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Louis Waeckel, Anne Kennel, Anne-emmanuelle Berger, Claude Lambert. Human leukocyte antigen-B27 typing by flow cytometry: Comparison of three CE-IVD methods. *Cytometry Part B: Clinical Cytometry*, 2022, 104 (6), pp.460-467. 10.1002/cyto.b.22102 . hal-04888912

**HAL Id: hal-04888912**

**<https://hal.science/hal-04888912v1>**

Submitted on 15 Jan 2025

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# Human leukocyte antigen-B27 typing by flow cytometry: Comparison of three CE-IVD methods

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## Abstract

**Background:** The human leukocyte antigen B27 (HLA-B27), associated with chronic inflammatory diseases is thus widely performed for diagnostic purposes. Genotyping by molecular biology is the current gold standard for HLA-B27 typing, but cheaper and faster flow cytometry methods have been developed.

**Methods:** In this report, we compare analytical performances of three CE-IVD flow cytometry kits: IOTest™ and DuraClone B27™ from Beckman Coulter and BD HLA-B27™ from BD Biosciences on a Navios™ cytometer as compared to molecular biology.

**Results:** Routine analyses could conclude for HLA-B27 in 197 patients (23.2%) and was not conclusive for 66 patients (7%, 8%). The experience revealed the needs to complete IOTest™ with a lineage marker (like CD3-APC) and a standardization procedure in detection of fluorescence. Comparative analysis considering 23/44 non-conclusive samples as negative, pointed out a 100% sensitivity and specificity of IOTest™ in determining genetically proved HLA-B27. Specificity of the BD HLA-B27™ kit was 94% (two false positives) sticking to the manufacturer instructions but raised to 100% and 94% sensitivity with a cutoff at 8.5 (225 on FACSDiva's scale).

**Discussion:** The DuraClone B27™ specificity was poor using the manufacturer cutoff but raised to 100% with a 8.0 cutoff instead of 15.

**Conclusion:** The three flow cytometry kits displayed satisfying performances but need adjustments. The DuraClone B27™ kit seems to be the best while the IOTest™ kit is not conclusive in 8% of cases. In most cases the use of molecular biology can be spared, but genotyping remains essential for patients whose HLA-B27 status cannot be determined with confidence by flow cytometry.

## KEYWORDS

HLA-B27 typing flow cytometry

## 1 | INTRODUCTION

The human leukocyte antigen B27 (HLA-B27) allele is strongly associated with ankylosing spondylitis and other chronic inflammatory

diseases (Berntson et al., 2013; Sibley, 2016). HLA-B27 prevalence in Caucasians ranges from 3% to 8% (Coates) but reaches up to 90% in patients suffering from ankylosing spondylitis (van der Linden & van der Heijde, 1995), IBD-associated spondyloarthritis (Mielants &

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Veys, 1985; Peluso et al., 2013), juvenile rheumatoid arthritis (Berntson et al., 2013; Rumsey et al., 2021), psoriatic arthritis (Queiro et al., 2016; Winchester & FitzGerald, 2020) reactive arthritis (Abou-Elella et al., 1995) or acute anterior uveitis (Chang et al., 2005). HLA-B27 screening is thus widely performed for diagnostic purposes.

The gold standard methods for HLA typing are microlymphocytotoxicity and molecular biology. However, they are costly, time-consuming and only available in highly specialized laboratories. Alternatively, whole blood HLA-B27 immunotyping by flow cytometry is faster and cheaper (Zeng et al., 2018). Leukocytes are identified according to their light scattering properties and according to their expression of specific surface markers such as CD45 (pan-leukocytes) or CD3 (T cells). The surface expression of HLA-B27 is detected using conjugated anti-B27 monoclonal antibodies, preferentially on CD45 bright lymphocytes or on CD3+ T cells as they display homogeneous and reproducible HLA-B27 fluorescence intensities.

But, microlymphocytotoxicity and flow cytometry experiments have shown that HLA-B27 is part of the HLA-B7 cross-reactive group (CREG) together with HLA-B13, B16, B17, B22, B37, B41, B42, B47, B48, B54, B55, B56, B60, B61, B73 and B81. Few monoclonal antibodies are commercially available for the immunodetection of HLA-B27 by flow cytometry. The most common clones are HLA-ABC-m3, GS145.2 and FD705. Unsurprisingly, they all cross-react with the HLA-B7 antigen although the FD705 clone exhibits the lowest cross-reactivity. (Fuller et al., 1990; Rodey et al., 1994; Wade et al., 2007). As a consequence, cells expressing HLA-B7 are partially labeled with anti-B27 antibodies, thus mimicking HLA-B27 expression. HLA-B7 being one of the most frequent alleles in Caucasians (González-Galarza et al., 2015), cross-reactivity between B27 and B7 might reveal problematic as it may lead to numerous false HLA-B27 positives.

Few CE-IVD approved flow cytometry kits are available for HLA-B27 immunotyping. Each one uses a different strategy to overcome this B7 caveat, whether by using anti-HLA-B7 antibodies and/or defining decision thresholds based on anti-HLA-B27 fluorescence intensities (MdfI) (Zeng et al., 2018) However, their respective analytical performances have never been compared.

In our clinical cytometry laboratory, we routinely type HLA-B27 using the CE-IVD IOTest™ kit (Beckman-Coulter; Fullerton, CA; ref: A07739). Two other CE-IVD flow cytometry kits are currently available for HLA-B27 immunotyping: the BD™ HLA-B27 (BD Biosciences, San Jose, CA; ref:340183) and the DuraClone B27™ (Beckman Coulter, ref:B36862). In this report, we sought to share our clinical experience with the IOTest™ kit and to compare its analytical performances of all three kits with the gold-standard molecular biology based typing.

## 2 | METHOD

### 2.1 | Samples selection

#### 2.1.1 | Routine immunophenotyping

In a first step, using the IOTest™ kit (Beckman-Coulter) we checked the distribution of HLA-B27 typing and inconclusive tests out of

848 tests performed on the basis of routine analysis for diagnosis, over a period of 24 months (2018–2019) in Saint-Étienne university hospital.

#### 2.1.2 | Comparison of the three CE-IVD flow cytometry kits

Then 44 samples from 40 patients and four external quality assessments (EQA) were prospectively selected to compare performances of the three flow cytometry kits. The selection was based on their IOTest™ routine test results. To ensure sufficient numbers of positive and inconclusive samples, we selected 11 clearly HLA-B27 positive and 23 inconclusive samples together with 10 clearly HLA-B27/HLA-B7 negative patients. Patient group was composed of 35 males and five females, and the mean age (+/- 1 SD) was of 55 ± 18 years old (ranging from 17 to 85 years old). All 44 samples were simultaneously tested by molecular biology and by the two other flow cytometry kits (BD™ HLA-B27 and DuraClone B27™).

### 2.2 | HLA typing by molecular biology

HLA typing by molecular biology was used as a reference method for the evaluation. HLA-B typing was performed with various molecular biology techniques depending on the initial clinical request: either NGS (Omixon® Kit Holotype 96/B) or SSO-PCR (OneLambda® Kit locus B HD) or SSP-PCR (Olerup® Kit Blow or B\*27) by the national HLA typing institute (Établissement Français du Sang Auvergne-Rhône-Alpes) according to the international guidelines. HLA typing results are expressed according to the WHO serological nomenclature.

### 2.3 | HLA typing by flow cytometry

HLA-B27 flow cytometry typing was performed on EDTA-anticoagulated whole blood using the three CE-IVD flow cytometry kits according to the respective manufacturer's instructions (Table 1):

IOTest™ HLA-B27-FITC/HLA-B7-PE (Beckman-Coulter, reference: A07739)

- Whole blood (50 µl) was incubated with 10 µl of a ready-to-use mix of anti-HLA-B27-FITC (clone HLA-ABC-m3 IgG2a) and anti-HLA-B7-PE (clone BB7.1 IgG1). This is a proportional adaptation of the 20 µl Antibody mix for 100 µl Blood as recommended by the manufacturer instructions. Saturation of each labelling was confirmed by testing serial ½ dilutions on one HLA-B7-B27+ and one HLA-B7 + B27- samples (not shown). An anti-CD45-ECD (2 µl, clone J33 IgG1, reference: A07784), not provided in the kit, was added in order to facilitate discrimination between leukocytes and residual red blood cells. After incubation for 15 minutes at room temperature in the dark, erythrolysis was performed using Versalyse™ lysing solution (reference: A09777) for 10 min (alternatively,

**TABLE 1** Comparative protocols and results of three CE-IVD commercially available kits for human leukocyte antigen-B27 (HLA-B27) immunotyping by flow cytometry. Reagents, protocols and performances of the tests were compared. MdFI (median fluorescence intensity)

	Beckman coulter IOTest™ B27	Beckman coulter DuraClone B27™	BD HLA-B27™
References	A07739	B36862	340,183
Leukocyte marker	None (CD45-ECD is not included in the kit)	CD3 PE-Dyomic649 (IgG1κ UCHT1)	CD3-PE (IgG1κ SK7)
Anti-HLA-B27	FITC-conjugated IgG2a HLA-ABC-m3 Liquid	PE-conjugated IgG2a HLA-ABC-m3 + IgG2b FD705 Lyophilized	FITC-conjugated IgG1κ GS145.2 Liquid
Anti-HLA-B7	PE-conjugated IgG1 BB7.1 Liquid	Unconjugated IgG1 BB7.1 Lyophilized	None
Calibration beads	None	Lyophilized	Liquid, used daily
Blood volume	50 µl	25 µl	50 µl
Incubation	20 min at room temp	20 min at room temp	20 min at room temp
Erythrolysis	Versalyse 10 min	Duralyse 15 min	FACS Lysing 10 min
PBS washing	4 ml then 300 g 10 min	None	4 ml then 300 g 10 min
<b>Flow cytometry HLA-B27 phenotyping</b>			
<b>HLA B27 negative genotype (n = 33)</b>			
HLA B7- B27- (n = 10)	10 B27 negative	10 B27 negative	10 B27 negative
HLA-B27 MdFI	0.34 [0.10–11.1]	1.82 [1.44–3.6]	0.66 [0.40–1.7]
HLA-B7 MdFI	1.6 [0.9–2.0]	nd	nd
HLA B18+ B81+ (n = 1)	1 inconclusive	1 B27 negative	1 B27 negative
HLA-B27 MdFI	3.28	3.45	2.04
HLA-B7 MdFI	5.76	nd	nd
HLA B7+ B27- (n = 22)	22 inconclusive	22 B27 negative	20 B27 negative
HLA-B27 MdFI	6.1 [1.9–22.4]	4.05 [2.88–7.64]	2.5 [1.0–4.9]
HLA-B7 MdFI	17.1 [10.2–33.7]	nd	nd
			2 B27 positive 6.9 & 8.3
<b>HLA B27 positive genotype (n = 11)</b>			
HLA B7- B27+ (n = 11)	11 B27 positive	11 B27 positive	11 B27 positive
HLA-B27 MdFI	39.0 [26.9–151]	16.3 [8.1–37.8]	10.3 [5.95–22.9]
HLA-B7 MdFI	1.6 [0.5–4.4]	nd	nd
HLA-B27 sensitivity	100%	100%	100%
HLA-B27 specificity	100%	100%	94%

the manufacturer instructions proposes another lysing solution as an example: e IOTest 3 Lysis Solution; Ref. A07799). The labelling was followed by washing in 4 ml of PBS; 300 g; 10 min and resuspension of the pellet in PBS-BSA 5%. Cell fixation, is another option proposed by the manufacturer instructions, we did not use as the labeled samples were always analyzed within 2 h.

BD™ HLA-B27 (BD Biosciences, reference: 340183)

- Whole blood (50 µl) was incubated with 30 µl of a ready-to-use mix of anti-HLA-B27-FITC (clone GS145.2 IgG1κ) and anti-CD3-PE (clone SK7 IgG1κ) for 15 min at room temperature in the dark. Then erythrolysis was performed using BD FACS Lysing Solution™

provided in the kit, for 10 min, followed by washing in 4 ml of PBS; 300 g; 10 min and paraformaldehyde 1% fixation. B530 fluorescence detection was calibrated daily using calibration beads provided in the kit.

DuraClone B27™ (Beckman-Coulter, reference: B36862)

- Whole blood (25 µl) was added to a DuraClone B27™ tube containing a dried ready-to-use mix of B575 fluorescent calibration beads and the following antibodies: two anti-HLA-B27-PE (clones FD705 IgG2b and HLA-ABC-m3 IgG2a), an anti-CD3-PE-Dyomics649 (clone UCHT1 IgG1κ) and an unconjugated anti-HLA-B7 (clone BB7.1 IgG1). The mix was then incubated for 30 min at

room temperature in the dark. Then, erythrolysis was performed using DuraLyse™ solution for 15 min. No washing step was performed (no-wash protocol). Note that, since the study has started, this kit has been temporarily removed from the market for quality assurance reasons.

All samples were analyzed within 4 h on the Navios™ cytometer using Navios™ software. Compensation of spectral overlaps were performed using VersaComp™ antibody capture beads (Beckman-Coulter, reference: B22804). Cytometer settings were checked daily using Flow-Check Pro™ and Flo-Set Pro™ fluorospheres (Beckman-Coulter, references: A63493 and A63492) according to the manufacturer's instructions and to our ISO 15189 procedures.

## 2.4 | Flow cytometry gating strategies

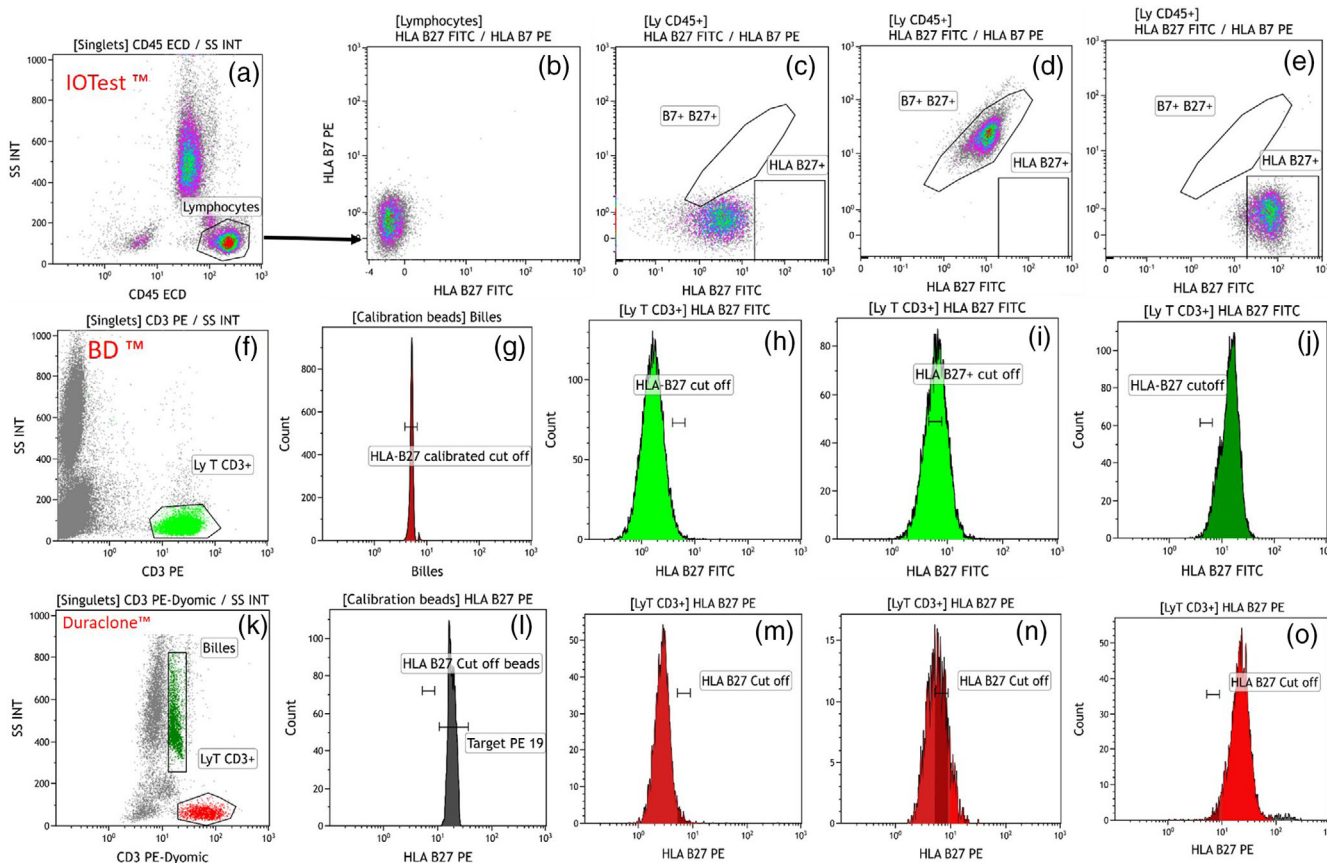
Sample and erythrolysis quality was checked on the Side Scatter (SSC)/Forward Scatter (FSC) dot plot and acquisition stability was checked on a time/SSC dot plot. Cell doublets were excluded using the FSC area/FCS height plotting. For each kit, representative gating strategy of HLA-B27 expression is depicted in Figure 1.

### 2.4.1 | IOTest™ HLA-B27-FITC/HLA-B7-PE

Lymphocytes were gated using a CD45-ECD/SSC dot plot (Figure 1a), and their HLA-B27 and HLA-B7 expression was analyzed on a HLA-B27-FITC/HLA-B7-PE dot plot (Figure 1b-e). HLA-B27 type was concluded when lymphocytes express high levels of HLA B27-FITC and no HLA-B7-PE. Double positive samples suggest the expression of HLA-B7 with the expression of HLA-B27 remaining undetermined. Some samples presented with moderate fluorescence intensity of HLA-B27-FITC (higher than HLA-B27 negative samples but lower than true HLA-B27 positive samples) and no HLA-B7-PE signal, suggesting other cross reactivities of HLA-B27-FITC antibody.

### 2.4.2 | Bd HLA-B27™

T lymphocytes were gated using a CD3-PE/SSC dot plot (Figure 1f) and HLA-B27 expression was standardized using fluorescent calibration beads provided in the kit. HLA-B27 positivity cut-off (Figure 1g) is provided by the manufacturer. For the lot we were using, the cut-off was a median fluorescence at 138 units for FACSCanto™ scale that is based on a 256 channels. The cut-off was not provided for a 10 channels based Beckman-Coulter Navios™ scale and we evaluated



**FIGURE 1** Representative gating strategies for human leukocyte antigen-B27 (HLA-B27) typing using the IOTest™, BD™ or DuraClone™ kits. Examples of negative (c, h, m), inconclusive (d, i, n) and positive (e, j, o) HLA-B27 are shown. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

it at 5.4 using a correction factor of 25.6 according to Solly (Solly et al., 2013). HLA-B27 MdFI was determined on CD3+ T cells population (Figure 1h-j). The manufacturer indicates a 10 channel zone (on BD Biosciences FACSCanto™ scale corresponding to values from 5.0 to 5.8 on Navios scale) around the decision threshold named “gray zone” where HLA-B27 positivity or negativity cannot be established with certainty because of cross reactivities to HLA-B7-related CREG.

### 2.4.3 | DuraClone B27™

T cells were selected on a CD3 PE-Dyomics649 (B695/30 nm)/SSC dot plot (Figure 1k). Beads were used to standardize the detection settings at target values of  $19 \pm 0.5$  for B575 and  $15 \pm 0.5$  for B675 given by the manufacturer (Figure 1l). A minimum of 3000 beads were acquired. HLA labeling was analyzed on a HLA-B27-PE (B575/30 nm) histogram (Figure 1m-n). Values in the “indeterminate zone”, defined by the manufacturer between 15 and 23, were not conclusive.

Flow cytometric data were analyzed using the Kaluza™ software (Beckman-Coulter) and statistics were performed with Microsoft™ Excel software. Results were expressed as median fluorescence intensity (MdFI)  $+ / - 1$  standard deviation and compared using Student's *t* test.

## 3 | RESULTS

### 3.1 | Routine immunophenotyping of HLA-B27 using the IOTest™ kit

Over a period of 2 years, 848 samples were routinely tested using the IOTest™ kit. One hundred ninety seven (23.2%) samples were immunotyped as HLA-B27 and 592 (69.8%) samples came out as HLA-B27 negative. The HLA-B27 status of 66 (7.8%) samples could not be determined because of double labeling with anti-HLA-B27 and anti-HLA-B7. Comparatively from HLA typing National Institute data base, 7.1% had HLA-B27 and 17.1% had HLA-B7 genotypes in the population from the same geographical area. Other most frequent genotypes from the CREG groups were the HLA-B13 (4.1%), HLA-B41 (2.2%), HLA-B55 (3.5%) while the remaining genotypes represented less than 1% of the population. In our routine analysis, 504 patients were females (59.4%) and 344 were males (40.6%), with a mean ( $+ / - 1$  SD) age of  $46.7 \pm 17.7$  years ranging from 1 to 91 years old. The majority of patients (59.1%) came from rheumatology and 25.9% of them were found HLA B27 positive. Other patients came from internal medicine (17% of the tests, with 18.1% of them being HLA B27 positive) and from ophthalmology (11% of the tests, with 30.1% being HLA B27 positive).

### 3.2 | Genotyping of 44 samples selected on IOTest™ (Table 1)

We have selected 11 samples positive on IOTest™. All were HLA-B27 confirmed on genotyping and none had HLA-B7 genotype (B27+ /

B7-) but one was B27 and B60 from the CREG group. Among the 23 samples selected because they were inconclusive on IOTest™, 22 had HLA-B7 genotype, one having HLA-B7+ / B13+ and one HLA-B7+ / B60 genotypes of CREG group. One had a HLA-B81+ CREG genotype with HLA-B18+ .

Among the 10 samples HLA-B27 negative on IOTest™, all were confirmed to express neither HLA-B27 nor B7 genotype but two had HLA-B13 and one had HLA-B81 CREG genotypes.

### 3.3 | IOTest™ kit immunolabeling shows little overlapping of fluorescence intensities regarding the genotypes

HLA-B27 immunolabeling was strong (HLA-B27 FITC MdFI 39.0 from 26.9 to 151) in the 11 samples of HLA-B27 genotype and was very low for HLA-B7 PE (MdFI 1.6 from 0.5 to 4.4) in complete agreement with the genotyping results. The immunodetection of HLA-B27 (0.34 from 0.10 to 11.1) and HLA-B7 (1.6 from 0.9 to 2.0) were negative on the 10 samples without HLA-B27 or HLA-B7 genotypes.

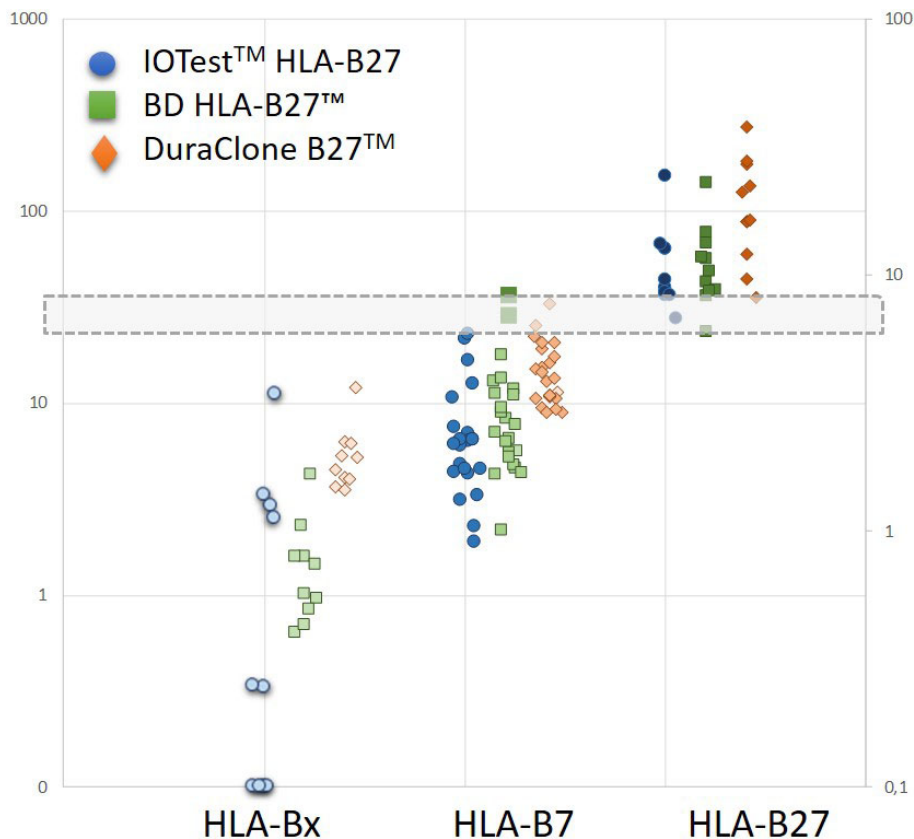
On the other hand, the 22 samples with HLA-B7 genotype, had intermediate levels of HLA-B27 immunolabeling (MdFI 6.1, from 1.9 to 22.4) significantly higher than HLA-B27 negative samples ( $p = 0.039$ ). These samples had a high HLA-B7 immunolabeling (17.1 from 10.2 to 33.7) while the one with HLA-B18+ / B81+ genotype had intermediate expression of HLA-B27 (MdFI 3.28) and HLA-B7 (MdFI 5.76). In sum, IOTest™ sensitivity was 100% and specificity was 100% as compared to molecular genotyping.

### 3.4 | Comparison of 44 samples selected on different flow cytometry kits (Table 1 and in Figure 2)

#### 3.4.1 | BD™ HLA-B27 kit

Thirteen samples came out HLA-B27 positive as their MdFI values of HLA-B27 FITC were above the cut-off of 5.8 that includes the “gray zone” according to the manufacturer. This included the 11 samples with the HLA-B27 genotype (MdFI 10.3 from 5.95 to 22.9). Two more samples had HLA-B27 intensities (MdFI 6.9 and 8.3) above the threshold but were HLA-B27 negative and HLA-B7 positive genotypes. Raising the cut off at 8.5, excludes all false positive at the cost bringing two real HLA B27 samples in the gray zone (respective MdFI = 5.95 and 8.19). Thirty one samples came out HLA-B27 negative as their HLA-B27 FITC MdFI values were below the cut-off of 5.4, including the 20 HLA-B7 positive samples, the 10 HLA-B27 negative / B7 negative samples and the one HLA-B81 positive. The HLA-B27 fluorescence intensity in HLA-B7 negative samples was low (MdFI = 0.66 from 0.40 to 1.7). Comparatively, the HLA-B27 MdFI had intermediate levels for the HLA-B81 sample (MdFI = 2.04) as well as for the 20 HLA-B7 samples (2.5 from 1.0 to 4.9). Compared to genotyping, sensitivity was 100% and specificity was 94% because of the two falsely positive samples.

**FIGURE 2** Human leukocyte antigen-B27 (HLA-B27) median fluorescence intensity (MdFI) values according to the flow cytometry kit and the HLA-B27/B7 genotype status ( $n = 44$ ). The x-axis corresponds to the HLA-B27 and HLA-B7 genotype status. The two y-axes correspond to the MdFI values. The gray rectangle corresponds to a proposed corrected gray zone for the BD HLA-B27™ kit (MdFI: 5.4–8.5) and for the DuraClone B27™ kit (MdFI: 6.5–8) on a 10 channels scale. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



### 3.4.2 | DuraClone B27™ kit

Using this method, 36 samples had HLA-B27 fluorescence intensity below 15 (the manufacturer cut-off including 33 HLA-B27 negative and 3 HLA B27 positive). Three more HLA B27 positive sample were below 23, the “indeterminate zone”. All samples with HLA-B27 fluorescence intensity above 23 were confirmed HLA-B27.

However, from preliminary analysis, we set up our own cut-off at eight. Using this cut-off, 11 samples were determined as HLA-B27 positive (B27 MdFI: 16.3 from 8.1 to 37.8) and 33 samples were determined as HLA-B27 negative, in complete agreement with the genotyping results.

Among the 33 samples qualified as HLA-B27 negative, the 22 samples with a B7 genotype displayed a higher HLA-B27 MdFI (4.05; from 2.9 to 7.6) compared to the 10 HLA-B7 negative samples (1.82 from 1.4 to 3.6) but still below the cut off. The sample with HLA-B81 genotype was not distinct (MdFI 3.45) from HLA-B27- B7- samples.

In sum, sensitivity and specificity were 100% and the kit never failed to conclude on the HLA-B27 status in this short group of tests. Still, a gray zone could be proposed from 6.5 to 8 instead of 15–23 as proposed by the manufacturer, on Navios scale (corresponding to 165–205 on FACSCanto scale).

## 4 | DISCUSSION

HLA-B27 typing by flow cytometry is faster and cheaper than genotyping but faces the issue of cross-reactivities between some HLA proteins

that share epitopes. In the IOTest kit™, cross-reactivity between HLA-B7 and HLA-B27 is monitored using monoclonal anti-HLA-B7 and anti-HLA-B27 antibodies respectively conjugated with PE and FITC.

Thus HLA-B27 type can be concluded with high confidence in case of single labelling of HLA-B27 at high intensity without HLA-B7 detection. As anti-HLA-B27 antibodies also bind to HLA-B7 or eventually to other HLA-B7 CREG molecules like HLA-B81, although the labelling intensity is lower as compared to HLA-B27 labelling, HLA-B27 cannot be surely excluded and must be checked in doubtful samples by alternative methods. Fortunately, this only concerns less than 10% of the routinely samples tested. We can expect that HLA-B27+/B7+ would have higher anti HLA-B27 labelling and could be distinguished from HLA-B27+/B7- profile but this combination is rare and we did not have the chance to check this with the genotype.

The HLA-B27 can be excluded in HLA-B27 negative labelling with good confidence but this require some precaution. Indeed, using the IOTest™ kit, we occasionally observed some false negative results. By measuring HLA-B27 labelling on the lymphocytes area defined on FSC-SSC dot plots, this can concern a great majority of red blood cells in cases where erythrolysis is not complete as it happens occasionally with some samples. For this reason, the IOTest™ kit, certainly lacks of precision and we recommend to add to the CE-IVD procedure a lineage marker such as CD3, preferentially conjugated with a fluorochrome that has minimal spectral overlap with FITC and PE, like APC as an example. In order to improve the quality assurance of the test that strongly relies on fluorescence intensity, we also recommend to add a standardization process of fluorescence detections by using calibrated beads daily.

With all these precautions, HLA-B27 can be confirmed in single positive labelling and confidently excluded in double positive labelling, avoiding the need for molecular biology testing in more than 90% of samples. In any cases there are doubts, like cases where HLA B27 fluorescence intensity is at intermediate level making decision difficult to make with high confidence (gray or indeterminate zone) and specially in case of double positive labelling with anti HLA-B7, it is then recommended to go for a check on an alternative method in order to assure a good specificity of the test.

Comparatively, the BD™ HLA-B27 kit, gives as good results as the IOTest™ although it is based on only a single labelling of HLA-B27. The analysis focus on the T cell population assuring a homogeneous labelling. The fluorescence detection is standardized using beads and fluorescence intensity targets that are provided in the kit. The decision threshold refers to a cutoff defined by the manufacturer, with a gray zone of incertitude. Unfortunately, the cutoff is only defined for instrument from the same company. Using a recent work, we could convert the cutoff value to the scale of detection of Beckman Coulter instruments, using a converting factor. Our results show that the gray zone should be larger, from 5.4 to 8.5 on Navios scale corresponding to 135–225 on FACSDiva's scale when fluorescence detection is standardized according to the manufacturer instructions.

Comparatively, DuraClone B27™ kit discriminates efficiently HLA-B27+ samples based on HLA-B27 labelling intensity that is internally standardized, knowing also that cross reactivity to HLA B7 is prevented with unconjugated anti-HLA-B7 antibody. In our experience, we got good results considering a cut-off value at 8.0 instead of 15 as proposed by the manufacturer. This needs to be validated on a larger number of samples.

HLA-B27 typing by flow cytometry possesses some caveats to bear in mind:

1 - Red Blood Cells (RBC) that do not express HLA molecules can outnumber lymphocytes. Lymphocytes and RBC are indistinguishable on a SSC/FSC dot plot, making the gating of lymphocytes difficult in case of partial erythrolysis. Thus, HLA-B27 expression on lymphocytes may falsely come out as negative. This explains the absolute need for using a lineage labelling, and CD3 is the most appropriate as it is used in two commercial kits. The analysis must then focus on a minimal number of CD3+ T cells.

2 - In most cases, HLA-B27 expression is clearly positive or negative. Still, there are cases where HLA-B27 positivity is equivocal. HLA-B7 CREG molecules can cross-react with anti-HLA-B27 antibodies, leading to false positivity. As an example, we showed that HLA-B81, a B-7 CREG protein, also interfere with the HLA-B27 but not with HLA-B7 staining. In cases of double labelling, anti HLA-B27 labelling of HLA-B7 molecules is less intense and could be differentiated from real HLA-B27 labelling. But other samples do have intermediate HLA-B27 labelling and no HLA-B7 labelling. The reason why needs to be explained but anti HLA-B7 labelling clearly do not exclude all CREG molecules other than HLA-B27 labelling. So, in this test, it is critical to consider HLA-B27 fluorescence intensity, explaining the need for standardization of fluorescence detection.

3 - But still, on daily practice and despite all mentioned precautions, there are always equivocal few cases with fluorescence intensity at intermediate level. Some manufacturers propose a “gray” or “indeterminate”, zone. Our results show that considering the gray zone provided by the manufacturer, few false positives were detected. As the aim of this test is to spare as many expensive tests as possible but taking minimal risks in false positive or false negative, we consider reasonable to take a wider “zone of risks” or gray zone as compared to the one proposed by the manufacturer. Samples falling into this fluorescence intensity zone should be considered as non-conclusive and referred to verification by micro-lymphotoxicity or molecular biology. This “zone of risks” must be determined not only by testing confirmed HLA-B27+ and HLA-B27 negative samples but also other HLA types from the HLA-B7 CREG that give cross reactivities to HLA-B27 antibody with intermediate fluorescence intensities.

Regarding the anti-HLA-B27 clones, Levering et al. (Levering et al., 2005) reported that GS145.2 clone, used in the BD™ HLA-B27 kit was more specific than HLA-ABC-m3 used in IOTest™ but this does not appears to be determinant in flow cytometry assays. This might explain the added value of using two clones in DuraClone B27™ (FD705 and HLA-ABC-m3).

In conclusion, our study demonstrates that the three HLA-B27 typing kits by flow cytometry are reliable for the diagnostic in 90% of the cases, sparing the more time consuming and more expensive use of molecular biology that remains necessary in any doubt on labelling intensity. The DuraClone B27™ kit appears to be the most efficient provided the cutoff is reduced as compared to what is provided by the manufacturer while the IOTest™ kit requires addition of a lineage selection and a standardization of fluorescence for Quality Assurance .

## ACKNOWLEDGMENT

To Adeline DEFAY and Nadine BARDEL for technical help in the analyses.

## CONFLICT OF INTEREST

Authors declare having no conflict of interest in performing and publishing this study.

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**How to cite this article:** Waeckel, L., Kennel, A., Berger, A.-E., & Lambert, C. (2023). Human leukocyte antigen-B27 typing by flow cytometry: Comparison of three CE-IVD methods. *Cytometry Part B: Clinical Cytometry*, 104(6), 460–467. <https://doi.org/10.1002/cyto.b.22102>