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Stepcount

Cat. Reference: 1399991218

Reagent provided:: 25 Stepcount tubes for 25 test

INTENDED USE.

Immunostep Stepcount tubes are designed for determining absolute counts of cells in peripheral blood, bone marrow, leukapheresis and culture medium samples using flow cytometry.

Stepcount tubes are a single-platform system for absolute counts, which can be used in combination with monoclonal antibodies (MoAb) conjugated with different fluorochromes, which make possible to identify cell subpopulations for which the absolute count is intended.

SUMARY

Procedure described in this data sheet is used for immunophenotyping techniques, and is based on known number lyophilized spheres that emit fluorescence at the wavelengths used in convencional flow cytometry.

PRINCIPLES OF THE PROCEDURE

The microspheres contained in the tubes are lyophilized and will be dissolved when the sample is added.

As the number of particles is know we can establish a relationship with any other population identified by the antibodies that we have incorporated.

Absolute counting of cells or cell subsets has a number of significant clinical applications as monitoring the disease status of HIV-infected patients, enumerating residual white blood cells in leukoreduced blood products, and assessing immunodeficiency in a variety of situations. The single-platform method (flow cytometry alone) has emerged as the method of choice for absolute cell enumeration. This technology counts only the cells of interest in a precisely determined blood volume. Exact cell identification is accomplished by a logical electronic gating algorithm capable of identifying lineage-specific immunofluorescent markers. Exclusion of unwanted cells is automatic. This extensive and detailed unit presents protocols for both volumetric and flow-rate determination of residual white blood cells and of leukocyte subsets. Keywords: absolute count; volumetric counting; flow rate cytometry; counting microsphere standards; single-platform absolute count; dual-platform absolute count; logical gate; lineage-specific markers; immunophenotyping; absolute residual WBC

REAGENT

Each box contain: 25 Stepcount tubes for 25 test.

Description: Closed transparent tubes with a metal screen, including a white matrix at the bottom.

Number of microspheres per tube: This value is found on the Stepcount tubes label and is different for each batch.

Particles mean diameter: 3.6 µm with a standard deviation of 0.25 µm

Excitable at wavelengths from 365 to 650 nm

Precautions

For use on Research only
Storage between 2° C and 8° C
Protect from direct light
Keep well closed
Use with a lyse/no-wash procedure.
No transfer the contents of the tube to another tube.
The sample must have protein content similar to whole blood to obtain correct values.

Protocol

1. For each patient sample, label a STEPCOUNT Tube with the reagent and sample identification number. Temper the sample and the tubes

2. Spin tubes in microfuge (short-spin) to ensure that spheres are at the bottom.

3. Pipette of the appropriate reagent just above the stainless steel retainer. Do not touch the pellet.
4. Pipette 50 µL of well-mixed, anticoagulated whole blood onto the side of the tube just above the white pellet.

NOTE: Avoid smearing blood down the side of the tube. If whole blood remains on the side of the tube, it will not be stained with the reagent.
Accuracy is critical. Use the reverse pipetting technique to pipette sample onto the side of

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the tube just above the retainer. Mix the sample until complete dissolution of the pellet.
5. Cap the tube and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature (20° to 25°C).

6. Add 450 µL red blood cell (RBC) lysis buffer (strongly recommended ammonium chloride) to the tube.

7. Cap the tube and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature. The sample is now ready to be analyzed on the flow cytometer.

NOTE: Vortex the samples thoroughly (at low speed) to resuspend beads and reduce cell aggregation before running them on the flow cytometer.

Adquisition and analysis.

Before acquiring samples, 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.

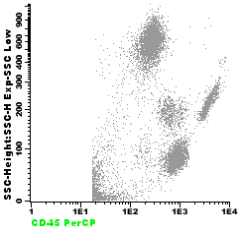
Before acquiring samples, set the Threshold or Discriminator in parameter FSC to minimize debris and ensure population of interest are included.

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

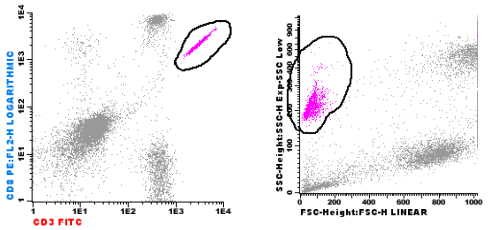
Acquire and store all events possible. It is recommended to acquire at a low or medium speed to avoid cell aggregates. If you cannot acquire the total sample, *acquire no more than 4 minutes without a stir sample.*

Example of CD3/CD8/CD45/CD4:

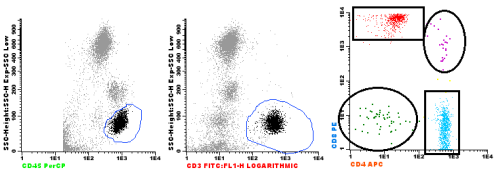
1. Gate the lymphocyte population from an FL3 vs SSC dot plot and adquire an adequate number of events. The population of Beads must be fully contained in this gate for a correct count.



2. Obtain the number of events in the absolute count bead region from an ungated FL1 vs FL2 dot plot.



3. Select the desired population and anoted the number of events.



4. The absolute number of the cell population of interest is determinated by dividing the number of cells of interest acquired by the number of beads acquired (FL1/FL2), and multiplying this result by the microsphere concentration (microsphere concentration is indicated in the label on the tube).

$$\text{Absolute Count of cells (cells/ µl)} = \frac{\text{Nº of event in population cell}}{\text{Nº of event in absolute count bead region}} \times \frac{\text{Nº of beads per test}}{\text{test volume}}$$

Storage

Store in the dark, at 2-8 °C. Do not use after expiration date stamped on vial. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the product is suspected, contact our Technical Services tech@immunostep.com
DO NOT FREEZE. Protect the fluorescent conjugates from the light.

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Performance characteristics

For all tests performed in the technical specifications has been used following combination of antibodies: CD3 FITC/ CD8 PE/ CD45 PerCP/ CD4 APC and CD45 FITC/CD34 PE. Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer, using Cell Quest acquisition software.

Accuracy:

We compared the results for the same sample, using the double platform and Stepcount. The samples are from healthy individuals with no apparent hematologic diseases.
For this study, 64 samples were tested. Data were analyzed to determinate the different between Doble Plaform and Stepcount. The results appear in the Figure 1 and 2

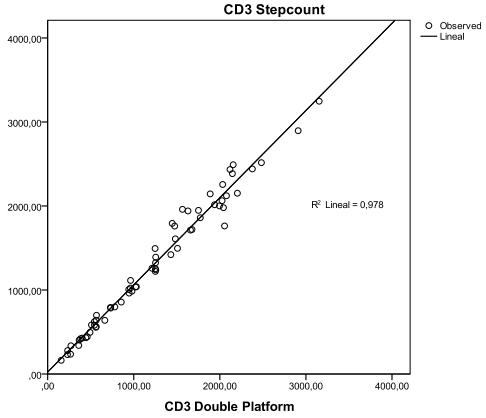


Figure 1. Doble Platform versus Stepcount to CD3+ cells

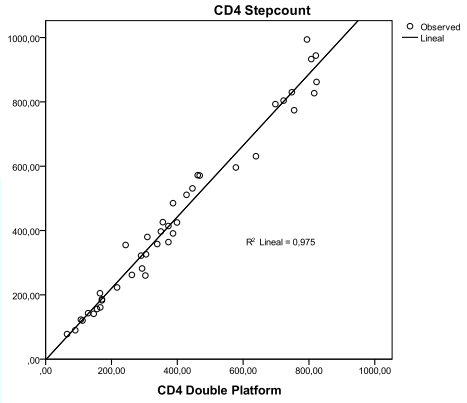


Figure 2. Doble Platform versus Stepcount to CD3+/CD4+ cells

	CORRELATION COEFFICIENT (R^2)	SLOPE	Y-INTERCEPT	95% LOWER CONFIDENCE LIMIT	95% UPPER CONFIDENCE LIMIT
CD3+	0.978	1.03	23.606	-32.820	80.032
CD3+/CD4+	0.975	1.11	-2.10	-28.429	24.113
Cd3+/CD8+	0.979	1.13	-16.585	-60.361	27.191

Figure 3. Doble Platform versus Stepcount to CD3+/CD8+ cells

	CORRELATION COEFFICIENT (R^2)	SLOPE	Y-INTERCEPT	95% LOWER CONFIDENCE LIMIT	95% UPPER CONFIDENCE LIMIT
CD3+	0.978	1.03	23.606	-32.820	80.032
CD3+/CD4+	0.975	1.11	-2.10	-28.429	24.113
CD3+/CD8+	0.979	1.13	-16.585	-60.361	27.191

Reproducibility:

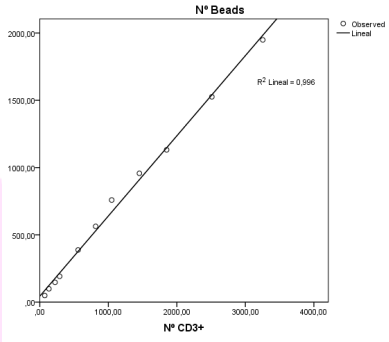
The reproducibility study was performed using three samples with content in lymphocyte high, medium and low respectively and testing each one in 10 different tubes.

	Subset	Mean	Dev. Tip.	Maxim.	Minim.	N total
Low	CD3	136,80	24,38	174	101	10
	CD4	38,40	6,20	45	25	10
	CD8	84,00	14,91	105	66	10
High	CD3	2846,30	248,55	3159	2411	10
	CD4	1598,20	169,72	1867	1340	10
	CD8	938,20	79,54	1051	802	10
Medium	CD3	400,00	35,64	467	344	10
	CD4	139,90	20,83	162	105	10
	CD8	224,00	21,81	269	185	10

Sensitivity or linearity:

To perform the sensitivity study, acquired a different number of beads for the same sample and calculate the number of Cells CD3+.

The sample was a peripheral blood of an individual without hematologic diseases diagnosed.



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	PEARSON CORRELATION	R2	SIGINIFICANCE OF CORRELATION LEVEL
CD3+	0.998	0.996	0.01

Comparison with the reference product in the market (Trucount, BD):

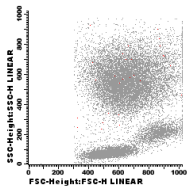
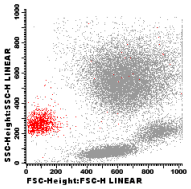
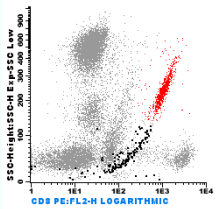
Statistical			
		event. CD34+ (External clinical site)	*event. CD34+* (Stepcount)
N	Valid	17	17
	Missing	0	0
Mean		1368,2353	1469,7388
Median		600,0000	599,27
Variance		3428498,261	3907456,955
Dev. Tip.		1851,62044	1976,72885
Minimum		21,40	22,51
Maximum		6558,00	6755,68
Percentile	25	90,3000	120,5550
	50	600,0000	508,5200
	75	2961,0000	3122,6350

Precision studies were performed comparing the results obtained from an external reference haematological department. For each sample two aliquots were made. The samples include both, leukapheresis and bone marrow sample from human donors.

References.

1. Frank Mandy, Bruno Brando. Enumeration of Absolute Cell Counts Using Immunophenotypic Techniques. Current Protocols in Cytometry. May 2001
2. Bruno Brando, Wolfgang Göhde, Jr. The "Vanishing Counting Bead" Phenomenon: Effect on Absolute CD34 Cell Counting in Phosphate-Buffered Saline-Diluted Leukapheresis Samples. Cytometry 43:154–160 (2001).
3. Jackson AL, Warner NL. Preparation, staining, and analysis by flow cytometry of peripheral blood leukocytes. In: Rose NR, Friedman H, Fahey JL, eds. Manual of Clinical Laboratory Immunology.3rd ed. Washington, DC: American Society for Microbiology; 1986:226-235

Troubleshooting guide.

PROBLEM	SOLUTION
Bead are not detected in the FSC/SSC dot plot. 	1. Decrease the threshold until the bead population appears. 2. Increase the value of FSC 
We can not clearly distinguish the population of areas 	1. Re-adding the lysis solution and allow more time to be sure that the tube has a transparent red color 2. Remove cellular debris by a gate in the region that shows the image 3. Identify the bead region in other fluorescence