BCP-ALL-MRD

EuroFlow

BCP-ALL
Minimal Residual Disease
Panel

Pacific Blue™	OC515™	FITC	PE	PerCP-Cyanine5.5	PE-Cyanine7	APC	APC-C750
CD20	CD45	CD81	CD66c/ CD123	CD34	CD19	CD10	CD38
CD20	CD45	CD81	CD73/ CD304	CD34	CD19	CD10	CD38

Ref: CYT-BCP-ALL-MRD

For research use only

BCP-ALL-MRD VIALS ARE A LYOPHILIZED PRODUCT. READ CAREFULLY THE FOLLOWING INSTRUCTIONS FOR RECONSTITUTION:

The lyophilized Minimal Residual Disease B-cell precursor Acute Lymphoblastic Leukemia (BCP-ALL-MRD) panel preserves the stability of the pre-mixed combination of antibodies. Reconstitute each lyophilized vial containing the pre-mixed combinations with distilled water as follows:

- Tube 1: 300 µl distilled water.
- Tube 2: 300 µl distilled water.

Mix well and let the solution at least 30 minutes.
Unused volume of reconstituted vial is stable during four weeks from date of reconstitution if it is stored in the dark at 2-8º C.

INTENDED USE

BCP-ALL-MRD panel is a two tube pre-mixed 8 colour antibody combinations designed for the accurate identification and discrimination of B-cell precursor (BCP) acute lymphoblastic leukemia (ALL) cells from normal/reactive BCP in bone marrow samples from treated BCP-ALL patients.
This reagent must be used by flow cytometry qualified personal.

SUMMARY AND EXPLANATION

In general, independently of the pathology, MRD is defined by the presence, even at low frequencies, of residual malignant cells in post therapeutic patients [1].

It has been shown that analysis of MRD is a powerful prognostic factor in ALL at diagnosis and relapse as well as before and after transplantation. The level of MRD has an important impact over patients outcome and overall survival. In the particular case of BCP-ALL, those patients with MRD levels <0.01% had a 5-year event-free survival higher than those with MRD levels between 0.01% and 0.1% [2].

MRD flow cytometry monitoring has demonstrated to be faster, cheaper and at least as sensitive and specific as the currently molecular MRD analysis based on RQ-PCR of rearranged Ig/TCR genes [3].

EuroFlow consortium has defined a combination of antibodies as the best suited method for MRD detection in BCP-ALL divided in two 8-color antibody tubes [3]. CD19, CD10, CD20, CD34, and CD45 were defined as backbone meanwhile CD66c, CD123, CD73 and CD304 have been selected because their frequent abnormal expression in BCP-ALL. CD66c is associated with Philadelphia chromosome and its expression in BCP-ALL patients combined with BCR-ABL positivity and hyperdiploid are important diagnosis and prognostic factors for BCP-ALL [4, 5]. The aberrant expression of CD123 has been shown relevant in MRD diagnosis (81.5% of ALL with hyperdiploid karyotype showed strong CD123 overexpression) [6]. Independently, CD73 and CD304 have shown an overexpression in BCP-ALL patients [7, 8]. In combination with the backbone markers, CD81 and CD38 were selected because of its better separation capacity between normal and BCP-ALL cells [3].

A prerequisite to improve sensitivity in MRD analysis is the acquisition of large number of cells. In this way, our fixative free ammonium chloride erythrocyte lysing solution, BulkLysis™, is the optimum reagent to obtain the best use of the sample.

REAGENT COMPOSITION

Material included

BCP-ALL-MRD kit is supplied as 2 different 8-color combinations containing enough volume for 20 tests containing:

- Fixative free ammonium chloride erythrocyte lysing solution (BulkLysis™).
- Following reagents for specific tandem compensation ready to use in liquid format sufficient for 5 tests (5µl/test):
  - CD45-OC515™

- CD34-PerCP-Cyanine5.5
- CD19-PE-Cyanine7
- CD38-APC-C750

- Tube 1 contains the following lyophilized antibodies mixture for surface staining (4 lyophilized vials of 5 tests each one):
  - Anti-human CD20-Pacific Blue™ antibody, clone 2H7, isotype: IgG2b.
  - Anti-human CD45-OC515™ antibody, clone: HI30, isotype: IgG1.
  - Anti-human CD81-FITC antibody, clone: M38, isotype: IgG1.
  - Anti-human CD66c-PE/ CD123-PE antibody, clone: KOR-SA3544 / AC145, isotype: IgG1/IgG2a.
  - Anti-human CD34-PerCP-Cyanine5.5 antibody, clone 581, isotype: IgG1.
  - Anti-human CD19-PE-Cyanine7 antibody; clone: HIB19, isotype: IgG1.
  - Anti-human CD10-APC antibody; clone: HI10a, isotype: IgG1.
  - Anti-human CD38-APC-C750 antibody; clone: LD38, isotype: IgG1.

- Tube 2: contains the following lyophilized antibodies mixture for surface staining (4 lyophilized vials of 5 tests each one):
  - Anti-human CD20-Pacific Blue™ antibody, clone 2H7, isotype: IgG2b.
  - Anti-human CD45-OC515™ antibody, clone: HI30, isotype: IgG1.
  - Anti-human CD81-FITC antibody, clone: M38, isotype: IgG1.
  - Anti-human CD73-PE/ CD304-PE antibody, clone: AD-2 / 12C2, isotype: IgG1/IgG2a.
  - Anti-human CD34-PerCP-Cyanine5.5 antibody, clone 581, isotype: IgG1.
  - Anti-human CD19-PE-Cyanine7 antibody; clone: HIB19, isotype: IgG1.
  - Anti-human CD10-APC antibody; clone: HI10a, isotype: IgG1.
  - Anti-human CD38-APC-C750 antibody; clone: LD38, isotype: IgG1.

	Fluorochrome	Pacific Blue™	OC515™	FITC	PE	PerCP-Cyanine5.5	PE-Cyanine7	APC	APC-C750
Tube 1	Antibody	CD20	CD45	CD81	CD66c/CD123	CD34	CD19	CD10	CD38
	Clone	2H7	HI30	M38	KOR-SA3544 / AC145	581	HIB19	HI10a	LD38
	Isotype	IgG2b	IgG1	IgG1	IgG1 / IgG2a	IgG1	IgG1	IgG1	IgG1
Tube 2	Antibody	CD20	CD45	CD81	CD73 / CD304	CD34	CD19	CD10	CD38
	Clone	2H7	HI30	M38	AD-2 / 12C2	581	HIB19	HI10a	LD38
	Isotype	IgG2b	IgG1	IgG1	IgG1 / IgG2a	IgG1	IgG1	IgG1	IgG1

All components contains sodium azide (NaN_3) ≤0.09% (m/v). Reagents are not considered sterile.

Material required but not included

- 3 laser-equipped flow cytometer (8 colors) and appropriate computer hardware and software.
- Test tubes suitable for obtaining samples in the flow cytometer used. Usually tubes with a rounded bottom for 6 ml, 12x 75 mm are used.
- Automatic pipette and tips
- 50 ml tubes
- Chronometer
- Vortex Mixer
- Centrifuge
- Washing buffer: phosphate buffered saline (PBS) containing ≤0.09% (m/v) sodium azide and bovine serum albumin (BSA) 0,5% (m/v).

STORAGE CONDITIONS

The reagent is stable until the expiration date shown on the label, when stored at 2-8º C. The reagents should not be frozen or exposed to direct light during storage or during incubation with sample. Keep all reagent vials in a dry place. Once opened, the vial must be stored in a vertical position to avoid any possible spillage.

WARNINGS AND RECOMMENDATIONS

1. For research use only.
2. Alteration in the appearance of the reagent, such as the precipitation or discoloration indicates instability or deterioration. In such cases, the reagent should not be used.
3. It contains ≤0.09% (m/v) sodium azide (CAS-Nr. 26628-22-8) as a preservative, but even so care should be taken to avoid microbial contamination of reagent or incorrect results may occur.
   Indication(s) of danger:

H302	Harmful if swallowed
Safety advice:	
P264	Wash thoroughly after handling.
P270	Do not eat, drink or smoke when using this product.
P301+P312	If swallowed, call a poison center or doctor/physician if you feel unwell.
P301+P330	If swallowed, rinse mouth.
P501	Dispose of contents/container in accordance with local/regional/national/international regulation.

4. All patient specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection [9], 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.
5. Use of the reagent with dilutions, incubation times or temperatures different from those recommended may cause erroneous results. Any such changes must be validated by the user.
6. Any serious incident relating to the product must be reported to Cytognos S.L. as well as the competent professional authority of the Member State in which the user is established.

PROCEDURE

Preparation

Sample must be collected in commercially available anticoagulant-treated tube (use of EDTA is recommended) [10, 11].

1. Determine the absolute count of leukocytes per µl of the sample to be processed.
2. Transfer the sample containing **at least** 10 x 10^6 nucleated cells for each MRD tube to be stained to a 50 ml tube (consider that during BulkLysis process some cells are unselectively lost). Do not use more than 2 ml of sample per 50 ml of lysing solution. If larger volumes of sample need to be processed (i.e. starting cells concentration is low), use several 50 ml tubes.
3. Fill the tube up to reach 50 ml volume with BulkLysis™ (CYT-BL) (diluted to 1X in distilled water and at room temperature - RT).
4. Mix well and incubate for 15 min in a roller or sample-shaker device.
5. Centrifuge at 800 g for 10 min and remove the supernatant using a Pasteur pipette or a vacuum system without disturbing the cell pellet. Typically, 300 µl of cell suspension should remain in the tube.
6. Add 2 ml of PBS + 0,09% (m/v) of NaN_3 + 0,5 % (m/v) of BSA and resuspend the cell pellet vigorously.
7. Complete the volume of the tube containing the cell suspension up to 50 ml final volume with PBS + 0,09% (m/v) of NaN_3 + 0,5 % (m/v) of BSA.
8. Mix well.
9. Centrifuge at 800 g for 5 min and remove the supernatant using a Pasteur pipette or a vacuum system, without disturbing the cell pellet.
10. Resuspend the cell pellet in 2 ml of PBS + 0,09% (m/v) of NaN_3 + 0,5 % (m/v) of BSA. Mix well and transfer this volume to a 5 ml "FACS tube".
11. Wash the 50 ml Falcon tube with 2 mL of PBS + 0,09% (m/v) of NaN_3 + 0,5 % (m/v) of BSA more to recover cells that might have left in the original tube. Add this volume to the 5 ml tube containing the rest of the sample transferred in step 10.
12. Centrifuge at 540 g for 5 min and remove the supernatant by decanting or using a Pasteur pipette. If the remaining cell volume is lower than 300 µl, PBS + 0,09% (m/v) of NaN_3 + 0,5 % (m/v) of BSA will be added to reach a volume of at least 300 µl.
13. In case multiple 50 ml tubes were used (because it was needed to lyse large sample volumes) the cell suspensions from the same sample should be combined at this moment, before adjusting cell concentration. Try to keep the final volume low, so that, in case that cell concentration needs to be adjusted as indicated in the next step, it can be easily done by diluting with the recommended buffer.
14. Adjust the final cells concentration to 1 x 10^5 cells/µl, by resuspending the pellet with PBS + 0,09% (m/v) of NaN_3 + 0,5 % (m/v) of BSA.
15. Adjust the volume in order to obtain 100 µl containing 10 x10^6 cells of the cell suspension per each tube to be stained/acquired.

Staining steps for BCP-ALL-MRD Tube1:

Reconstitute the lyophilized vial using 300 µl distilled water, mix well and place it in a roller for at least 30 minutes.
Add 50 µl of antibody mixture. Then add the cell suspension (10 x 10^6 cells per tube).
Mix well. For optimal staining conditions, if needed, complete with PBS until a final volume of 200 µl.
Incubate for 30 min at RT protected from light.
Add 2 ml of fixative lysing solution.
Mix well.
Incubate for 10 min at RT protected from light.
Centrifuge for 5 min at 540 g.
Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 100 µl residual volume in each tube.
Add 2 ml of PBS+0,5% (m/v) BSA + 0,09% (m/v) NaN_3 to the cell pellet.
Mix well.
Centrifuge for 5 min at 540 g.

Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 100 µl residual volume in each tube.
Resuspend the cell pellet in 500 µl PBS + 0,5% (m/v) BSA (without NaN_3).
Acquire the cells immediately after staining or (if not immediately acquired) store at 4°C (for 1h maximum) until measured in the flow cytometer.
Acquire the sample in medium flow rate.

Staining steps for BCP-ALL-MRD Tube2:
Reconstitute the lyophilized vial using 300 µl distilled water, mix well and place it in a roller for at least 30 minutes.
Add 50 µl of antibody mixture. Then add the cell suspension (10 x 106 cells per tube).
Mix well. For optimal staining conditions, if needed, complete with PBS until a final volume of 200 µl.
Incubate for 30 min at RT protected from light.
Add 2 ml of fixative lysing solution.
Mix well.
Incubate for 10 min at RT protected from light.
Centrifuge for 5 min at 540 g.
Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 100 µl residual volume in each tube.
Add 2 ml of PBS+0,5% (m/v) BSA + 0,09% (m/v) NaN_3 to the cell pellet.
Mix well.
Centrifuge for 5 min at 540 g.
Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 100 µl residual volume in each tube.
Resuspend the cell pellet in 500 µl PBS + 0,5% (m/v) BSA (without NaN_3).
Acquire the cells immediately after staining or (if not immediately acquired) store at 4°C (for 1h maximum) until measured in the flow cytometer.
Acquire the sample in medium flow rate.

Important Recommendations
In order to achieve optimal results, it is needed to follow the EuroFlow Standard Operating Protocol for Cytometer Setup [12]. You will find a complete guide (Cytometer Setup SOP) on the web site www.EuroFlow.org, which includes recommendations for FSC, SSC and target voltage PMT settings, compensation setup and instrument performance monitoring.

Flow cytometry analysis
Cytognos recommends the use of the analysis software Infinicyt™ [13], which provides a revolutionary approach for data integration and multidimensional analysis of flow cytometry data. Its innovative features make the analysis and interpretation of the results easier, faster and more accurate. Infinicyt™ comprises exclusive tools that allow for a better identification and description of the different cell populations. It is being developed as part of the EuroFlow™ project "Flow cytometry for fast and sensitive diagnosis and follow-up of haematological malignancies", in which highly qualified cytometry professionals are involved. Infinicyt™ includes a wide variety of analysis tools and exclusive graphics which allow analysis with a reference picture, or calculate new virtual parameters. You will find complete information about Infinicyt™ on the web site: www.infinicyt.com.

LIMITATIONS
It is advisable to acquire stained samples as soon as possible to optimize results. Non-viable cells may show unspecific staining. Prolonged exposure of samples to lytic reagents may cause white cell destruction and targeted population cell loss.
When using whole blood lysing procedures some red blood cells may not lyse, for instance if there are nucleated red blood cells or if abnormal protein concentration and haemoglobinopathies are observed. This may cause misleading results since unlysed red blood cells are counted as leucocytes.
Results obtained by flow cytometry may be erroneous if cytometer laser is misaligned or if gates are incorrectly set.
Knowledge of antigen normal expression pattern and its relation to other relevant antigens is paramount to carry out an adequate analysis.

QUALITY CONTROL
- Pipettes precision and cytometer calibration should be verified to obtain optimal results.
- In multicolour panels, fluorochromes emit in wavelengths that can show certain spectral overlap which must be corrected by electronic compensation. Optimal compensation levels can be established by analysing cells from healthy individuals stained with mutually exclusive monoclonal antibodies conjugated with appropriate fluorochromes.
- This product has been manufactured in accordance with standards of production and quality system of the ISO 13485:2012 standard.

REFERENCES

1. Andy C. Rawstron, J. Anthony Child, Ruth M. de Tute, Faith E. Davies, Walter M. Gregory, Sue E. Bell, Alexander J. Szubert, Nuria Navarro-Coy, Mark T. Drayson, Sylvia Feyler, Fiona M. Ross, Gordon Cook, Graham H. Jackson, Gareth J. Morgan, and Roger G. Owen. Minimal Residual Disease Assessed by Multiparameter Flow Cytometry in

Multiple Myeloma: Impact on Outcome in the Medical Research Council Myeloma IX Study. J of Clinical Oncology. 31(20): 2540-2547 (2013).
2. Borowitz MJ, Wood BL, Devidas M, Loh ML, Raetz EA, Salzer WL, et al. Prognostic significance of minimal residual disease in high risk B-ALL: a report from Children's Oncology Group study AALL0232. Blood 2015; 126 (8):964-971.
3. Theunissen P, Mejstrikova E, Sedek L, van der Sluijs-Gelling AJ, Gaipa G, et al. Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. Blood 2016; pii: blood-2016-07-726307.
4. Kalina T, Vaskova M, Mejstrikova E, Madzo J, Trka J, Stary J, et al. Myeloid antigens in childhood lymphoblastic leukemia: clinical data point to regulation of CD66c distinct from other myeloid antigens. BMC Cancer 2005; 5:38.
5. Kiyokawa N, Iijima K, Tomita O, Miharu M, Hasegawa D, Kobayashi K, et al. Significance of CD66c expression in childhood acute lymphoblastic leukemia. Leuk Res 2014; 38 (1):42-48.
6. Djokic M, Bjorklund E, Blennow E, Mazur J, Soderhall S, Porwit A. Overexpression of CD123 correlates with the hyperdiploid genotype in acute lymphoblastic leukemia. Haematologica 2009; 94 (7):1016-1019.
7. Wang W, Gao L, Li Y, Li ZL, Gong M, Huang FZ, et al. The application of CD73 in minimal residual disease monitoring using flow cytometry in B-cell acute lymphoblastic leukemia. Leuk Lymphoma 2015:1-8.
8. Solly F, Angelot F, Garand R, Ferrand C, Seilles E, Schillinger F, et al. CD304 is preferentially expressed on a subset of B-lineage acute lymphoblastic leukemia and represents a novel marker for minimal residual disease detection by flow cytometry. Cytometry A 2012; 81 (1):17-24.
9. Protection of Laboratory Workers from occupationally acquired infections. Second edition; approved guideline (2001). Villanova PA: National Committee for Clinical Laboratory Standards; Document M29-A2.
10. Procedures for the collection of diagnostic blood specimens by venipuncture- approved standard; Fifth edition (2003). Wayne PA: National Committee for Clinical Laboratory Standards; Document H3-A5.
11. Clinical applications of flow cytometry: Quality assurance and immunophenotyping of lymphocytes; approved guideline (1998). Wayne PA: National Committee for Clinical Laboratory Standards; Document H42-A.
12. Kalina T, Flores-Montero J, van der Velden VHJ, Martin-Ayuso M, Böttcher S, Ritgen M, et al. EuroFlow standardization of flow cytometry instrument settings and immunophenotyping protocols. Leukemia, 2012 Sep; 26(9):1986-2010. doi:10.1038/leu.2012.122.
13. Cytognos. 2011. Infinicyt™ Flow Cytometry Software. Version 1.7. Cytognos S.L. Salamanca, Spain. http://www.infinicyt.com

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 (YYYY-MM)
	Storage temperature limitation
	Keep out of sunlight
	Consult instructions for use
	For research use only
	Batch code
	Catalogue number
	Manufacter

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
www.cytognos.com