CD20 (L27)

Monoclonal mouse anti-human reagent for identification of cells expressing the CD20 antigen

Form	Catalog No.	Form	Catalog No.
FITC	345792	APC	340908
PE	345793	APC-Cy7	335829
PerCP	345794	APC-H7	641414
PerCP-Cy5.5	332781	V450	655872
PE-Cy7	335828		

1/2014 23-5067-05

IVD

CE

Becton, Dickinson and Company
BD Biosciences
2350 Qume Drive
San Jose, CA 95131 USA

EC REP

Benex Limited
Pottery Road, Dun Laoghaire,
Co. Dublin, Ireland
Tel +353.1.202.5222
Fax +353.1.202.5388

BD Biosciences
European Customer Support
Tel +32.2.400.98.95
Fax +32.2.401.70.94
help.biosciences@europe.bd.com

Becton Dickinson Pty Ltd,
4 Research Park Drive,
Macquarie University Research Park,
North Ryde NSW 2113, Australia

Becton Dickinson Limited,
8 Pacific Rise, Mt. Wellington,
Auckland, New Zealand

bdbiosciences.com
ClinicalApplications@bd.com

1. INTENDED USE

CD20 is intended for in vitro diagnostic use in the identification of cells expressing the CD20 antigen, using a BD FACS™ brand flow cytometer.

The flow cytometer must be equipped to detect light scatter and the appropriate fluorescence, and be equipped with appropriate software (such as BD CellQuest™, BD CellQuest™ Pro, BD FACSDiva™, or BD FACSCanto™ clinical software) for data acquisition and analysis. See the cytometer user's guide for instructions.

Applications

Expression of the CD20 antigen in the characterization of hematologic neoplasia.[1]

2. COMPOSITION

CD20, clone L27, is derived from hybridization of mouse Sp2/0 myeloma cells with spleen cells from BALB/c mice immunized with the LB lymphoblastoid cell line. CD20 is composed of mouse IgG_1 heavy chains and kappa light chains.

Each of the following reagents is supplied in phosphate-buffered saline (PBS) containing gelatin and 0.1% sodium azide. Concentrations are listed in Table 1.

Table 1 Bottling concentrations

Form	Amount provided	Conc[a] (µg/mL)
FITC	100 µg in 2.0 mL of PBS	50
PE	25 µg in 1.0 mL of PBS	25
PerCP	50 µg in 2.0 mL of PBS	25
PerCP-Cy™5.5[b]	10 µg in 1.0 mL of PBS	10
PE-Cy™7	50 µg in 0.5 mL of PBS	100

Table 1 Bottling concentrations

Form	Amount provided	Conc[a] (µg/mL)
APC	20 µg in 0.5 mL of PBS	40
APC-Cy7[c]	25 µg in 0.5 mL of PBS	50
APC-H7	50 µg in 0.5 mL of PBS	100
BD Horizon™ V450	50 µg in 0.5 mL of PBS	100

a. Conc = concentration
b. Cy™ is a trademark of GE Healthcare. This product is subject to proprietary rights of GE Healthcare and Carnegie Mellon University, and is made and sold under license from GE Healthcare. This product is licensed for sale only for in vitro diagnostics. It is not licensed for any other use. If you require any additional license to use this product and do not have one, return this material, unopened, to BD Biosciences, 2350 Qume Drive, San Jose, CA 95131, and any money paid for the material will be refunded.
c. APC-Cy7: US Patent 5,714,386

Antibody purity is as follows:

- FITC: ≤5% free fluorophore at bottling, as measured by size-exclusion chromatography (SEC)
- PE, PerCP, PerCP-Cy5.5, PE-Cy7, APC, APC-Cy7, APC-H7, V450: ≤20% free fluorophore at bottling, as measured by SEC

3. STORAGE AND HANDLING

The antibody reagent is stable until the expiration date shown on the label when stored at 2°C–8°C. Do not use after the expiration date. Do not freeze the reagent or expose it to direct light during storage or incubation with cells. Keep the outside of the reagent vial dry.

Do not use the reagent if you observe any change in appearance. Precipitation or discoloration indicates instability or deterioration.

4. REAGENTS OR MATERIALS REQUIRED BUT NOT PROVIDED

- Disposable 12 x 75-mm capped polystyrene test tubes
- Micropipettor with tips
- Vortex mixer
- Centrifuge
- BD FACS™ lysing solution (10X) (Catalog No. 349202)

  For dilution instructions and warnings, see the reagent instructions for use (IFU).
- BD CellWASH™ (Catalog No. 349524) or a wash buffer of PBS with 0.1% sodium azide
- Stain buffer solution consisting of PBS plus 0.2% bovine serum albumin (BSA) with 0.1% sodium azide
- BD CellFIX™ (Catalog No. 340181) or 1% paraformaldehyde (PFA) solution in PBS with 0.1% sodium azide

  Store at 2°C–8°C in amber glass for up to 1 week.
- BD™ Stabilizing Fixative (3X) (Catalog No. 339860)

  For dilution instructions and warnings, see the reagent IFU.
- BD Calibrite™ beads

  See bdbiosciences.com for information.
- BD FACS™ 7-color setup beads (Catalog No. 335775)
- BD™ Multicolor CompBeads (Catalog No. 644204)
- BD FACSDiva™ CS&T IVD beads (Catalog No. 656046 and 656047)

- BD FACS brand flow cytometer

  See the appropriate cytometer user's guide for information.

5. SPECIMEN(S)

Reagents can be used for immunophenotyping by flow cytometry with a variety of specimen types, including peripheral blood, bone marrow aspirates or biopsies, and other body fluids or tissues. Each type of specimen can have different storage conditions and limitations that should be considered prior to collection and analysis.[2,3]

Samples with large numbers of nonviable cells can give erroneous results due to selective loss of populations and to increased nonspecific binding of antibodies to nonviable cells. Viability of samples should be assessed and a cutoff value established. A cutoff value of at least 80% viable cells has been suggested.[2]

WARNING All biological specimens and materials coming in contact with them are considered biohazards. Handle as if capable of transmitting infection[4,5] and dispose of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Wear suitable protective clothing, eyewear, and gloves.

6. PROCEDURE

Staining the samples

1. Add the appropriate volume of CD20 fluorochrome-conjugated monoclonal antibody to 100 µL of whole blood in a 12 x 75-mm capped polystyrene test tube.

   See the appropriate vial label for volume.

2. Vortex gently and incubate for 15 to 30 minutes in the dark at room temperature (20°C–25°C).
3. Add 2 mL of 1X BD FACS lysing solution to each tube.
4. Vortex the tubes gently and incubate for 10 minutes in the dark at room temperature.
5. Centrifuge the tubes at 300*g* for 5 minutes.
6. Aspirate the supernatant.
7. Add 2 mL of buffer to each tube according to the fluorochrome you are using (Table 2). A "Yes" in both columns indicates that either buffer can be used.

Table 2 Recommended buffer

Fluorochrome	Stain Buffer	BD CellWASH
FITC	Yes	Yes
PE	Yes	Yes
PerCP	Not tested	Yes
PerCP-Cy5.5	Yes	Yes
PE-Cy7	Yes	Yes
APC	Yes	Yes
APC-Cy7	Not tested	Yes
APC-H7	Yes	Yes
V450	Not tested	Yes

8. Centrifuge the tubes at 300*g* for 5 minutes.
9. Aspirate the supernatant.
10. Add 0.5 mL of buffer to each tube according to Table 2 and analyze the samples immediately or add fixative according to Table 3.

Adding fixative

1. Add 0.5 mL of fixative according to Table 3. A "Yes" in both columns indicates that either fixative can be used.

   CAUTION For multicolor analysis using a PE conjugate, use BD CellFIX or 1% paraformaldehyde solution.

Table 3 Compatible fixative

Fluorochrome	BD CellFIX (or 1% PFA) solution	1X BD Stabilizing Fixative
FITC	Yes	No
PE	Yes	No
PerCP	Yes	No
PerCP-Cy5.5	Yes	No
PE-Cy7	Yes	Yes
APC	Yes	No
APC-Cy7	Yes	Yes
APC-H7	Yes	Yes
V450	Yes	No

2. Vortex the tubes gently.
3. Incubate the tubes at 2°C–8°C in the dark according to Table 4.

Table 4 Recommended incubation procedure

	BD CellFIX (or 1% PFA) solution	1X BD Stabilizing Fixative
Incubation time	60 minutes	30 minutes
Additional steps	1. Centrifuge the tubes at 300*g* for 5 minutes. 2. Aspirate the supernatant. 3. Add 0.5 mL of stain buffer to each tube and vortex gently.	None

   CAUTION Some APC-Cy7 conjugates, and to a lesser extent PE-Cy7 and APC-H7 conjugates, show changes in their emission spectra with prolonged exposure to paraformaldehyde or light. For overnight storage of stained cells, wash and resuspend in buffer without paraformaldehyde after 1 hour of fixation.

   CAUTION Do not store PE conjugates in BD Stabilizing Fixative because this can cause a decrease in staining intensity.

   CAUTION Prolonged exposure of cells to paraformaldehyde can lead to increased autofluorescence in the violet channels.

4. Mix the tubes thoroughly before analysis.

Store at 2°C–8°C until analyzed. We recommend analyzing within 24 hours of staining.

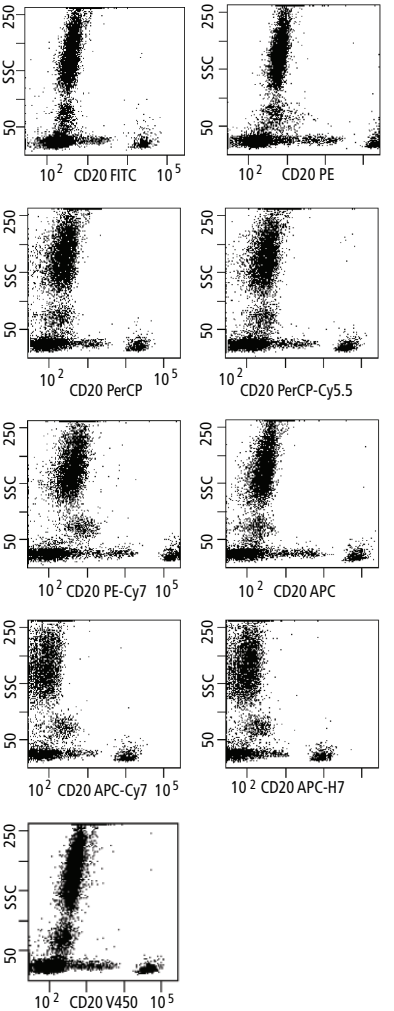
Analytical results

Abnormal numbers of cells expressing this antigen or aberrant expression levels of the antigen can be expected in some disease states. It is important to understand the normal expression pattern for this antigen and its relationship to expression of other relevant antigens in order to perform appropriate analysis.

Flow cytometry

Vortex the cells thoroughly at low speed to reduce aggregation before running them on the flow cytometer.[6] Acquire and analyze list-mode data using appropriate software. Before acquiring samples, adjust the threshold to minimize debris and ensure that the populations of interest are included. Figure 1 displays representative data performed on peripheral blood leucocytes and gated on lymphocytes. Laser excitation is at 405 nm, 488 nm, and 635 nm.

Figure 1 Representative data analyzed with a BD FACS brand flow cytometer



Internal quality control

Cytometer setup depends on the fluorochrome and cytometer type you are using in your experiment. See Table 5 for the setup method appropriate for the cytometer and specific labeled fluorochrome reagent you are using. Setup methods follow Table 5.

Table 5 Setup method recommended for cytometer and fluorochrome combinations

Fluorochrome	BD FACS Canto II	BD FACS Canto	BD FACS Calibur
FITC	1, 2, 4	2	3
PE	1, 2, 4	2	3
PerCP	2	2	3
PerCP-Cy5.5	1, 2, 4	2	3
PE-Cy7	1, 2, 4	2	NA
APC	1, 2, 4	2	3
APC-Cy7	2, 4	2	NA
APC-H7	1, 4	2	NA
V450	4	NA	NA

Setup Method 1

Recommended when using a 6-color reagent combination that includes APC-H7:

1. Use BD FACS 7-color setup beads and BD FACSCanto clinical software to set photomultiplier tube (PMT) voltages and to check cytometer sensitivity.
2. Link the lyse/wash (LW) setup from BD FACSCanto clinical software to an experiment in BD FACSDiva v7.0 or later software.

   See the BD FACSDiva™ Software Reference Manual for Version 7.0 for more information.
3. Select **Overwrite** in the **Cytometer Settings Mismatch** dialog to link PMT voltages.

   This will change the parameter name from **APC-H7** to **APC-Cy7**. Parameters that do not match will be removed.

   CAUTION If you select **Apply** in the **Cytometer Settings Mismatch** dialog, the PMT voltages for APC-Cy7 will not be linked from the LW setup and the current PMT voltage settings and parameter name will not change.
4. Write down the APC-Cy7 parameter PMT voltage, unlink from the LW setup, and change the **APC-Cy7** parameter label to **APC-H7**.
5. Enter the APC-Cy7 PMT voltage you wrote down in the APC-H7 parameter.
6. Use BD Multicolor CompBeads and BD FACSDiva software to set fluorescence compensation.

   See the *BD™ Multicolor CompBeads* IFU for more information.

Setup Method 2

Recommended when using a 6-color reagent combination that includes APC-Cy7:

1. Use BD FACS 7-color setup beads and BD FACSCanto clinical software to set PMT voltages and fluorescence compensation, and to check cytometer sensitivity.
2. Link the LW setup from BD FACSCanto clinical software to an experiment in BD FACSDiva software.

3. Select **Overwrite** in the **Cytometer Settings Mismatch** dialog to link PMT voltages and compensation settings for all parameters. Parameters that do not match will be removed.

   NOTE If you are using the BD default 4-2H-2V laser configuration, selecting **Overwrite** will change the parameter name from **APC-H7** to **APC-Cy7**.

   CAUTION If you select **Apply** in the **Cytometer Settings Mismatch** dialog, only the settings for parameters that match those in the LW setup will be linked. If you are using the 4-2H-2V laser configuration and you select **Apply**, the PMT voltages and compensation settings for APC-Cy7 will not be linked. The parameter name will remain as **APC-H7**.

Setup Method 3

Use BD Calibrite beads and BD FACSComp™ software to set PMT voltages and fluorescence compensation, and to check cytometer sensitivity.

Setup Method 4

Recommended when using an 8-color reagent combination that includes violet fluorochromes:

1. Use BD FACSDiva CS&T IVD beads and BD FACSDiva v7.0 or later software to set PMT voltages. See the BD FACSDiva CS&T IVD beads reagent IFU for more information.
2. Use BD Multicolor CompBeads and BD FACSDiva software to set fluorescence compensation.

See the cytometer user's guides and IFUs for more information.

We recommend running a control sample daily from a normal adult subject or a commercially available whole blood control to optimize cytometer settings and as a quality control check of the system.[7]

7. PERFORMANCE CHARACTERISTICS

Specificity

The CD20 antigen is a phosphoprotein with a molecular weight of 35 or 37 kilodaltons (kDa), depending on the degree of phosphorylation.[8] The antigen is not glycosylated.[8]

The CD20 antigen is expressed on B lymphocytes synchronous with the expression of surface IgM.[8,9] The antigen is present on both resting and activated B lymphocytes but is lost before differentiation into plasma cells.[8] The CD20 antigen is found in both mantle-zone and germinal-center areas of secondary follicles of lymphoid tissue and can be expressed on follicular dendritic cells (FDCs) in germinal centers.[8] Low-level expression of the CD20 antigen has been detected on a subpopulation of T lymphocytes.[10]

Sensitivity

Sensitivity is defined as resolution of the $CD20^+$ population from the $CD20^-$ population. Sensitivity was measured by evaluating a range of antibody concentrations. Each concentration of reagent was tested on whole blood cells. The separation of $CD20^+$ from $CD20^-$ was determined for each sample and averaged within each concentration. The bottled antibody concentration for each reagent provided optimum sensitivity in resolving the $CD20^+$ cells from the negative. See Table 1.

Reproducibility

CD20 was submitted to the Third International Workshop and Conference on Human Leucocyte Differentiation Antigens. Participating laboratories evaluated clone L27 as part of a blind panel of antibodies and reported consistent results.[11]

Repeatability

To determine the repeatability of staining with each reagent, samples were stained with multiple lots of reagents. The different samples used in the evaluation provided an average mean fluorescence intensity (MFI) value as shown in Table 6. For each sample, two different lots of reagents generated a pair of results. Individual standard deviations (SDs) were determined from the paired results for each sample. Individual SDs were combined to derive a pooled SD for each reagent that provides an estimate of within-sample repeatability.

Table 6 Repeatability of MFI of B lymphocytes across different lots and across multiple donors (N)

Fluorochrome	N[a]	Average MFI	Pooled SD	Pooled %CV[b]
FITC	8	520.2	58.46	11.24
PE	3	2,773.9	304.98	10.99
PerCP	28	542.0	51.84	9.57
PerCP-Cy5.5	5	2,055.9	418.67	20.37
PE-Cy7	4	2,181.5	192.15	8.81
APC	3	4,063.5	1,368.09	33.67
APC-Cy7	4	1,205.3	137.41	11.40
APC-H7[c]	64	9,639.3	315.77	3.28
V450[c]	64	26,746.7	480.6	1.8

a. N = number of samples
b. CV = coefficient of variation
c. Data collected as median fluorescence intensity.

Accuracy

Accuracy data was collected on BD FACScan™ and BD FACSCalibur™ flow cytometers using BD CellQuest software, v3.1 or later, to determine $CD20^+$ lymphocytes. Results from CD20 PerCP-Cy5.5 reagent were compared to results from CD20 PE reagent. See Table 7.

Table 7 Regression analysis of CD20 PerCP-Cy5.5 reagent vs CD20 PE reagent

Fluor[a]	N[b]	r	Slope	Intercept	Range (%)
CD20	53	0.97	0.91	–0.01	3.2–29.4

a. Fluor = fluorochrome
b. N = number of samples

8. LIMITATIONS

- For In Vitro Diagnostic Use.
- Conjugates with brighter fluorochromes (PE, APC) will give greater separation than those with other dyes (FITC, PerCP). When populations overlap, calculation of the percentage of cells positive for the marker can be affected by the choice of fluorochrome.
- Use of monoclonal antibodies in patient treatment can interfere with recognition of target antigens by this reagent. This should be considered when analyzing samples from patients treated in this fashion. BD Biosciences has not characterized the effect of the presence of therapeutic antibodies on the performance of this reagent.
- Single reagents can provide only limited information in the analysis of leukemias and lymphomas. Using combinations of reagents can provide more information

than using the reagents individually. Multicolor analysis using relevant combinations of reagents is highly recommended.[3]

- Since reagents can be used in different combinations, laboratories need to become familiar with the properties of each antibody in conjunction with other markers in normal and abnormal samples.
- Reagent performance data typically was collected using EDTA-treated blood. Reagent performance can be affected by the use of other anticoagulants.

WARRANTY

Unless otherwise indicated in any applicable BD general conditions of sale for non-US customers, the following warranty applies to the purchase of these products.

THE PRODUCTS SOLD HEREUNDER ARE WARRANTED ONLY TO CONFORM TO THE QUANTITY AND CONTENTS STATED ON THE LABEL OR IN THE PRODUCT LABELING AT THE TIME OF DELIVERY TO THE CUSTOMER. BD DISCLAIMS HEREBY ALL OTHER WARRANTIES, EXPRESSED OR IMPLIED, INCLUDING WARRANTIES OF MERCHANTABILITY AND FITNESS FOR ANY PARTICULAR PURPOSE AND NONINFRINGEMENT. BD'S SOLE LIABILITY IS LIMITED TO EITHER REPLACEMENT OF THE PRODUCTS OR REFUND OF THE PURCHASE PRICE. BD IS NOT LIABLE FOR PROPERTY DAMAGE OR ANY INCIDENTAL OR CONSEQUENTIAL DAMAGES, INCLUDING PERSONAL INJURY, OR ECONOMIC LOSS, CAUSED BY THE PRODUCT.

TROUBLESHOOTING

Problem	Possible Cause	Solution
Poor resolution between debris and lymphocytes	Cell interaction with other cells and platelets	Prepare and stain another sample.
	Rough handling of cell preparation	Check cell viability; centrifuge cells at a lower speed.
	Inappropriate instrument settings	Follow proper instrument setup procedures; optimize instrument settings as required.
Staining dim or fading	Cell concentration is too high at the staining step	Check and adjust the cell concentration or sample volume; stain a fresh sample.
	Insufficient reagent	Repeat staining with increased amount of antibody.
	Cells not analyzed within 24 hours of staining	Repeat staining with fresh sample; analyze promptly.
	Improper medium preparation (sodium azide omitted)	Use sodium azide in staining medium and washing steps.
Few or no cells	Cell concentration is too low	Resuspend fresh sample at a higher concentration; repeat staining and analysis.
	Cytometer malfunctioning	Troubleshoot the cytometer.

REFERENCES

1. Marti GE, Faguet G, Bertin P, et al. CD20 and CD5 expression in B-chronic lymphocytic leukemia. *Ann NY Acad Sci*. 1992;651:480-483.
2. Rothe G, Schmitz G. Consensus protocol for the flow cytometric immunophenotyping of hematopoietic malignancies. *Leukemia*. 1996;10:877-895.
3. Stelzer GT, Marti G, Hurley A, McCoy P Jr, Lovett EJ, Schwartz A. US-Canadian consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: standardization and validation of laboratory procedures. *Cytometry*. 1997;30:214-230.
4. *Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline–Third Edition*; Wayne, PA: Clinical and Laboratory Standards Institute; 2005. M29-A3.
5. Centers for Disease Control. Perspectives in disease prevention and health promotion update: universal precautions for prevention of transmission of human immunodeficiency virus, hepatitis B virus, and other bloodborne pathogens in health-care settings. *MMWR*. 1988;37:377-388.

6. 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.
7. *Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition*. Wayne, PA: Clinical and Laboratory Standards Institute; 2007. H42-A2.
8. Dörken B, Möller P, Pezzutto A, Schwartz-Albiez R, Moldenhauer G. B-cell antigens: CD20. In: Knapp W, Dörken B, Gilks WR, et al, eds. *Leucocyte Typing IV: White Cell Differentiation Antigens*. New York, NY: Oxford University Press; 1989:46-48.
9. Loken MR, Shah VO, Dattilio KL, Civin CI. Flow cytometric analysis of human bone marrow. II. Normal B-lymphocyte development. *Blood*. 1987;70:1316-1324.
10. Hultin LE, Hausner MA, Hultin PM, Giorgi JV. CD20 (Pan-B cell) antigen is expressed at a low level on a subpopulation of human T lymphocytes. *Cytometry*. 1993;14:196-204.
11. Ling NR, Maclennan ICM, Mason DY. B-cell and plasma cell antigens: new and previously defined clusters. In: McMichael AJ, ed. *Leucocyte Typing III: White Cell Differentiation Antigens*. New York, NY: Oxford University Press; 1987:302-335.