PERFECT-COUNT Microspheres™

REF: CYT-PCM-50

For in vitro diagnostic use

50 tests

For Professional use only

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	Use by YYYY-MM
	Temperature limitation
	Consult instruction for use
IVD	***In vitro* diagnostic medical device**
LOT	**Batch code**
REF	**Catalog number**
	Manufacturer

1. INTENDED USE

Perfect-Count Microspheres™ is designed for determining absolute counts of cells in peripheral blood, bone marrow, leukapheresis and culture medium samples using flow cytometry.

Perfect-Count Microspheres™ is a microbead-based single-platform system for absolute counts, which can be used in combination with monoclonal antibodies (MoAb) conjugated with different fluorochromes, which make it possible to identify the cell subpopulations for which the absolute count is intended.

2. SUMMARY AND EXPLANATION

In recent years, the enumeration of absolute levels of cells and their subsets has been shown to be relevant in different research settings as well as for clinical diagnosis laboratories. Absolute counting of cells or cell subsets by flow cytometry is an established technique in the enumeration of CD4+ and CD8+ T-lymphocytes for monitoring of patients with human immunodeficiency virus (HIV+) infection, in the enumeration of CD34+ haematopoietic stem and progenitor cells in patients who are candidates for bone marrow autotransplantation, and in the enumeration of residual leucocytes as part of the quality control of leukoreduced blood products (1-3).

The absolute counting of cell subpopulations by flow cytometry can be calculated using a double platform technique in which the information provided by the flow cytometer (FC) and by a haematological counter are combined, or using a single platform technique for which only the flow cytometer is used. The single platform technology has emerged as the method of choice for absolute cell enumeration in clinical applications. The advantage of this strategy is the inherent capacity to positively identify all cells of interest exclusively, and thus exclude contaminating cells (1). The identified cell number is extracted directly from the original blood volume. The blood volume can be determined by volumetric methods or methods based on known quantities of fluorescent microspheres that act as a reference, although methods based on the use of reference particles can be applied to any flow cytometer regardless of its manufacturer (2).

3. PRINCIPLES OF THE PROCEDURE

Procedures described in this insert apply to immunophenotyping applications in which the use of Perfect-Count Microspheres™ is combined with monoclonal antibodies (MoAb), which allow to positively identifying the cells of interest.

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. Variations of the ratio between beads type A and B warns about problems during sample preparation and/or flow cytometry acquisition which could invalidate final results. This system can be used as a double reference standard, which firstly assures the accuracy of the assay and secondly ensures accurate calculation of the number of cells 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 at the end of this technical data sheet. The calculation of the absolute number of the cell population of interest can be made using the following formula:

$$\textbf{Absolute count (Cells/µl)} = \frac{\textbf{Number of cells of the target subpopulation counted}}{\textbf{Total number of microspheres counted (R1*R2)}} \times \textbf{Num of Perfect-Count/µl (value specified by manufacturer)}$$

4. REAGENT

Perfect-Count Microspheres™ 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 488 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.

5. 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 reagent is 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 reagent is stored in conditions different from those recommended, the user must validate such conditions.
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. Perfect-Count Microspheres™ contains 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).
   - 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. 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.
7. 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.
8. 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.
9. 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%.
10. The bead count of Perfect-Count Microspheres™ 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.
11. Avoid evaporation and leakage of Perfect-Count Microspheres™ and samples to prevent erroneous results.

12. Preparation methods involving washing should not be used, since this could result in unknown cell loss, leading to incorrect results for the absolute count. For this reason, the use of QUICKLYSIS™ (CYT-QL-1) erythrocyte lysing buffer is recommended, since it requires no further washing and contains no fixatives (5, 6).
13. Stained and lysed samples must be acquired from the flow immediately after the addition of Perfect-Count Microspheres™.
14. The method's sensitivity depends on the number of events acquired. To obtain a precise absolute count value it is advisable to stop acquisition when 1.000 to 20.000 events (1) have been acquired in the region of total microspheres.
15. Use of the reagent with incubation times or temperatures different from those recommended may cause erroneous results. The user must validate any such changes.

6. STORAGE CONDITIONS:

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

7. SPECIMEN COLLECTION AND PREPARATION:

Whole blood sample must be taken aseptically by means of a venipuncture (7) in a sterilized tube for blood collection containing an appropriate anticoagulant (use of EDTA is recommended). Aphaeresis samples should be obtained according to the manufacturer's specifications. Store anticoagulated blood samples at 20-25ºC until ready for staining. It is advisable to test blood samples within the 24 hours after their extraction and aphaeresis within the 6 hours after they are collected.

Cryopreserved, haemolysed samples, or samples with suspended cell aggregates should not be rejected.

Optimal staining using CYTOGNOS monoclonal antibodies are achieved with white blood cell counts in the range of 4-10 x 10^6 cells/ml.

- White blood cell counts exceeding 10 x 10^6 cells/mL require dilution with autologous plasma or PBS to obtain a concentration of cells in the range of 4-10 x 10^6 cells/mL. It is important to consider the dilution factor employed for calculation of the absolute counts.
- White blood cell counts lower than 4 x 10^6 cells/mL cannot be concentrated since Perfect-Count Microspheres™ is a direct absolute count quantitation method.

8. PROCEDURE

8.1. Material provided

Perfect-Count Microspheres™, Ref: CYT-PCM-50 contains reagent sufficient for 50 determinations

8.2. Reagents and material required but not provided

- Flow cytometer: Perfect-Count Microspheres™ are designed for use on a flow cytometer equipped with a 488 nm Argon ion laser for fluorescence excitation, and appropriate computer and software.
- Combination of monoclonal antibodies conjugated with fluorochrome, which make it possible to identify the cell subpopulations for which the absolute count by flow cytometry is intended.
- Non wash Erythrocyte lysing buffer (the use of QUICKLYSIS™ reagent is recommended)(5,6)
- Disposable 6 mL test tubes of 12x 75 mm suitable for flow cytometry.
- Precision micropipettes (100 µL) and tips
- Cronometre
- Vortex Mixer
- Parafilm

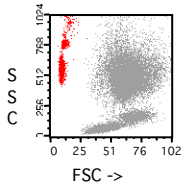
8.3. Sample preparation

1. Verify the accuracy of the pipette. Pipette calibration can be performed using distilled water (1 µL of distilled water = 1mg) and a precision weighing scale. In point 5.9 (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 sample to each test tube. Points 7 and 8 of Warnings and Recommendations make reference to this reverse pipetting technique.
4. Stain samples following specific instruction in the appropriated MoAb package insert. Mix gently in the Vortex and incubate for 10 minutes at room temperature in the dark. Add 2 ml of QUICKLYSIS™ lysing buffer to each tube. Mix and incubate in the dark for 10 minutes at room temperature.
5. Immediately prior to using Perfect-Count Microspheres™, mix the vial manually for 30-40 seconds (do not use Vortex). With the same pipette used for dispensing the sample, add 100 µL (the same volume as the one used previously for the sample addition) of Perfect-Count Microspheres™ 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.

8.4. Flow Cytometric Analysis

A. Cytometer Setup

Verify the cytometer is correctly aligned and standardized for light scatter (FSC and SSC parameters must be set on linear amplification) and fluorescence intensity (FL1, FL2, FL3 FL4 parameters must be set on logarithmic amplification) and colour compensation has been set following the instructions of the cytometer manufacturer. Before acquiring samples, set the Threshold or Discriminator in parameter FSC to minimize debris and ensure population of interest are included. Perfect-Count Microspheres™ population is coloured in red in the following figure.



B. Sample Acquisition

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

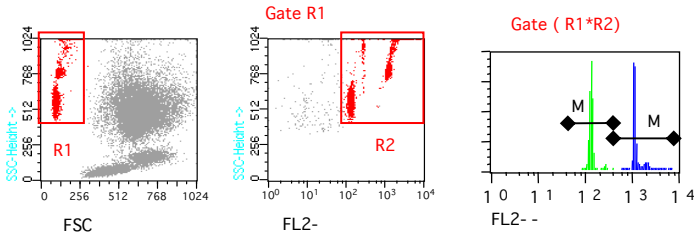
Acquire and store all events. To ensure reliable statistics events acquisition should be stopped when 1000 to 20.000 events have been collected in the Perfect-Count Microspheres™ region (1).

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

C. Data Analysis

On the acquisition and analysis template or protocol, the appropriate diagrams will be used to discriminate the target cell population in each study, and the following additional figures for the analysis of the results obtained with Perfect-Count Microspheres™:

- A FSC/SSC dot plot where is defined an R1 region to select the total microspheres
- A FL2/SSC dot plot gating the R1 region where is defined an R2 region for a cleaner selection of the total of Perfect-Count Microspheres™.
- A FL2 histogram acquiring the events included in regions R1 and R2 (gating R1*R2) where is defined two linear regions (M1 and M2), to identify and select A microspheres (with lower intensity for FL2) and B microspheres (with higher intensity for FL2), respectively. In the statistic chart corresponding to this histogram are featured the data referred to the total microspheres acquired and the percentage of A and B type spheres detected. These data will be used later in the calculation of results.



The discrimination of the cell population for which the absolute count is intended, should be done following the instructions of the reagent used for that purpose (the combination of monoclonal antibodies used). The number of events in the region containing the cell population of interest, will be used later in the calculation of results.

8.5. 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 the cell population of interest is determined by dividing the number of cells of interest acquired by the number of Perfect-Count Microspheres™ acquired (R1*R2), and multiplying this result by the microsphere concentration (microsphere concentration is indicated in point 14 about Lot Specific Data).

$$\text{Absolute count (Cells/µl)} = \frac{\text{Number of cells of the target subpopulation counted}}{\text{Total number of microspheres counted (R1*R2)}} \times \text{Number of Perfect-Count/µl (value given by manufacturer)}$$

- If the sample has been diluted, the result of this formula must be corrected with the dilution factor applied.

8.6. Quality Control

- Perfect-Count Microspheres™ is the only available single platform method for absolute counting 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. Perfect-Count Microspheres™ 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.
- Refer to the specific MoAb data sheet to know reagent quality control procedures.

- For optimal results, ensure that the pipettes and the cytometer are calibrated according to the frequency recommended by the manufacturers.

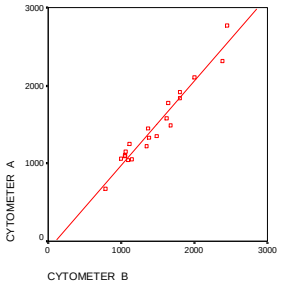
9. LIMITATIONS

1. 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 Microspheres™. Find detailed information in points 7-9 of *Warnings and Recommendations*.
2. It is advisable to test blood samples within the 24 hours after their extraction and aphaeresis within the 6 hours after they are collected.
3. Preparation methods requiring a wash step should not be used due to an unknown loss of cells, leading to incorrect results for the absolute count. For this reason, the use of QUICKLYSIS™ (CYT-QL-1) erythrocyte lysing buffer is recommended, since it requires no further washing and contains no fixatives (5, 6).
4. Stained and lysed samples must be acquired from the flow cytometer immediately after the adding Perfect-Count Microspheres™.
5. Samples should be mixed manually immediately prior to running on the flow cytometer to ensure thorough resuspension of cells and microspheres.
6. To ensure reliable statistics events acquisition should be stopped when 1.000 to 20.000 events have been collected in the Perfect-Count Microspheres™ region (1).
7. The numbers of Perfect-Count Microspheres™ varies by lot. It is critical to use the specified numbers of microspheres and the proportion between the different types of microspheres shown on the *Lot Specific Data* for the particular lot being used.
8. Results obtained by flow cytometry may be erroneous if the cytometer is misaligned or the gates are improperly set.

10. PERFORMANCE CHARACTERISTICS

10.1. Precision

A study was performed with 20 samples acquired in two different flow cytometers to assess stain-to-stain precision. The degree of correlation obtained was r2=0.97. Results are shown in the following figure.



Y= -100.764 + 1.081 x

10.2. Accuracy

Comparison of the absolute CD4+ T-lymphocyte counts derived from Perfect-Count Microspheres™ and TruCount/MultiTEST method, using MultiSET software was performed on 104 HIV+ cases (3). The results of the regression analysis are shown on the following chart:

N	Mean difference CD4 cells/µl	SD	Correlation Coefficient (r^2)	Slope	Y-Intercept	95 % CI levels
104	27	47	0.99	0.9781	-12,61	9

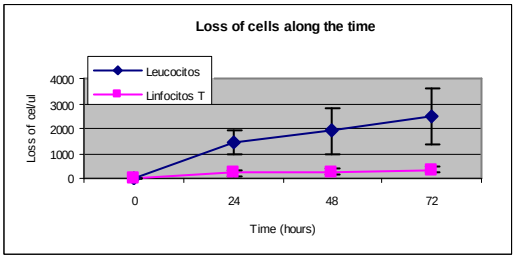
10.3. Reproducibility

A study using three replicate measurements of T-lymphocyte (CD3+), B-lymphocyte (CD19+) and NK cells (CD56+) counts were made to assess reproducibility. The resuts appear in the following table:

	T-LYMPHOCYTES CD3+			B-LYMPHOCYTES CD19+			NK CELLS CD56+		
Sample	MEAN Cel/µL	SD	CV	MEAN Cel/µL	SD	CV	MEAN Cel/µL	SD	CV
1	1479	79,65	5,38	271	15,14	5,58	88	9,71	11,03
2	1970	67,21	3,41	348	16,09	4,62	118	9,84	8,33
3	2120	14,22	0,67	432	19,69	4,55	134	11,54	8,61
4	1159	20,55	1,77	299	3,21	1,07	172	6,65	3,86
5	1317	19,51	1,48	139	4,72	3,39	204	14	6,86

10.4. Stability

To check the stability of the sample, the results were evaluated for the same sample after processing immediately after extraction, 24 hours after extraction, 48 hours after extraction and 72 hours after extraction. The results of absolute loss of leucocytes and lymphocytes along the time are shown on the following figure. It is advisable to test blood samples within the 24 hours after their extraction.



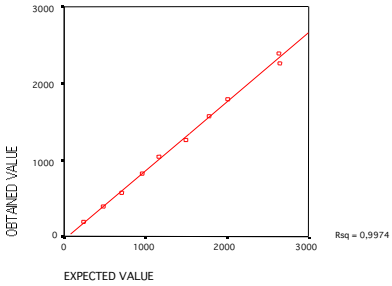
To check the stability of the processed sample the results were evaluated for the same sample on acquiring it immediately, 2 hours, 4 hours, 6 hours and 8 hours after its preparation. The study was performed in duplicated samples stored at room temperature and stored at 2-8ºC. The following tables show the result of internal quality control system, the total number of beads acquired and the values obtained for absolute CD3+ T-lymphocytes, CD19+ B-Lymphocytes and CD56+ NK cells. We conclude from this data that Perfect-Counts Microspheres™ must be added and mixed immediately prior to sample acquisition

SAMPLE STORED AT 2-8ºC	INTERNAL QC SYSTEM	TOTAL BEAD COUNT	ABSOLUTE CD3+ COUNT	ABSOLUTE CD19+ COUNT	ABSOLUTE CD56+ COUNT
0 HOURS	OK	1251	1362	220	87
2 HOURS	OK	1149	1616	239	81
4 HOURS	NO VALID	1003	1932	276	125
6 HOURS	NO VALID	974	2073	279	124
8 HOURS	NO VALID	857	2528	404	203

SAMPLE STORED AT ROOM TEMPERATURE	INTERNAL QC SYSTEM	TOTAL BEAD COUNT	ABSOLUTE CD3+ COUNT	ABSOLUTE CD19+ COUNT	ABSOLUTE CD56+ COUNT
0 HOURS	OK	1345	1360	207	80
2 HOURS	NO VALID	1016	1823	278	105
4 HOURS	NO VALID	725	2904	422	99
6 HOURS	NO VALID	771	2931	520	476
8 HOURS	NO VALID	770	2913	533	560

10.5. Linearity

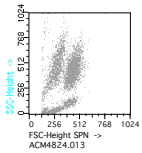
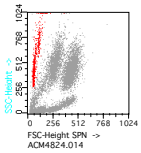
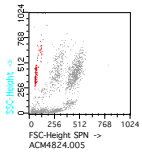
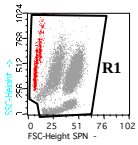
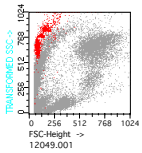
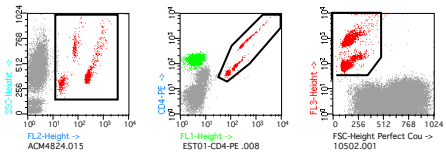
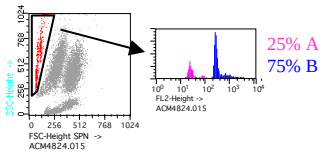
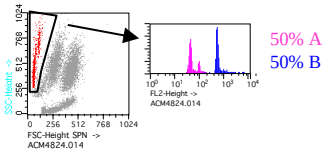
Measurement of 10 serial dilutions was made of as concentrated normal whole blood sample to achieve a range of T-lymphocytes (CD3+) concentrations. The following figure shows excellent correlation obtained (r^2=0,997).



11. REFERENCES

1. Mandy F and 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. 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).
4. Protection of Laboratory Workers from occupationally acquired infections. Second edition; approved guideline (2001). Villanova PA: National Committee for Clinical Laboratory Standards; Document M29-A2.
5. Menéndez P, et al. Comparison between a lyse-and-then-wash method and a lyse-non-wash technique for the enumeration of CD34+ hematopoietic progenitor cells. Cytometry (Comm. Clin. Cytometry) 34: 264-271 (1998)
6. Gratama JW, Menéndez P, Kraan J, Orfao A. Loss of CD34+ hematopoietic progenitor cells due to washing can be reduced by the use of fixative-free erythrocyte lysing reagents. J Immunol. Methods 239: 13-23 (2000)
7. Procedures for the collection of diagnostic blood specimens by venipuncture-approved standard; Fifth edition (2003). Wayne PA: National Committee for clinical laboratory standards. NCCLS document H3-A5.

12. TROUBLESHOOTING GUIDE

PROBLEM	SOLUTION
Perfect-Count Microspheres™ are not detected in the FSC/SSC dot plot. 	1.- Decrease the threshold or discriminator value until 0.  2.-Check that the acquisition corresponds to the total of events instead to a drawn region.
Most of the events acquired corresponds to debris and died cells. 	1.-Repeat the acquisition after some minutes to wait for the lysing solution effect. 2.-Drawn a region excluding debris and rest of cells and acquire the sample gating this region. In this way, the acquisition is enriched in alive cells.  Acquire Gating R1
Difficulty to define Perfect-Count™ in the FSC/SSC dot plot because microspheres are mixed with cells. 	As microspheres are fluorescent particles it is possible to identify them in basis to other fluorescences. Depending on the markers we have in the sample there are some alternative solutions to identify them. In this figure are shown some examples. 
The relative proportion of the microspheres does not agree with the reference ranges of the batch. 	1.-Check that the Perfect-Count™ vial has been properly mixed before preparing the sample, and once prepared check that the sample has been properly mixed before the acquisition. 2.- Check that the threshold is low enough for not to “cut” one of the types of beads.  3.- Check the acquisition process has been performed at medium or low speed. 4.-If the problem persists, repeat the acquisition in other cytometer to discard a problem in the pressures of the machine. 5.-If this problem continues and you are obtaining a wrong proportion, please contact to our support technical service. support@cytognos.com

13. 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.

14. LOT SPECIFIC DATA