



Streamlining basophil activation testing to enable assay miniaturization and automation of sample preparation

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Conclusions: A whole blood based and simplified procedure for BAT is proposed. It relies on a dry antibody

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formulation technology and requires only a few manual steps to be completed. This procedure can also be transposed in a 96 well plate format, fully automated and miniaturized, when sample volume reduction, throughput increase or unattended sample preparation is required.

1. Introduction

The prevalence of allergy has increased significantly over the last two decades (Sanchez-Borges et al., 2018) and it is now considered that allergy represents a considerable health and economic burden worldwide. Both adults and children are affected and higher rates are usually observed in countries with a westernized lifestyle. As an example, the prevalence of oral food challenge (OFC)-proven allergy before the age of 5 is 3.6% in Denmark, 4% in the United Kingdom and Australia, and 6.8% in Norway (Loh and Tang, 2018). While the exact reasons of such an increase are not fully understood yet, a deeper understanding of the involved mechanisms has led to advances in research for allergy prevention and management. As a result, recommendations for allergy prevention (Kumfer and Commins S.P. Primary Prevention of Food Allergy, n.d.; Togias et al., 2017; Muraro et al., 2014) have been evolving and a multitude of therapeutic avenues are currently being tested (Burks et al., 2017; Nadeau et al., 2011; Wood et al., 2016; Dantzer and Wood, 2018; Gasser and Eggel, 2018; Orengo et al., 2018).

As a direct result of these trends, the need for tests for both initial diagnosis of allergy and its monitoring during immunotherapy has been increasing. The allergy testing toolbox is composed by a variety of approaches, either *in vivo* or *in vitro*. Among them, allergen challenges (oral, nasal etc...) remain the gold standard despite the fact that severe reactions may occur and that they are not exempt of falsely negative or positive results (Van Erp et al., 2014; Van der Valk et al., 2015; Sicherer and Sampson, 2018). Skin prick tests (SPT) and allergen specific IgE (sIgE) measurements, which reflect IgE sensitization are also broadly

used in the field but their sensitivity and specificity are not always satisfactory. Indeed, even if sensitization is needed for an allergic reaction to occur, it is not always sufficient to lead to a clinical reaction as high sIgE levels do not always mean that a person will experience allergic reactions (van Veen et al., 2013; Beyer et al., 2015). In contrast, it is also possible that a person with undetectable or very low sIgE titers experiences severe allergic reactions (Sicherer and Sampson, 2018; Lopez de Oliveira et al., 2013).

The basophil activation test (BAT) is another path to allergy testing. Based on the use of flow cytometry, it is an *ex vivo* functional test that enables the study of basophil reactivity against a given allergenic source. Leveraging the use of whole blood as a starting sample, basophil reactivity is studied in BAT with not only cellular players but also many additional soluble components (IgG4s, IgA, metabolites, cytokines, etc.) (Santos et al., 2015b). Acknowledging that BAT provides complementary information to the IgE sensitization tests, the relevance and the utility of BAT have already been established and abundantly demonstrated in the literature (Hoffmann et al., 2015; Santos et al., 2014; Santos et al., 2015a; Santos and Shreffler, 2017; Brandstrom et al., 2015; Sénéchal et al., 2019; Klingebiel et al., 2019; Santos and Lack, 2016). In spite of this, its use in routine practice or clinical research is currently hampered by practical aspects such as the required flow cytometry expertise and the cumbersomeness of existing workflows. An additional specificity of BAT is the need for dose-responses as an important prerequisite to gather the full capabilities of the assay, thereby opening the way to the determination of basophil sensitivity (Hoffmann et al., 2015). Therefore, and depending on the type of laboratories

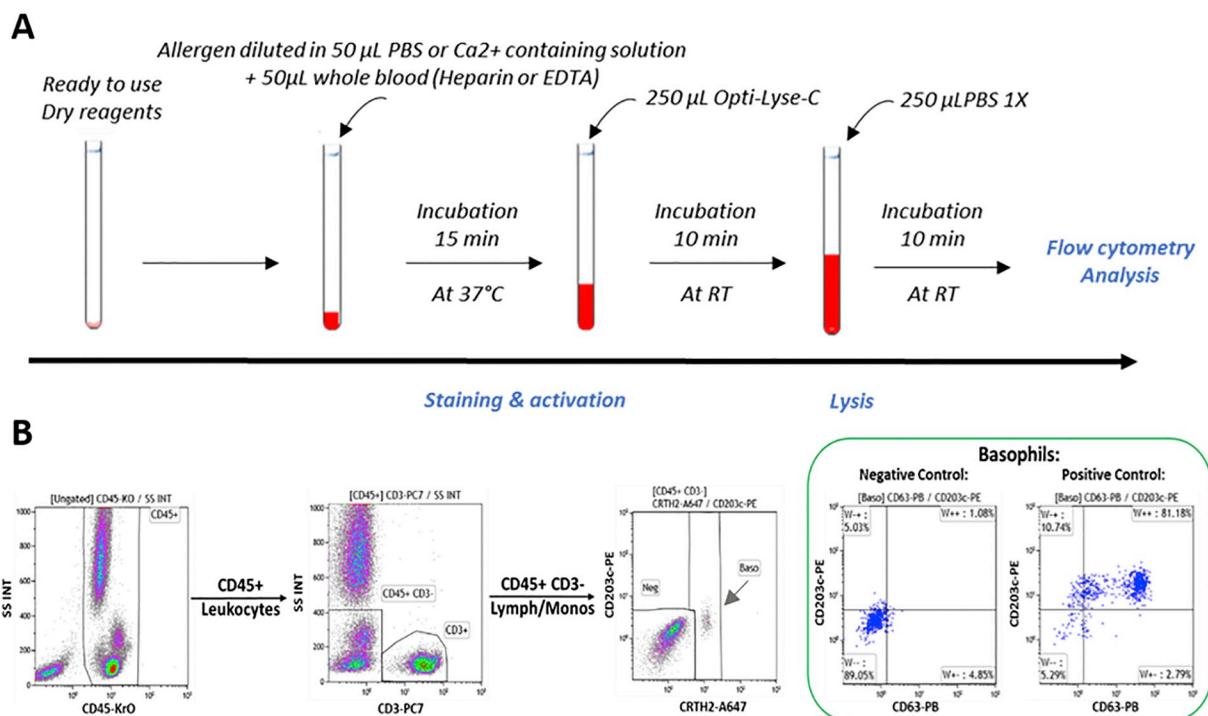


Fig. 1. Schematic of streamlined BAT workflow and gating strategy.

(A): BAT procedure, relying on only 4 pipetting steps and without the implementation of any centrifugation step. In the case of heparinized whole blood, only PBS is used as the level of Ca²⁺ does not need to be adjusted back to physiological conditions.

(B): On leukocytes (CD45+) T Cells are excluded from the low SS population of interest and basophils identified with anti-CRTH2 staining. The basophil activation phenotype is finally characterized with CD203c and CD63.

concerned, the implementation of BAT testing can be cumbersome. As an added limitation, it is important to mention the volume of blood required, especially when pediatric patients are considered and/or when it is envisioned to test multiple allergen sources on the same blood sample, in a similar fashion as in specific IgE based component resolved diagnosis (van Hage et al., 2016).

As a consequence of these different points, rather than being used throughout the field of allergology, BAT remains limited to specialized laboratories. This represents a major limitation and it is generally acknowledged that a broader distribution and further use of BAT would highly benefit the allergy testing and research field (Santos and Shreffler, 2017; Santos and Lack, 2016). One of the most important questions and expectations around the use of BAT in initial diagnosis and/or allergy monitoring is whether BAT could someday be considered as a potential surrogate to OFC (Santos et al., 2014; Santos et al., 2015a; Santos and Lack, 2016). If so, a broader use of BAT would constitute a valuable path toward decreasing the number of OFC performed every year.

In this context, we set out to improve the common procedures for flow cytometry-based BAT assays. The first objective was to design a simplified, standardized but yet powerful BAT workflow that could be compatible with flow cytometry naïve or non-expert users and with the requirements of clinical research. Leveraging these foundations, it was subsequently demonstrated that the developed procedure could be miniaturized and fully automated on a 96 well plate format.

2. Materials and methods (788 words)

2.1. Flow cytometry experiments

BAT prototype assays from Beckman Coulter (Marseille, France) were used throughout the study. A proprietary dry reagent formulation technology was used to prepare ready-to-use and room temperature stable reagents. As a function of the experiments, flow cytometry staining reagents (CD45-KrO (Clone J33), CD3-PC7 (Clone UCHT1), CRTH2-A647 (Clone BM16), CD203c-PE (Clone 97A6) and CD63-PB (CLB-Gran/12) all from Beckman Coulter, Marseille, France) alone or in combination with either anti-IgE (Clone E124.2.8, Beckman Coulter, Marseille, France) or allergen extracts from raw allergenic sources (milk, pellitory, peanut or pollen cypress protein extracts) were dried together in the considered test tubes or 96 well plates. Water-soluble protein extracts of milk, Curly® (peanut containing snack from Vico, Vic-sur-Aisne, France) and cypress pollen were prepared in-house and used throughout the study. Herein, Curly® has been chosen as the peanut source as it is one of the peanut containing food commonly used for OFC in our department. Milk extract was prepared by dissolving the desired quantity of skimmed and dried milk from Régilait (Saint-Martin-Belle-Roche, France) in phosphate buffered saline (PBS). For Curly® extract, the starting material was first reduced into powder using a pestle and a mortar. Water soluble materials, including proteins were then extracted into PBS, pH = 7.4 by overnight rotation at 4 °C and the supernatant finally recovered post-incubation after centrifugation (10,000 g, 4 °C, 20 min). For cypress, *Cupressus sempervirens* (Cups) pollen grains (10%, w/v) (Allergon AB, Ångelholm, Sweden) were incubated overnight in PBS, pH = 7.4, at 4 °C under rotation. After centrifugation (18,000 g, 4 °C, 20 min), the supernatant was dialyzed against distilled water and lyophilized. The lyophilized extract was dissolved in distilled water and stored as aliquots at −20 °C until use. Protein concentration (about 2 mg.mL^{−1}) was evaluated by Bradford method (Bradford MM) according to the manufacturer's recommendations. A commercial pellitory extract (from Bühlmann Laboratories, AG, Switzerland) was also used for some experiments.

Peripheral blood samples were collected into EDTA tubes and stored at 18–25 °C until sample preparation. BAT was performed less than 24 h after blood drawing. For each whole blood sample, considered conditions (control conditions, test conditions, replicates, dry and liquid

reagents, etc.) were always assayed in parallel.

2.1.1. Manual BAT procedure

The fully optimized protocol is depicted in Fig. 1A. Dry reagents were first reconstituted by tube vortexing with 50 µL of a calcium containing buffer (Activation buffer, CE, Beckman Coulter, Marseille, France) and 50 µL of EDTA anticoagulated whole blood. The activation solution contains enough calcium to set back the level of free calcium to physiological conditions (see supplementary information). It also contains heparin to avoid coagulation but no IL-3 as it would influence the basal level of CD203c (Bühning et al., 2004). Heparinized blood could conversely be used, however, in that case, no activation buffer is required and is replaced by PBS. Tubes were placed at 37 °C in a water bath for 15 min to allow cell staining and activation. While shorter or longer durations could also be used, herein, a 15 min incubation time was chosen as it does provide a suitable compromise to reach both a reduced turnaround time and satisfactory basophil activation levels (Fig. S2). Erythrocytes were subsequently lysed by the addition of 250 µL of lysis reagent (Opti-Lyse-C, CE-IVD, Beckman Coulter, Marseille, France) and incubated 10 min at room temperature (18–25 °C). 250 µL of PBS were added to each tube and a final 10 min incubation period was performed to ensure proper lysis before analysis on a Navios (3 lasers, 10 colors, CE-IVD) Beckman Coulter flow cytometer (Indianapolis, USA). 575/30, 755/LP, 660/20, 450/50 and 550/40 optical filters have been used for PE, PC7, A647, PB and KrO respectively with PE and PC7 excited at 488 nm, A647 at 638 nm and PB and KrO at 405 nm. Of note, panel design and gating procedure were optimized so that no centrifugation step before flow cytometry analysis nor compensation for data interpretation would be required. Although a Navios instrument has been used in this experiment, any other instruments presenting at least 3 lasers and an appropriate filter combination could have been used.

2.1.2. Automated BAT procedure

Depending on the experiment considered, 20 µL, 10 µL or 5 µL of whole blood were used for each condition tested. 96 well plates containing dry and room temperature stable staining reagents (prototypes from Beckman Coulter, Marseille, France) were prepared in house using a proprietary methodology. A classical robotic liquid handler (Biomek NXP, Beckman Coulter) containing both an orbital shaker and a Peltier heating unit was used throughout the study to automate the sample preparation. In the final configuration, the single remaining manual step was to load the robotic workstation with the ready-to-use 96 well plates and the required liquid reagents (whole blood, activation solution, Opti-Lyse C and PBS). Including incubation periods, 45 min (hands-off time) in total were required for the automated sample preparation to complete. Obtained samples were finally analyzed on a plate loader equipped cytometer (Cytoflex, 3 lasers, 13 colors, RUO, Beckman Coulter, Indianapolis, USA) without the implementation of any centrifugation step prior to cytometry analysis.

2.2. Ethics statement

BAT was performed on control donors and on excess whole blood from patients receiving allergy work-up in the APMH departments. Under the French law, ethics committee approval and patient consent were not required for this type of non-interventional study, provided the patients had received information and retained the right to oppose the use of excess sample and anonymized medical data (Loi n° 2012–300 du 5 mars, 2012).

2.3. Data expression and statistical analysis

All acquired flow cytometry data were analyzed using Kaluza software (RUO, Beckman Coulter, Indianapolis, USA). As showed in Fig. 1B basophils were gated as side scatter (SS)^{Low}/CD45⁺/CD3[−]/CRTH2⁺

cells and both CD203c and CD63 were used to characterize basophil activation status. Means and coefficients of variation (CVs) on cell detection were calculated for each subpopulation. For this, Kaluza analysis data were exported to Excel software (Microsoft, Redmond, USA) and 95% confidence intervals (CI95%) were calculated based on Chi-squared distribution. Analyse-it (Analyse-it Software, Ltd., Leeds, UK) was used to determine correlational data. The effective concentrations at which 50% of the maximal basophil activation was obtained (EC50) were calculated using GraphPad (GraphPad Software, San Diego, California) curve fitting models with the “log(Agonist) vs. response – Variable slope” equation of a non-linear regression:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC50} - X) * \text{HillSlope}))}$$

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3. Results

3.1. General assay strategy

BAT assays were conducted using the streamlined methodology described in Fig. 1A and the approach depicted in Fig. 1B and based on the CRTH2 positivity was adopted to identify basophils. Leukocytes were initially gated using SS *versus* CD45 expression. As CRTH2 is not only expressed on basophils but also on eosinophils and Th2 lymphocytes, CD3+ cells as well as high SS cells were excluded to avoid any contamination and basophils were finally identified by their expression of CRTH2. To characterize the activation phenotype of basophils, both CD203c and CD63 were monitored with resting basophils identified as CD203c^{Low}CD63[−]. As discussed in the literature, these two activation markers are complementary and it is important to consider both of them to comprehensively characterize the activation status of basophils (Ebo et al., 2012). Using a flow cytometer equipped with 3 lasers and 10 fluorescence detectors, no compensation was required with the proposed panel and gating strategy. As seen in Fig. 1B, in spite of the absence of a centrifugation step, the population of interest could be properly gated and characterized. In case the cytometer run time needs to be decreased, a final wash step could also be introduced (data not shown).

3.2. Transition to dry staining reagents

We first checked that the general assay attributes were not altered by the transition to the dry reagent format. For this purpose, five whole blood samples from non-allergic donors were tested under negative and positive control conditions. Similar profiles were obtained using dry and liquid reagent formats (Fig. S1). We further evaluated whether the concept of dry reagents could be extended to allergenic sources so that pre-built dose-response curves could be prepared. Results obtained with dry *versus* liquid reagents in a peanut and milk allergic donor using water-soluble extracts produced in house were compared (Fig. S2).

Similar profiles were obtained with both types of reagents, demonstrating the feasibility of a dry formulation that would combine staining reagents and activators such as anti-IgE or complex mixtures of allergenic and non-allergenic proteins.

In order to finely characterize the agreement between the two reagent formats, a Bland-Altman plot analysis (Fig. 2) was performed. A regression analysis was also performed (Fig. S4). These two analyses demonstrate that neither the general attributes (MFI and percentages of activated basophils) of the assay nor the numbers of basophils detected were significantly altered by the transition to the dry reagent format. Indeed, a high level of agreement was observed for all parameters studied with no obvious proportional bias detected.

In the case of Figs. 2A and 4SA, the whole basophil population, irrespective of its activation phenotype, has been considered to build the plots.

Correlations between results obtained with liquid and dry reagents on 5 non-allergic donors with negative (unstimulated) and positive control (anti-IgE) and an allergic donor to peanut and milk with control conditions and different allergenic protein extract sources. (A): Mean fluorescence intensity of basophils CD203c-PE and CD63-PB expressions. (B): Number of activated basophils (CD203c+/CD63+). (C): Percentage of activated basophils (CD203c+/CD63+).

3.3. Assay performance

After having verified that the general assay attributes were not altered by the transition to the dry reagent format, intra-assay and inter-operator variabilities were assessed. Intra-assay variability test was performed with one operator, three different whole blood samples, five replicates and with both negative (unstimulated) and positive (anti-IgE) control conditions. Inter-operator assay variability was subsequently assessed with five operators, three different whole blood samples, two activation status (both negative and positive controls), with each condition performed in triplicate.

For both assays, CVs on cell detection were monitored (Fig. 3A and B). To this end, leukocytes (CD45⁺), CD3⁺ T cells (CD45⁺ CD3⁺), eosinophils (SS^{High}/CRTH2⁺/CD3[−]), low SS CD3[−] cells, and basophils (%Baso/CD45⁺) were considered. CVs ranging from 0.43% to 7.65% were obtained with the highest variability observed on basophils due to the lower frequency of this population. For the donors considered here, basophil percentages ranged from 0.17% to 0.64% or 0.15 to 0.44% of all leukocytes for the intra-assay or inter-operator study, respectively. All CVs obtained were below 10%, even in the case of the inter-operator assay.

Intra-assay variability of full dose-response curves with both staining reagents and allergens dried together in the reagent tubes was also assessed. For this purpose, in addition to control conditions, reactivity to eight different concentrations (from 4.4 pg/mL to 44 µg/mL) of a cypress pollen extract was tested in triplicate in a whole blood sample from a donor with known reactivity to cypress pollen. Under these conditions, the obtained mean proportions of CD63+/CD203c+ basophils ranged from 6.03% to 93.86% (Fig. 3C).

To assess the repeatability of the assay under these conditions, CVs

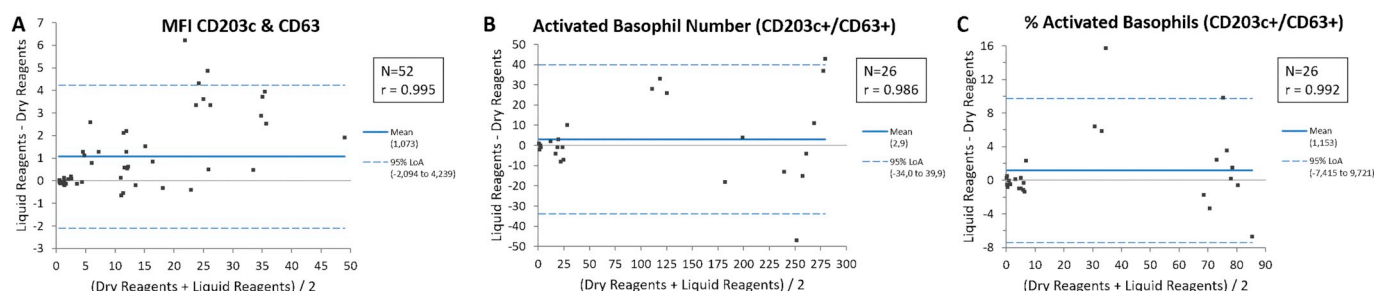


Fig. 2. Agreement between data obtained with dry or liquid reagent format.

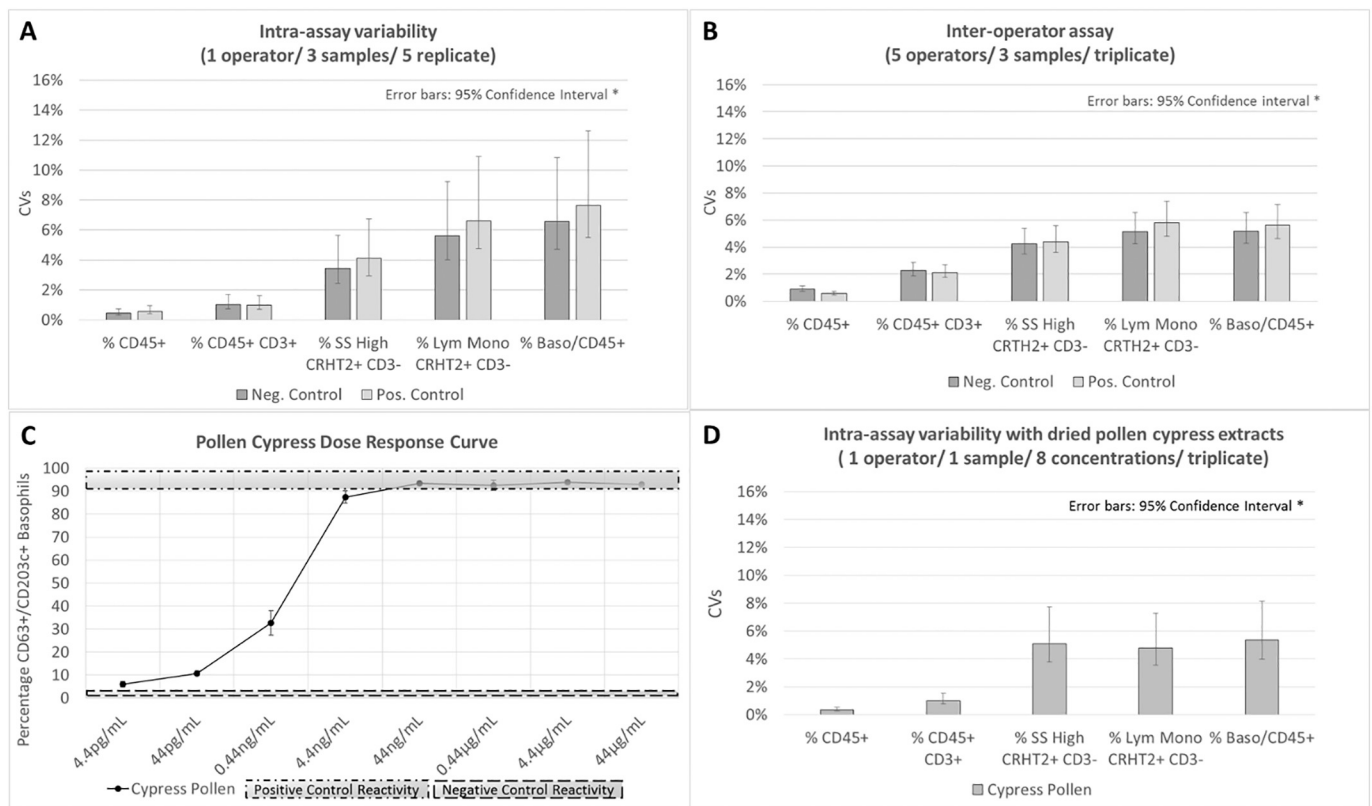


Fig. 3. Evaluation of assay performances.

(A): Intra-assay test and (B): inter-operator test variability under control conditions (Neg Ctl: unstimulated; Pos Ctl: stimulated with anti-IgE). (C): Dose response curve for a cypress pollen allergic individual when tested in triplicate with cypress pollen extracts. Greyed out zones in graph represent variabilities observed for negative and positive control conditions. (D): Intra-assay variability with dried cypress pollen extracts. (CD45⁺), CD3⁺ T cells (CD45⁺ CD3⁺), eosinophils (SS^{High}/CRTH2⁺/CD3⁻), lymphocytes and monocytes CD3⁻ cells, and the percentage of basophils within total leukocytes (%Baso/CD45⁺) were assessed.

*: 95% CI were calculated based on Chi-squared distribution.

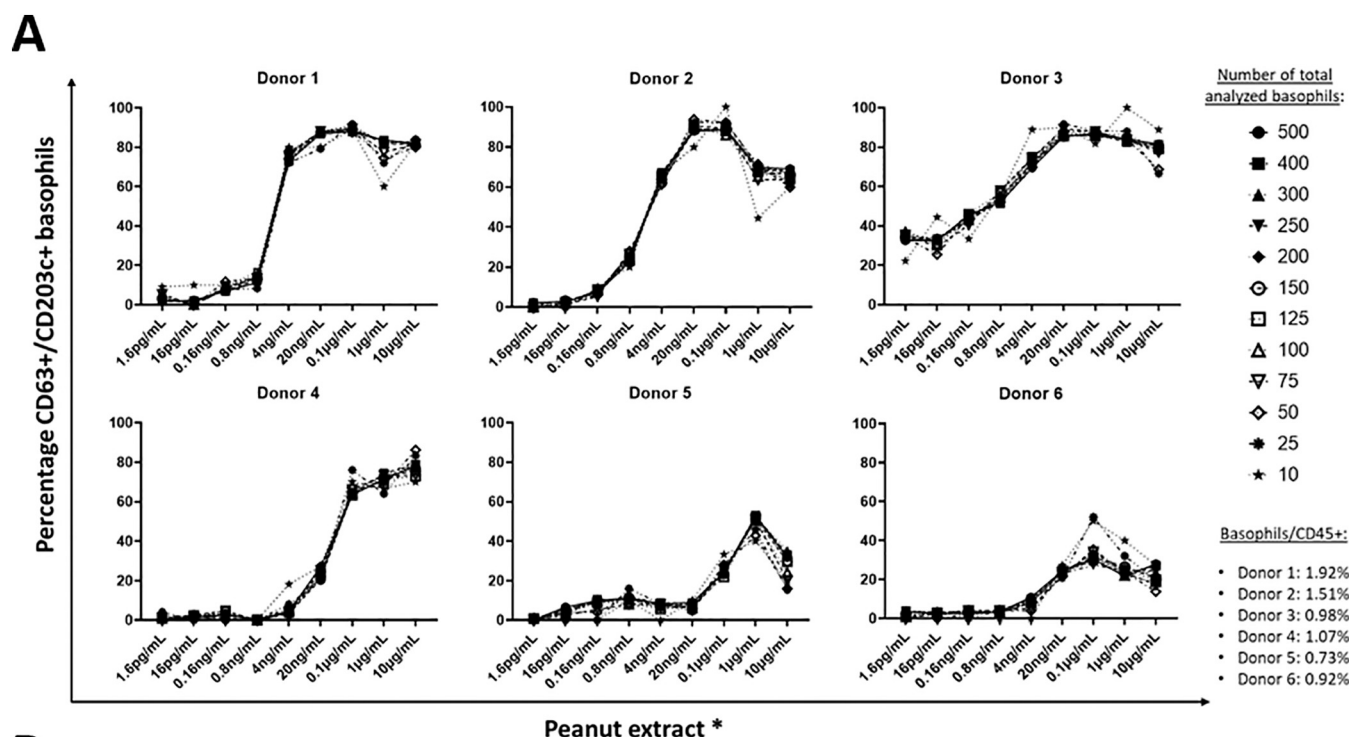
on the percentages of gated cells were assessed. As shown in Fig. 3D, they ranged from 0.35% in the case of the total leukocytes population to 5.35% in the case of basophils.

3.4. BAT feasibility with a minimal number of analyzed basophils

In BAT, a significant number of basophils are usually acquired and analyzed so that statistically representative data can be obtained. It is commonly advised to consider at least 500 basophils to obtain reliable results. Having the ambition to miniaturize and transpose the BAT assay into the 96 well plate format, we wondered what would be the impact of a decrease in the number of analyzed basophils. To this extent, we considered 6 different whole blood samples with *ex vivo* reactivity to peanut proteins. This particular set of samples was chosen to illustrate the heterogeneity typically encountered in a cohort of allergic individuals. Percentages of basophils varied from 0.7% to 1.9% of the total leukocyte population with a maximum *ex vivo* reactivity to peanut proteins ranging from 34% to 90%. Basophil reactivity to nine different concentrations of peanut containing snack extracts (from 1.6 pg/mL to 10 µg/mL) was then studied using the manual tube test format. During these experiments, more than 500 basophils per tube were initially acquired in each of the tested conditions. Following this and as depicted in Fig. 4, this dataset was iteratively analyzed *in silico* considering decreasing numbers of total basophils (from 500 to 10 total basophils) so that the impact of a decrease of total basophil numbers on measurement accuracy could be assessed. For each basophil number, a dose-response curve representing the evolution of the percentage of CD203c⁺/CD63⁺ basophils as a function of the peanut extract protein concentration was constructed. For each patient, all obtained dose response

curves were then overlaid (Fig. 4A). It can graphically be observed that similar dose-response curve profiles were obtained when considering total basophil counts down to 50. Below this threshold, significant differences between the dose response curves could easily be grasped from the figures. To quantify this observation, the effective concentrations at which 50% of the maximal activation occurred were then determined (EC50) for each of the previously built dose response curves. CVs on EC50s were then calculated considering iteratively for each donor various ranges of total basophil counts (Fig. 4B). CVs ranging from 0.74% to 19.10% were obtained for total basophil counts between 500 and 50, demonstrating that dose response curves could still be characterized relatively well within this range. When total basophil counts of 25 and 10 were included for the calculation of the CVs, values above 50% were obtained for several donors, confirming that the EC50 measurement accuracy is significantly decreased when total basophil counts are too low.

Basophil reactivity to the proteins contained in a peanut containing snack extract (from 1.6 pg/mL to 10 µg/mL) was studied in 6 different whole blood samples. More than 500 basophils were initially acquired in each of the tested conditions. An iterative approach was then adopted to assess the impact of a decrease of the total basophil number (from 500 to 10 basophils) on the definition of dose response curves. Corresponding dose-response curves (A) were constructed and CVs on EC50s were calculated across the different acquisition conditions (B). * Peanuts containing snacks extract.



B

Range of Basophil Counts	CVs (%) on EC50 Values					
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
From 10 to 500 basophils	12.13	5.05	18.80	14.05	30.17	54.15
From 25 to 500 basophils	6.92	4.83	19.11	13.16	18.6	49.79
From 50 to 500 basophils	5.58	4.50	19.10	8.64	18.28	16.09
From 75 to 500 basophils	5.92	4.38	17.07	8.96	9.57	14.26
From 100 to 500 basophils	6.32	3.93	15.37	7.99	3.34	7.87
From 200 to 500 basophils	4.87	4.95	10.16	7.39	1.51	8.79
From 300 to 500 basophils	3.86	7.01	1.73	8.59	0.74	8.46

Fig. 4. *In silico* analysis.

3.5. Automation and miniaturization of BAT assay: transposition to 96-well plate format

After the *in silico* demonstration that reduced numbers of basophils can be used to build a dose response curve, we checked whether the BAT procedure could be transposed and miniaturized into the 96 well plate format. To this end, plates containing dried reagents were prepared in house and a fully automated sample and liquid handling method was developed on a robotic platform.

In addition to the liquid handling parameters, other aspects such as mixing and temperature control had to be properly optimized. As indicated in Fig. S3, red blood cell lysis efficiency was indeed significantly

affected by the mixing conditions. During these experiments, it was also found that the basophil reactivity could be affected as well (Table S1). Mixing steps of 10 s at 1000 rpm were found to provide the best results. Under these defined settings, the basophil population could be easily discriminated and its activation status properly characterized (fig. S5).

While a water bath was used to control the temperature when implementing BAT in a tube format, a Peltier system was used for the 96 well plate format. Our setup presented an air gap of a few mm between the bottom of the wells and the metallic contact surface of the Peltier, it was thus important to verify the effectiveness of the temperature control. To this extent, we considered two sets of BAT experiments, all performed with the same whole blood sample. The first set consisted in

tube format BAT experiments with water bath temperature control and the second one in plate format BAT experiments with Peltier temperature control. Various incubation temperatures (25, 33, 37 and 45 °C with tubes and 38, 42 and 46 °C with plates) and both negative and positive control conditions were considered with each tested situation performed in triplicate (fig. S4). Based on these results, a command of 42 °C on the Peltier system was used going forward.

With these defined settings, eight different concentrations of a pellitory extract (from 0.01 pg/mL to 100 ng/mL) were considered to study the *ex vivo* reactivity of a whole blood sample originating from a donor with a known reactivity to pellitory. To this end, 96 well plates containing dry and ready-to-use staining reagents and allergenic extracts were prepared in house. The single manual step corresponded to load the dry reagent plate and the required liquid reagents (whole blood and lysis reagents) into the robotic workstation. Including incubation periods, a total of 45 min hands-off time was required for the automated sample preparation to proceed. Various conditions involving the use of various amounts of whole blood per point (20 µL, 10 µL and 5 µL) were tested with each point performed in triplicate. To this end, no other modifications than proportionally adjusting the quantity and/or volume of reagents (staining antibodies, lysis solution, PBS) to the volume of blood samples were implemented. As shown in Fig. 5, similar dose response curves, with very similar EC50s were obtained (respectively 1,13 ng/mL, 1,49 ng/mL and 1,14 ng/mL for 20 µL, 10 µL and 5 µL of whole blood) in spite of significantly different numbers of basophils acquired in each condition (an average of 463, 275 and 62 basophils acquired using either 20 µL, 10 µL or 5 µL of whole blood respectively).

Eight different concentrations of pellitory extract (from 0.01 pg/mL to 100 ng/mL) were tested in triplicate on a whole blood sample originating from a donor with a known reactivity to pellitory using either 20 µL, 10 µL or 5 µL of whole blood per tested point. Respectively, under these conditions, an average of 463, 275 and 62 basophils were acquired. The mean values of each tested condition are represented in the graph.

4. Discussion

With the increase of studies reporting potential applications of BAT, new questions and perspectives have emerged. In this work, to enable workflow simplification and standardization, we used a dry reagent formulation stable at room temperature, compatible with allergens from water-soluble extracts and fluorochrome-conjugated antibodies. In the simplest configuration tested, only staining reagents (fluorochrome-conjugated antibodies) were dried and the relevant allergens were added in liquid format prior to the addition of whole blood. It was further demonstrated that non-conjugated anti-IgEs or allergen-containing protein extracts could be dried together with staining reagents, allowing for ready-to-use test tubes, even in the context of dose-response curve generation. The choice of fluorochromes to which staining antibodies are conjugated was also carefully optimized: i) yielding a 5 color assay that does not require any compensation, compatible with a typical flow cytometer equipped with 3 lasers ii) allowing proper basophil identification iii) and detailed activation status characterization leveraging both CD203c and CD63 activation markers. Consequently, a streamlined and standardized procedure could be proposed. On top of providing an easier path to BAT, it is shown that this simplification also benefits the robustness of the assay, as demonstrated by very low intra-assay and inter-operator variability without impairing performance characteristics.

Further leveraging these foundations, we also demonstrated here for the first time that BAT can not only be miniaturized and transposed to the 96 well plate format but also fully automated using a robotic workstation, reducing hands-on time to as little as 5 min. When working in a dose response curve fashion with eight different concentrations of an allergenic extract, it was demonstrated that it is

possible to conduct the assay with as little as 5 µL of whole blood per tested point, providing that a minimum of basophils could be acquired. In our configuration, total numbers of acquired basophils as low as 50 enabled a proper characterization of EC50s. While this lower limit will need further studies with larger cohort sizes to be carefully determined, the perspective of decreasing significantly the total number of basophils acquired per test potentially enhances the compatibility of BAT with pediatric studies for which the quantity of blood might be limited. Interestingly, this also opens the path toward BAT based component resolved allergy diagnosis.

In a general context where the true potential of BAT for allergy diagnosis and monitoring is investigated (Hemmings et al., 2018), we believe that the approaches discussed in this report can contribute to a further democratization of the assay beyond expert laboratories. If verified, such a trend could facilitate the implementation of BAT in more clinical studies, thereby enabling the gathering of more data to finally yield a better understanding of the possible role of BAT in the allergy testing landscape.

As of now, it is in the context of a multi-technique approach that the use of BAT is the most promising. It is indeed unlikely that BAT could replace any of the techniques more commonly used in the context of diagnosis or monitoring of allergy, such as SPT or IgE quantitation. It has been shown however that BAT has the potential to provide a unique set of information, complementary to the results obtained with more classical tests, thereby providing a potential path toward multi-methodological approaches which would improve sensitivity and specificity (Santos et al., 2014; Santos et al., 2015a). Santos and Lack even provocatively asked whether BAT could in some cases be considered as a food challenge in a test tube (Santos and Lack, 2016). While answering to this question is very complex, a desirable long-term goal is indeed to reduce the number of OFCs so that this gold standard - but yet expensive and potentially dangerous approach - would only be used to confirm the absence of reactivity already observed with *ex vivo* and/or *in vitro* assays.

To confirm or disprove these important hypotheses, additional studies with larger cohorts of patients are required. In this context, the availability of BAT assays that combine, as the approaches described herein, ease of use, reproducibility and standardization capabilities will be crucial. It is only when these features will be provided that we can expect a more frequent use of BAT in the field of allergology.

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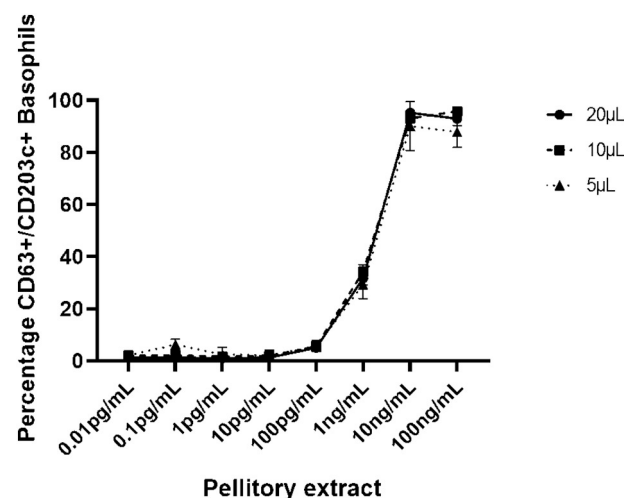


Fig. 5. Automated BAT with minimal whole blood sample volumes.

supported by the Public Assistance of Marseille Hospitals (Junior AORC 2016, NCT03265262, principal investigators A. Carsin and J. Vitte).

Declaration of Competing Interest

R. Arif-Lusson is a PhD student and is currently employed by Beckman Coulter Inc. J-M. Busnel is an employee of Beckman Coulter Inc. The other authors have no conflicts of interest to disclose in relation to this work. Unrelated to this work: J. Vitte has received speaker fees from MEDA Pharma, Mylan and Thermo Fisher, consultancy fees from Sanofi, reimbursement from Thermo Fisher for attending the 2015 EAACI congress, and from Stallergènes for attending the 2013 ISMA congress. Thermo Fisher has supported Dr. Thomas Romain, a student from J. Vitte lab, for attending the 2016 and 2017 EAACI congresses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jim.2020.112793>.

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Supplementary data

In order to be able to work with EDTA anticoagulated blood samples and as described in the materials and methods section, an activation buffer was used. The intent of this activation buffer is to set back the ionized calcium concentration to a level compatible with the *in vitro* activation of basophils. To illustrate this, the concentration of free calcium was measured in 5 different anticoagulated blood samples after a 1 to 1 dilution into the considered activation solution. To this end, a potentiometric approach was used on a GEM 4000 analyzer from Werfen (Munich, Germany). An average free calcium concentration of 3.1 mmol/L was found on these samples with a coefficient of variation of 11.0%.

Furthermore, an experiment aiming at assessing whether BAT results obtained with heparinized blood significantly differ from those obtained with EDTA anticoagulated blood was performed. In the case of 3 non-allergic donors, both heparinized and EDTA anticoagulated whole blood samples were considered with both negative and positive control tubes and each point performed in triplicate. To this end, the manual BAT procedure described in paragraph 2.1 was implemented. Of note and as described in the materials and methods section, while 50 μ L of EDTA anticoagulated blood were initially mixed with 50 μ L of activation solution, a PBS solution was used instead of the latter in the case of heparinized blood. Corresponding results, as shown in Figure S1, demonstrate that highly similar results are obtained with both workflows.

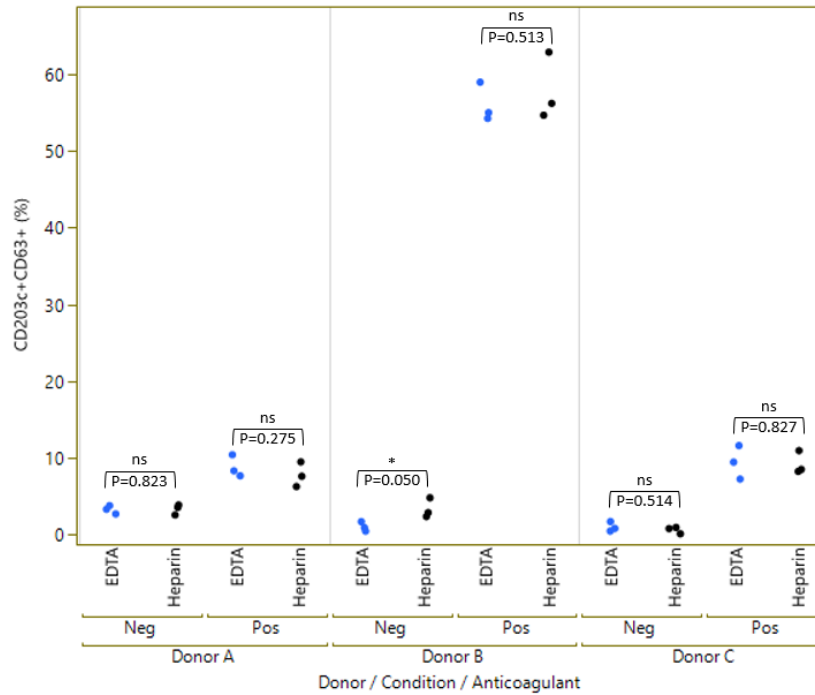


Figure S1 – Influence of anticoagulant on BAT results

Whole blood samples from 3 non-allergic donors and liquid staining reagents were used with each experiment performed in triplicate. Both negative and anti-IgE positive controls were considered. While 50 μ L of EDTA anticoagulated blood were initially mixed with 50 μ L of activation solution, a PBS solution was used instead of the latter in the case of heparinized blood. Brackets show differences between groups with the corresponding raw p-values (Mann-Whitney U test).

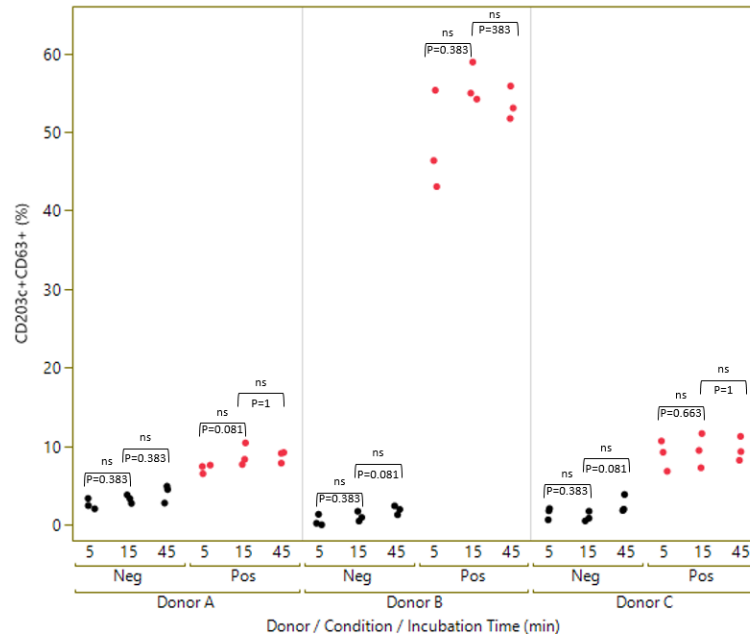


Figure S2 – Influence of incubation time on basophil activation status.

EDTA anticoagulated whole blood samples from 3 non-allergic donors and liquid staining reagents were used with each experiment performed in triplicate. Both negative and anti-IgE positive controls were considered. Brackets show differences between groups with the corresponding raw p-values (Mann-Whitney U test). No significant differences between groups were found.

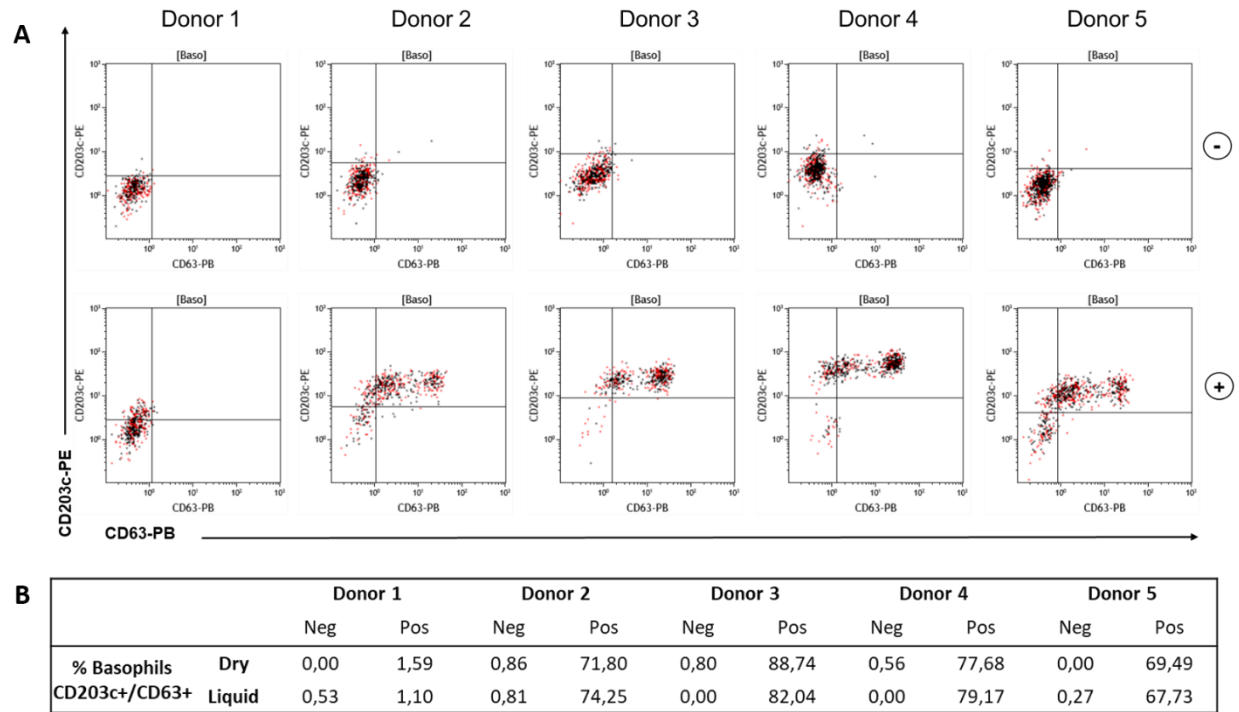
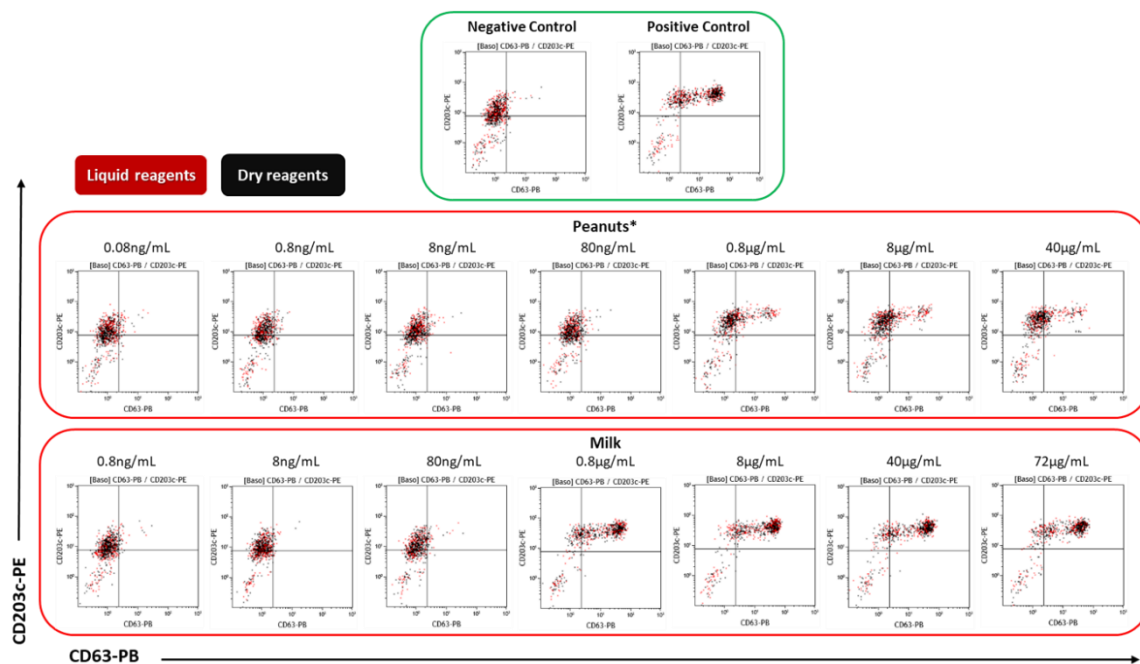


Figure S3 - Comparison of basophil activation status between dry and liquid reagents on 5 non-allergic donors with negative (unstimulated) and positive control (anti-IgE). **A:** Dot-plots corresponding to dry (black dot plots) and liquid reagents (red dot plots) have been overlaid. **B:** Percentages of activated basophils (CD203c+/CD63+) obtained with dry and liquid reagents

A



B

		Neg	Pos	Peanuts 0.08ng/mL	Peanuts 0.8ng/mL	Peanuts 8ng/mL	Peanuts 80ng/mL	Peanuts 0.8μg/mL	Peanuts 8μg/mL	Peanuts 40μg/mL	Milk 0.8ng/mL	Milk 8ng/mL	Milk 80ng/mL	Milk 0.8μg/mL	Milk 8μg/mL	Milk 40μg/mL	Milk 72μg/mL
% Basophils	Dry	5,54	72,25	5,71	4,85	6,17	7,04	26,65	27,49	30,19	4,95	3,12	6,52	70,38	80,63	74,72	77,94
CD203c+/CD63+	Liquid	4,58	68,88	8,03	5,13	5,88	5,66	42,37	33,92	36,03	3,98	3,23	5,36	80,22	80,05	78,29	78,13

Figure S4 - Comparison of results obtained with liquid and dry reagents (staining reagents and allergens). **A:** Basophil dot-plots corresponding to the different tested conditions. **B:** Percentages of activated basophils (CD203c+/CD63+) obtained with dry and liquid reagents. * Peanut containing snack.

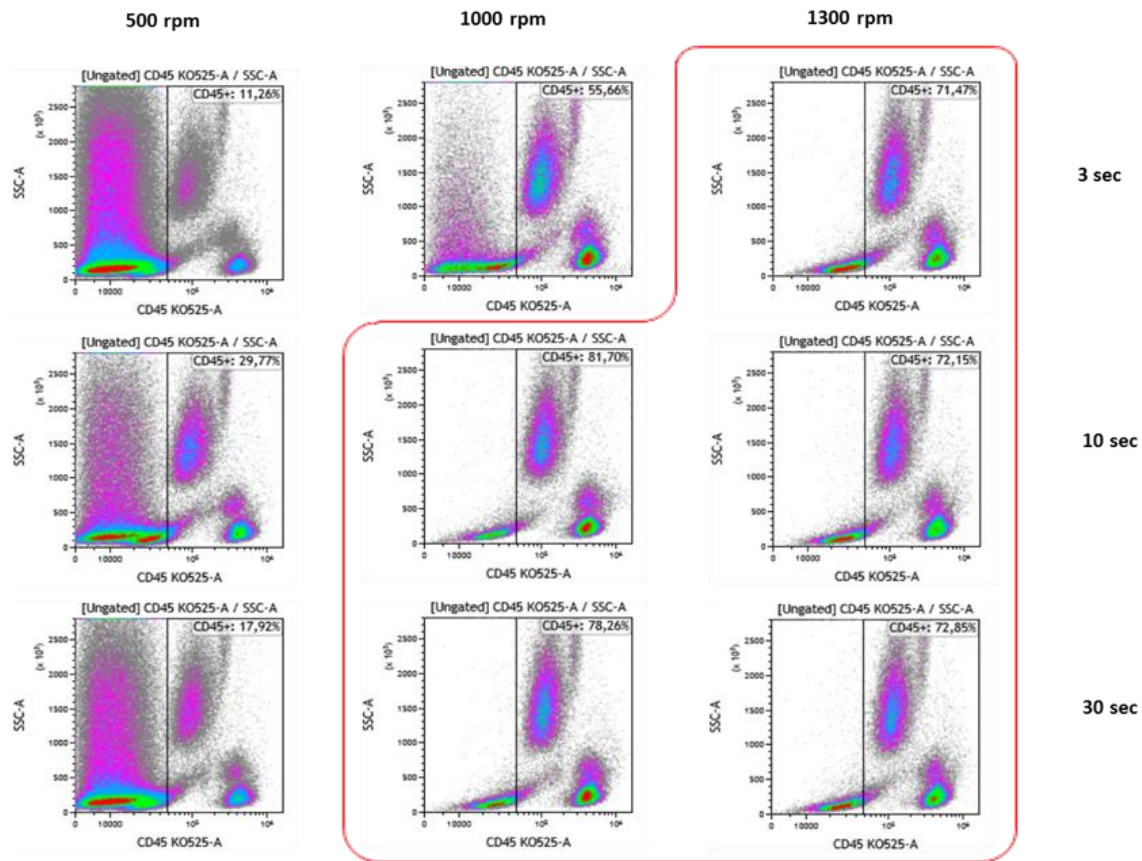


Figure S5: Impact of the mixing settings step on erythrocytes lysis efficiency

Different mixing duration and speed were applied on the Biomek orbital shaker system to evaluate the impact of the mixing step on the lysis efficiency of erythrocytes.

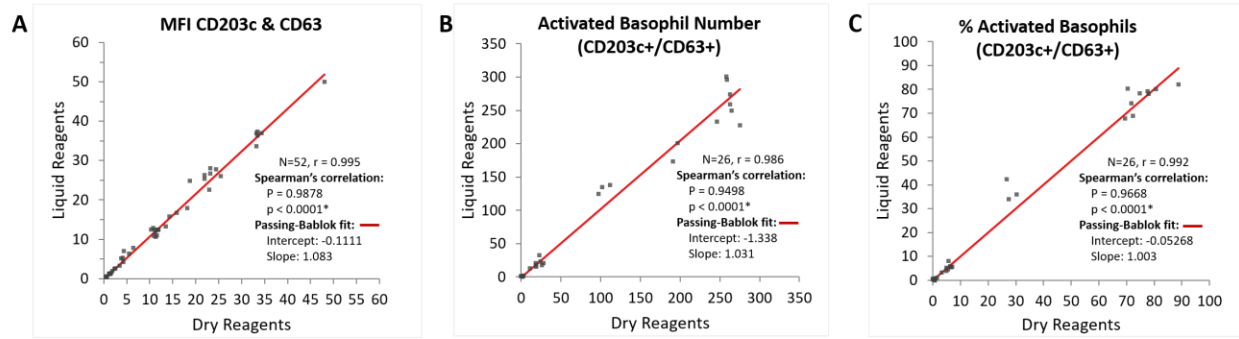


Figure S6: Correlational data between dry versus liquid reagent format.

Correlations between results obtained with liquid and dry reagents on 5 non-allergic donors with negative (unstimulated) and positive controls (anti-IgE) and an allergic donor to peanut and milk with control conditions and different allergenic protein extract sources. **(A):** Mean fluorescence intensity of basophils CD203c-PE and CD63-PB expressions. Spearman's correlation $\rho = 0.9878$ with $p < 0.0001^*$. **(B):** Number of activated basophils (CD203c+/CD63+). Spearman's correlation $\rho = 0.9498$ with $p < 0.0001^*$. **(C):** Percentage of activated basophils (CD203c+/CD63+). Spearman's correlation $\rho = 0.9668$ with $p < 0.0001^*$.

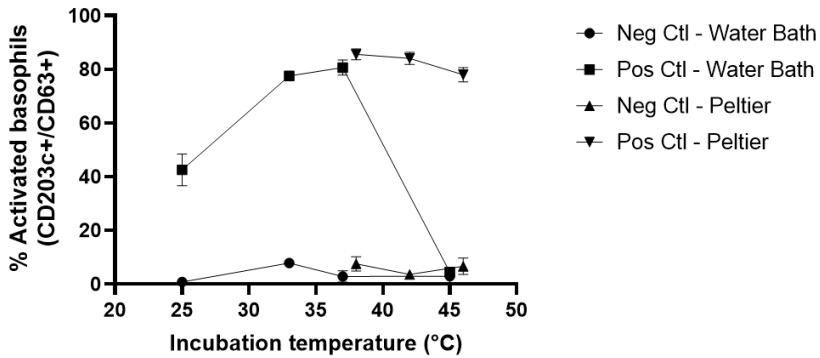


Figure S7: Setting up the peltier system for an optimized incubation period

BAT assays were conducted in triplicate with whole blood from non-allergic donor under negative and positive control conditions, either in manual tube format using a water bath incubation step or in automated 96 well plate format using a peltier for incubation step, under different temperature incubation (25, 33, 37 and 45°C on water bath and 38, 42 and 46°C on peltier). The mean values of each tested condition are represented.

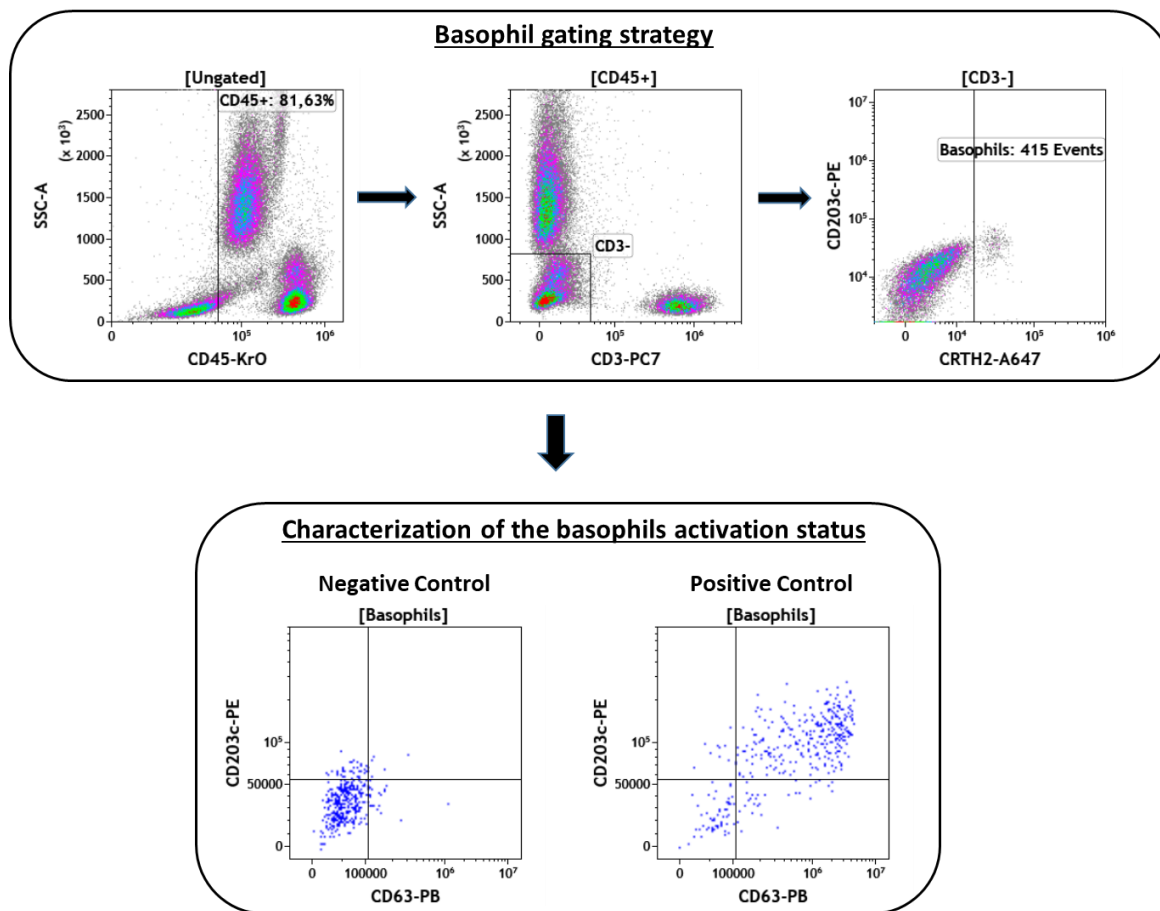


Figure S8: Gating strategy example using the 96 well plate format.

		500 rpm		1000 rpm		1300 rpm	
		%	CV	%	CV	%	CV
3 sec	%CD45+ cells	49,70	62,73%	67,90	10,45%	70,00	5,05%
	% Act. basophils	76,34	2,02%	76,89	5,96%	78,05	13,97%
10 sec	%CD45+ cells	58,62	25,58%	78,41	4,75%	69,25	9,11%
	% Act. basophils	81,51	2,90%	80,57	4,84%	74,44	8,39%
30 sec	%CD45+ cells	56,83	35,55%	77,54	5,02%	68,98	6,58%
	% Act. basophils	78,69	4,90%	79,76	3,95%	71,10	5,15%

Table S1: Impact of the mixing settings steps on erythrocyte lysis efficiency and basophil reactivity.

To better assess the impact of the orbital shaker settings on the erythrocyte lysis efficiency and basophil reactivity, the recruitment of the CD45+ cells (% CD45+ cells) as well as the percentage of activated basophils (%Act. basophils) were monitored. For this purpose, whole blood from a non-allergic donor was tested in triplicate under positive control condition (anti-IgE stimulation). The mean values of each tested condition are presented in this table.

Percentage of CD203c+/CD3+ Basophils									EC50
Cypress Pollen Extract:	4.4pg/mL	44pg/mL	0.44ng/mL	4.4ng/mL	44ng/mL	0.44µg/mL	4.4µg/mL	44µg/mL	ng/mL:
Replicate a	7,27	9,97	29,61	89,64	93,66	95,00	93,18	93,79	0,83
Replicate b	5,04	10,42	29,40	84,50	95,54	90,85	93,49	92,77	0,91
Replicate c	5,78	11,83	38,78	88,25	94,32	91,91	94,90	92,11	0,65

Table S2: Raw data of the dose-response to cypress pollen extract. Each dose response was performed in triplicate and the EC50 determined for each replicate.