JT, Barnett D. Perfect-Count : A novel approach for the single platform enumeration of absolute CD4+ T-lymphocytes. Cytometry Part B (Clinical Cytometry) 57B:47-52 (2004).
6. Protection of Laboratory Workers from occupationally acquired infections. Second edition; approved guideline (2001). Villanova PA: National Committee for Clinical Laboratory Standards; Document M29-A2.
7. Brando B, Barnett D, Arroz MJ. Low level leucocyte counting (LLLC) in blood products. J Biol Regul Homeost Agents 17: 234-240 (2003).
8. Bull BS, Breton-Corius J. Morphology of the erythron. In Beutle E, Lichman M, Coller B, Kripps T eds. Williams Hematology, Fifth Edition. New York: MacGraw-Hill, Inc 1995:349.
9. Dumont LJ, Dzik Wh, Rebulla P, Brandwein H. Practical guidelines for process validation and process control of white cell reduced blood components: report of the biochemical excellence for safer transfusion working party of the International Society of Blood Transfusion. Transfusion 36: 11-20 (1996).

WARRANTY

This product is warranted only to conform to the quantity and contents stated on the label. There are no warranties that extend beyond the description on the label of the product. Cytognos's sole liability is limited to either replacement of the product or refund of the purchase price.

EXPLANATION OF SYMBOLS

	**Use by (use by YYYY-MM)**
	**Storage temperature limitation**
	**Consult instruction for use**
IVD	***In vitro* diagnostic medical device**
LOT	**Batch code**
REF	**Code number**
	**Manufacturer**

LOT SPECIFIC DATA

PRODUCED BY

CYTOGNOS SL
Polígono La Serna, Nave 9
37900 Santa Marta de Tormes
Salamanca (España)
Phone: + 34-923-125067
Fax: + 34-923-125128
Ordering information: admin@cytognos.com
Technical information: support@cytognos.com