

Basophil allergen threshold sensitivity, CD-sens, IgE-sensitization and DBPCFC in peanut-sensitized children

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Abstract

Background: Immunoglobulin E (IgE)-sensitization to peanut is common and can indicate an allergy. A positive test needs to be confirmed by a double-blind, placebo-controlled food challenge (DBPCFC), which is regarded as 'the gold standard'. The aim of the study was to evaluate the basophil allergen threshold sensitivity (CD-sens) and antibodies to peanut allergen components in relation to DBPCFC in the diagnoses of peanut allergy in children.

Methods: Thirty-eight children with suspected peanut allergy underwent a DBPCFC. CD-sens to peanut and Ara h 2 were analysed as well as IgE-antibody to peanut and some of its allergen components (Ara h 1, 2, 3, 8 and 9).

Results: Twenty-five children had a positive DBPCFC, and 92% of these were positive in CD-sens to peanut and Ara h 2. Two children with a positive DBPCFC were classified as 'low-responders' and were not further evaluated. Children positive in DBPCFC had higher CD-sens values to peanut (median 1.3; range 0.4–29, $n = 21$) compared with children negative in DBPCFC (median 0; range 0–0.5, $n = 13$) ($P < 0.0001$). A positive DBPCFC correspond with increased levels of IgE-antibody to Ara h 1, 2 and 3 compared with those with a negative challenge ($P < 0.0001$ for all). All children with a negative CD-sens were negative in DBPCFC.

Conclusion: In this study, a negative CD-sens to peanut excluded peanut allergy. Both tests, CD-sens to peanut and immunoassay for IgE-antibody to the peanut components, appear to be safe, time saving and cost-effective complements to DBPCFC.

Peanut allergy is common, potentially life threatening and often life long. Immunoglobulin E (IgE)-sensitization to peanut has been reported in 7–11% of children in Western countries (1–3), and the prevalence of peanut allergy in children varies between 0.75% and 3% (1, 4). Symptoms vary from mild oral symptoms to severe anaphylaxis or even fatal reactions (5, 6). The negative impact on quality of life is significant in families with an allergic child (7, 8).

To diagnose peanut allergy and to assess who will react mildly or severely to peanuts is difficult. Food allergy is usually diagnosed by case history, skin prick test (SPT) and IgE-antibody (IgE-ab) quantification, and low levels of IgE-ab to peanut have been detected already at 6 months of age (9). Thus, in many cases, a positive test needs to be confirmed by

a double-blind, placebo-controlled food challenge (DBPCFC), which is regarded as 'the gold standard' (10, 11). However, DBPCFC is time consuming and associated with risks (12), and thus, there is a need for complimentary diagnostic tools (13, 14).

It is now possible to analyse IgE-ab to different allergen components [component resolved diagnostics (CRD)] in various food allergens. Thus, IgE-ab against peanut storage proteins (Ara h 1, 2 and 3) are associated with severe allergic reactions (15–17), while IgE-ab to Ara h 8, a structural homolog to the major allergen in birch (17), is associated with mild or no symptoms (2).

In IgE-mediated allergy, mast cells and basophils are sensitized with IgE-ab. By triggering basophils with decreasing

concentrations of allergen, the lowest triggering dose, CD-sens can be determined (18, 19). Stimulation of basophils has been introduced earlier in the diagnosis of food allergy (20, 21).

The aim of the present study was to evaluate CD-sens and IgE-ab to peanut allergen components in relation to DBPCFC in the diagnosis of peanut-allergic children.

Methods

Study population

Forty-three children, aged 4–19 years, who were referred to Sach's Children's Hospital, Stockholm, Sweden, for oral peanut challenge were invited to participate. The inclusion criteria were suspected peanut allergy and peanut IgE-ab (≥ 0.35 kU_A/l) or a positive skin prick test (SPT) (≥ 3 mm) to peanut. Exclusion criteria were antihistamines or oral steroids taken within 4 days prior to the challenge or history of anaphylaxis to peanut confirmed in medical records (A detailed description of the patients in Table S1).

Five children were excluded from the study. One did not complete the study, two did not follow the study protocol, and two other children were found not to fulfil the inclusion criteria. Thus, 38 children were included in the present study.

The study was approved by the ethics committee in Stockholm, Sweden, and the parents provided written consent.

Double-blind, placebo-controlled food challenge

Double-blind, placebo-controlled food challenge was performed by experienced personal using a challenge medium containing 11% peanut and 7% fat in increasing doses every 30 min in five steps from 1 mg to 5 g. Symptoms were scored according to Astier *et al.* (6) (Table 1). A negative test was

Table 1 Symptom score according to Astier *et al.* (6) used to evaluate clinical reactions in DBPCFC

Symptom score	Symptoms
0	No symptoms
1	Abdominal pain that resolved without medical treatment, rhino conjunctivitis or urticaria <10 paplers, rash
2	One organ involved Abdominal pain requiring treatment Generalized urticaria Non laryngeal angioedema Mild asthma (cough, fall of peak expiratory flow <20%)
3	Two organs involved (of symptoms mentioned under 2)
4	Three organs involved (of symptoms mentioned under 2) or asthma requiring treatment or laryngeal oedema, or hypotension
5	Cardiac and respiratory symptoms requiring hospitalization in the intensive care unit

DBPCFC, double-blind, placebo-controlled food challenge.

defined as no objective allergic symptoms during two hours after the DBPCFC was completed. Up to three challenges were performed; the first two were either placebo or active in random order, and the third challenge was for ethical reasons always active. Information about the child's health was collected, a peripheral venous catheter inserted, and a clinical examination was performed before challenge.

In this publication, we compare the results of the first active challenge and the blood sample drawn at the same occasion.

Blood sampling

Blood samples were collected before the challenges and stored at +4°C for a maximum of 24 h before cell analyses. Serum was separated and stored at –20°C pending analyses.

Basophil analyses

Basophils were stimulated (18, 19) with decreasing concentrations of an extract in phosphate-buffered saline (PBS) of crude peanut (peanut) and recombinant Ara h 2 (18, 19) (A detailed description of the method in Data S1). The same batch of peanut was used as in all oral challenges. Anti-FcεRI (Bühlmann Laboratories AG, Schönenbuch, Switzerland) and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma Chemical Co, St. Louis, MO, USA) were used as positive controls and RPMI (a cell culture media developed at Roswell Park Memorial Institute) as negative control. Leucocytes were stained for CD63 and CD203c (Immunotech, Marseille, France) and counted in a Navios flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). The cut-off determining a positive test was set to 5% of CD63-positive basophils.

Individuals with basophils, which after anti-FcεRI stimulation, that is the positive control, responded with 0–5% CD63-upregulation, were regarded as non-responders. For individuals with a response between 5% and 16% (low-responders), the results should be interpreted with caution. The cut-off of 16% was calculated (mean 76% –3 SD) from the positive controls of an in-house reference material of 264 allergic children and adults.

Definition of CD-sens

Basophil allergen threshold sensitivity was measured as the lowest allergen concentration giving 50% (LC50) of maximum CD63% up-regulation of the dose–response curve. CD-sens is defined as the inverted value for LC50 multiplied by 100 and was used to describe the patient's allergen sensitivity.

Serological analyses

IgE and IgE-ab to peanut and the recombinant allergen components Ara h 1, 2, 3, 8 and 9 were determined in serum with ImmunoCAP® (Thermo Fisher Scientific, Uppsala, Sweden), according to the instructions of the manufacturer. A positive test was defined as an IgE-ab level > 0.1 kU_A/l.

Statistics

The results are presented as median and range. Differences between patients with positive and negative challenge were tested with Wilcoxon's rank-sum test. Spearman's rank-order correlation (r_s) was used to assess the relation between the cumulative amount of peanut tolerated before reaction at challenge and the severity of the reaction. The percentage of IgE-ab of total IgE was calculated and designated 'IgE-ab fraction'. Significance was considered at a P -value of <0.05 . No adjustment for multiple testing has been performed. Thus, significant results should be regarded as descriptive and explorative. Statistical analyses were carried out using SAS v9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics

Of the 38 included children, 21 were boys. The median age of the whole group was 12 (range 4–19) years. Twenty of the enrolled children had a convincing history of peanut allergy, and eighteen had been identified by routine clinical work-up and claimed that they had never eaten peanuts. Twenty-two children had a positive (>3 mm) SPT to peanut, and 34 had IgE-ab to peanut (>0.35 kU_A/l) when included in the study.

DBPCFC to peanut

Twenty-five children (66%) reacted with positive and 13 children (34%) with negative DBPCFC (Table 2). Of the 25 reacting, ten claimed they had never eaten peanuts, and of the 13 nonreacting children, eight had the same claim while five reported a previous allergic reaction to peanut.

The symptoms were scored according to Astier *et al.* (6) and were mild to moderate (grade 1–3) in fifteen (60%) of the reacting children. Nine children (36%) had severe reactions (grade 4), and one child (4%) was observed in the intensive care unit (grade 5) (Table 2). No child reacted to placebo.

No association was found between the threshold dose of peanut at challenge and the severity of the reaction ($r_s = 0.21$, $P = 0.32$) (Table 2).

Table 2 Severity score in challenged patients

DBPCFC	<i>n</i> (%)	Severity score	<i>n</i> (%) by DBPCFC	Eliciting dose (g), median (range)
Negative	13 (34)	Grade 0	13 (100)	
Positive	25 (66)	Grade 1	4 (16)	5.111 (0.011–6.111)
		Grade 2	8 (32)	0.111 (0.001–1.111)
		Grade 3	3 (12)	1.111 (0.111–6.111)
		Grade 4	9 (36)	1.111 (1.111–6.111)
		Grade 5	1 (4)	1.111 (1.111–1.111)

DBPCFC, double-blind, placebo-controlled food challenge.

Table 3 CD-sens in relation to DBPCFC after stimulation with peanut and Ara h 2 number of patients and (%) is given

CD-sens	DBPCFC + (%)	DBPCFC – (%)
Peanut		
Total	24 (65)*	13 (35)
Negative	0 (0)	10 (77)
Positive	22 (92)†	3 (23)
Low responders	2 (8)	
Ara h 2		
Total	25 (66)	13 (34)
Negative	0 (0)	10 (77)
Positive	23 (92)†	3 (23)
Low responders	2 (8)	

DBPCFC, double-blind, placebo-controlled food challenge.

*One child was not tested in CD-sens (peanut).

†One child was positive in CD-sens, but the CD63 expression barely reached the cut-off; hence, a CD-sens value could not numerically be calculated.

CD-sens

Ninety-two per cent (22 of 24) of the children with a positive DBPCFC were positive in CD-sens after stimulation with peanut and 92% (23 of 25) after stimulation with Ara h 2 (Table 3) (Fig. 1). Two children were low responders, having a low response to the positive control, anti-FcεRI, ($<16\%$) to allow further evaluation. Seventy-seven per cent with a negative DBPCFC were negative in CD-sens after stimulation

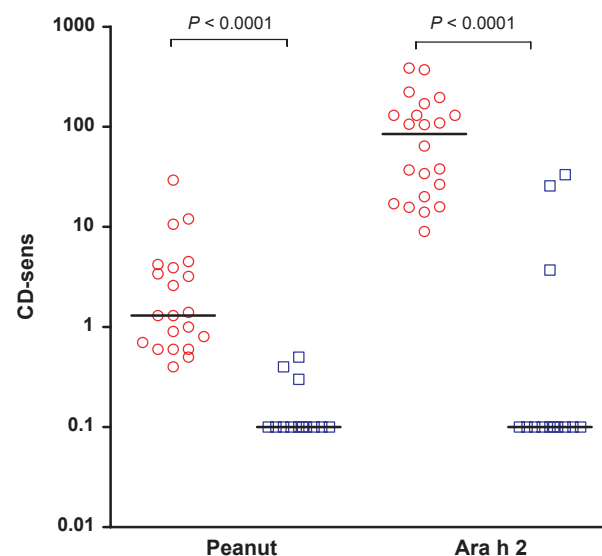


Figure 1 CD-sens to peanut and Ara h 2 in children with a positive (○) or negative (□) DBPCFC. Four children were excluded from the figure. One child was not tested in CD-sens (peanut), two children were low responders, and another child was positive in CD-sens, but the CD63 expression barely reached the cut-off; hence, a CD-sens value could not numerically be calculated. Horizontal bars, median.

with peanut and Ara h 2. Three children negative in challenge had positive values in CD-sens to both peanut and Ara h 2, and all of them had also IgE-ab to these allergens. However, all children negative in CD-sens were negative in DBPCFC (Table 3) (Fig. 1) (A supplement consisting of all CD-sens dose-response curves in Fig. S1).

Children with a positive DBPCFC had significantly higher levels of peanut CD-sens, 1.3 (range 0.4–29.3) than negative children 0 (range 0–0.5) ($P < 0.0001$). The value for CD-sens to Ara h 2 differed also significantly between children positive, 84.5 (range 9.0–385.0) and negative 0 (range 0–33.2) in DBPCFC ($P < 0.0001$) (Fig. 1). One child was positive in CD-sens, but the CD63 expression barely reached the 5% cut-off; therefore, a CD-sens value could not be calculated.

IgE-ab to peanut and peanut components

The median peanut serum IgE-ab level in the whole group at the time of challenge was 36.5 (range 0.1–1022) kU_A/l. Children positive at DBPCFC had significantly higher levels of peanut IgE-ab, 114 (range 1.0–1022) kU_A/l, compared with those negative at the DBPCFC, 1.0 (range 0.1–46.9) kU_A/l ($P < 0.0001$) (Fig. 2).

Children with a positive DBPCFC had also significantly higher levels of IgE-ab to Ara h 1, 2 and 3 compared with those negative at the challenge ($P < 0.0001$ for all) (Fig. 2). No significant differences were found for the levels of IgE-ab to Ara h 8 and 9 between children positive and negative in DBPCFC (Fig. 2). Two children positive in DBPCFC had low IgE-ab levels (0.6 and <0.1 kU_A/l) to Ara h 2. Four children negative in DBPCFC had IgE-ab to Ara h 2 (0.1–1.9 kU_A/l), and three of them had a positive CD-sens.

The IgE-ab fraction was calculated, and children positive in DBPCFC had a median IgE-ab fraction of 26% (range 1–72%) for peanut and 11% (range 0–55%) for Ara h 2, while those negative in DBPCFC had 0% (range 0–10%) and 0% (range 0–2%), respectively ($P < 0.0001$ and <0.0001).

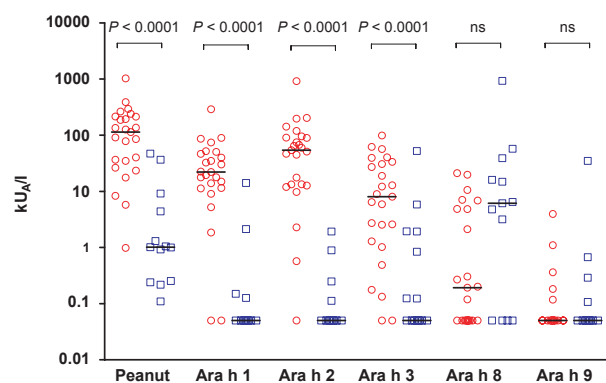


Figure 2 Immunoglobulin E-ab to peanut and peanut components in children with a positive (○) or negative (□) DBPCFC. Horizontal bars, median, ns, not significant.

Discussion

In this study, we show that CD-sens and CRD are useful diagnostic tools in the evaluation of a peanut allergy. We demonstrate that a majority (92%) of the children with a positive DBPCFC is positive in CD-sens after basophil stimulation with both peanut and Ara h 2. We also show that all children negative in CD-sens to peanut allergens are negative in DBPCFC. Furthermore, IgE-ab to Ara h 1, 2 and 3 are associated with peanut allergy.

The diagnosis of peanut allergy has so far been based on case history, presence of IgE-ab and a positive SPT. On an individual basis, the levels of IgE-ab or SPT wheal size to peanut can predict neither an allergic reaction nor its severity. The probability for a reaction increases with elevated levels of peanut IgE-ab (22). However, in Northern Europe where serological (IgE-ab) cross-reactivity between peanut and deciduous trees is common, this is a problem when diagnosing peanut allergy (2, 23).

When objectively confirming a food allergy, an oral food challenge is demanded, and DBPCFC is regarded as 'the gold standard' (10, 11). DBPCFC is an attempt to mimic real-life exposure under standardized conditions. However, the evaluation of the reaction in an oral provocation is complicated (13), and in contrast to bronchial allergen titration challenge for airborne allergens (19, 24), there is no objective definition of a positive oral provocation. Additionally, the grade of a positive provocation is very difficult to determine and quantify as both the severity and type of symptoms need to be considered in conjunction with the threshold challenge dose.

This is illustrated in the present study where 66% of the challenged children reacted to active substance, but no associations could be shown between the amount of peanut and the severity of the reaction (Table 2).

Evaluation of basophil reactivity after food-allergen stimulation has been suggested in food-allergy diagnosis (20, 21). In the present study, we evaluated the lowest dose of peanut allergens triggering the basophils, CD-sens (18, 19). This method is a well-documented marker for basophil sensitivity. In contrast to methods measuring basophil reactivity (20, 21), CD-sens will give additional information about the allergen sensitivity, i.e. how much allergen the basophils will tolerate before they react. CD-sens has been launched as a complement to allergen provocation tests in allergic rhino-conjunctivitis (19) and asthma (24).

All children positive in CD-sens to peanut were also positive in CD-sens to Ara h 2. It should be noted that the CD-sens value completely depends on the concentration and the purity of the allergen extract. It is not possible to compare extracts of different allergens as they are not, despite how they are marketed, standardized. A benefit of using a recombinant allergen like Ara h 2 is the knowledge of the exact protein content. However, pure proteins can sometimes be difficult to handle because of the extreme low protein concentrations needed and solubility. The advantage of using a crude peanut extract is that the same batch could be used for both CD-sens and DBPCFC. We suggest that stimulation

with peanut is sufficient when using CD-sens in the diagnostic work-up in peanut allergy.

Two children with a positive DBPCFC had basophils with a low response, <16%, to anti-FcεRI, which was used as the positive control, i.e. they are 'low responders'. The term 'non-responders' is well documented (20, 21) and 'low responders' have also been discussed previously (21). In the present study, 92% of the children with a positive DBPCFC had a positive CD-sens to peanut and Ara h 2, and if the low responders were excluded, the concordance between CD-sens and DBPCFC was 100%. Thus, among patients with a convincing history of peanut allergy who are non- or low-responders in CD-sens, an oral peanut challenge has to be recommended.

In contrast to the study by Rubio *et al.* (21), the present study shows that a negative CD-sens seems to be reliable for predicting tolerance as all the children with a negative CD-sens were negative in DBPCFC. Furthermore, children positive in DBPCFC had significantly higher CD-sens values than those negative in DBPCFC. This is in line with a recent publication showing positive correlations between basophil stimulation and cow's milk allergy (21). In our study, three children negative in DBPCFC were weakly positive in CD-sens to peanut. Interestingly, the same children also had IgE-ab to Ara h 2 > 0.1 (range 0.2–1.9) kU_A/l. The positive CD-sens in these IgE-sensitized children may be an early sign of a developing peanut allergy and should be clinically followed-up.

For CD-sens, the technical reporting limit was used as cut point in the discrimination between tolerant and intolerant patients. It is possible to find cut points for CD-sens and for IgE-ab using, for example ROC analysis that compared with the technical reporting limit has a lower number of misclassified patients in relation to DBPCFC. At these cut points, the performance for e.g. CD-sens to peanut and IgE-ab to Ara h 2 are very similar. But the not equal number of patients for the tests caused by the low responders without result and patients with positive but not quantifiable result in CD-sens make a direct comparison between the methods complicated. Also as a result of the limited number of patients in this study, the cut points are very unreliable. ROC-curves for CD-sens to peanut and Ara h 2 and IgE-ab to peanut and Ara h 2 with corresponding listings of results in Data S2.

In the present study, we show that children with a positive DBPCFC have significantly higher levels of IgE-ab to Ara h 1, 2 and 3 compared with those negative in DBPCFC. This is in line with a previous report where peanut allergy has been confirmed with food challenge (16). In particular, IgE-ab to Ara h 2 has previously been linked to peanut allergy, which is confirmed by our study. Ara h 8 is a peanut component cross-reactive with deciduous trees, and individuals who, for example are birch allergic can therefore appear positive for IgE-ab to peanut. In our study, we did not see any significant difference between the IgE-ab levels to Ara h 8 in relation to the outcome of DBPCFC. As there are birch-allergic children in both groups, these findings are expected.

There is no treatment today for peanut allergy except avoidance, but in the future, anti-IgE-ab may be used as

therapy. An earlier study has shown that a small IgE-ab fraction, <1% clinically relevant IgE-ab of IgE, is important for successful anti-IgE treatment (25). We have seen that the IgE-ab fraction differs significantly between children positive and negative in DBPCFC. Children positive in DBPCFC have significantly larger IgE-ab fractions, 25% to peanut and 11% to Ara h 2 than those negative. This suggests that the fraction size should be considered before starting treatment with anti-IgE in patients with food allergy.

The strength of this study is that all children underwent DBPCFC, their IgE-ab levels to peanut, Ara h 1, 2, 3, 8 and 9 were quantified, and CD-sens to both peanut and Ara h 2 were measured. Although this is rather a small study, we believe that the results are of general importance as they are in line with other studies on peanut allergy (15, 16).

In summary, in this study, a negative CD-sens to peanut excludes peanut allergy. Both diagnostic tests, CD-sens to peanut and IgE-ab to the peanut components, especially to Ara h 2, appear to be safe, time saving and cost-effective complements to DBPCFC.

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Authors contributions

S.G.O Johansson, Anna Nopp, Magnus Borres and Caroline Nilsson conceived the idea and designed this study. Susanne Glaumann has under supervision from Caroline Nilsson performed the DBPCFC. Anna Nopp performed the laboratory work-up, and Magnus Rudengren carried out the statistic analyses together with Susanne Glaumann. The manuscript has been written by Susanne Glaumann together with all the contributing authors.

Conflict of interest

Magnus Borres and Magnus Rudengren are employed at Thermo Fisher Scientific, Uppsala, Sweden. All other authors have declared they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article found at: www.wileyonlinelibrary.com

Figure S1. Dose-response curves, CD-sens.

Table S1. Patient positive in DBPCFC.

Data S1. CD-sens method.

Data S2. ROC curves for CD-sens to peanut, CD-sens to ara h 2, IgE-ab to peanut and IgE-ab to ara h 2 in relation to DBPCFC.

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Patient positive in DBPCFC

Patient negative in DBPCFC

numberPatient	sex	(years)Age	1st active Symptoms challenge	Severity score	(g)Amount peanut	peanutCD-sens	Ara h 2CD-sens	(kU _A /L)peanutIgE-ab	(kU _A /L)Ara h 1IgE-ab	(kU _A /L)Ara h 2IgE-ab	(kU _A /L)Ara h 3IgE-ab	(kU _A /L)Ara h 8IgE-ab	(kU _A /L)Ara h 9IgE-ab
5	female	13.8	No symptoms	0	6.111	<0.1	<0.1	1.0	<0.1	<0.1	<0.1	39.1	<0.1
6	female	18.2	No symptoms	0	6.111	<0.1	<0.1	1.0	<0.1	<0.1	<0.1	16.0	<0.1
10	male	14.7	No symptoms	0	6.111	<0.1	<0.1	1.3	<0.1	<0.1	<0.1	6.1	0.7
11	male	7.7	No symptoms	0	6.111	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	6.5	<0.1
12	male	9.9	No symptoms	0	6.111	<0.1	<0.1	0.2	<0.1	<0.1	1.9	57.3	<0.1
13	male	9.2	No symptoms	0	6.111	<0.1	<0.1	0.9	0.1	<0.1	0.1	14.8	0.1
14	female	13.7	No symptoms	0	6.111	<0.1	<0.1	0.3	<0.1	<0.1	<0.1	<0.1	<0.1
16	female	6.0	No symptoms	0	6.111	<0.1	<0.1	36.6	0.1	0.1	0.1	929.0	0.3
18	male	14.7	No symptoms	0	6.111	0.4	3.7	0.2	<0.1	0.2	<0.1	<0.1	<0.1
21	female	10.1	No symptoms	0	6.111	<0.1	<0.1	46.9	0.1	<0.1	5.8	0.1	0.1
26	female	6.8	No symptoms	0	6.111	<0.1	<0.1	4.4	14.1	<0.1	0.8	4.8	34.9
27	male	11.7	No symptoms	0	6.111	0.5	33.2	1.0	<0.1	0.9	<0.1	3.2	<0.1
29	male	14.1	No symptoms	0	6.111	0.3	25.6	9.1	2.1	1.9	1.9	<0.1	<0.1

1 OBJECTIVE

The objective with this document is to describe how to measure basophil allergen threshold sensitivity from CD63 up-regulation (CD63-sensitivity = CD-sens).

PROCEDURE

1.1 Short description of method

Human basophils can be challenged *in vitro* with allergens. The resulting up-regulation of a cell surface activation marker, CD63 can be studied by means of flow cytometric analysis of a cell population identified by the basophil specific marker CD203c.

Heparinized venous blood is used as source of basophils. The blood is kept at +4°C for a maximum of 24 hours before testing. Briefly, a small volume of blood (100 µl) is incubated at +37°C for 20 min with several dilutions of an allergen. An antibody, cross-linking the FcεRI, is used as positive control.

First a phycoerythrin (PE) conjugated anti-CD203c mAb is used for identification of basophils. As a second step the cell surface expression of CD63 is determined using a fluorescein isothiocyanate (FITC) conjugated anti-CD63 mAb. The CD203c antibody has been shown to be specific for basophils and does not stain other white blood cells (H.J Buhning et.al. Blood: 1999 7, 94, 2343-2356).

Following allergen stimulation the two conjugated antibodies are added and incubated for 25 min at +4°C. This is followed by lysis of the red blood. The remaining white blood cells are washed and resuspended in phosphate buffered saline (PBS). Surface CD63 expression is measured by two-colour flow cytometric analysis.

The lowest allergen concentration giving 50% (LC50) of the maximal CD63 up-regulation is defined as the threshold sensitivity.

1.2 Protocol

Stimulation procedure:

1. Collect peripheral blood samples aseptically into sterile evacuated blood collection tubes with sodium-heparin as anticoagulant. The blood samples should be stored at +4°C until processing.
2. Specimens should optimally be less than 24 hours old for processing. However, if required samples not older than 48 hours can be retested.
3. Equilibrate the blood sample to room temperature and ensure homogeneity by putting the blood tube on a rocking table for five minutes.
4. Dilute the allergen. Prepare ten-fold dilutions (if possible, also a 1/3 step, i.e. half a 10-log, between) of the allergen covering a broad concentration range (approximately, 8-10 dilution steps). It is necessary to dilute the allergen concentration properly so that the CD sensitivity can be calculated.
5. Set up and mark appropriate numbers of plastic tubes for staining.
6. Carefully add 100 µl of blood to each tube. Avoid contamination of the tube walls with blood.

7. Add stimuli; 100 μ l of each allergen dilution to each tube.
8. Add 25 μ l of the positive control, anti-Fc ϵ RI and 100 μ l RPMI (negative control) to two separate tubes.
9. Gently vortex each tube and incubate at +37°C for 20 min.
10. Put the tubes on ice for 5 min.

Staining procedure:

11. Add 20 μ l of the conjugated anti-CD63 and anti-CD203c to each tube.
12. Gently vortex each tube and incubate for 25 min on ice, protected from light.
13. Add 2 ml of lysing solution to each tube, vortex gently.
14. Incubate at +4°C for approx. 5 minutes.
15. Centrifuge for 5 minutes at 300g and carefully remove the supernatant. Avoid aspiration of the white blood cell pellet.
16. Add 2 ml of PBS to each tube.
17. Centrifuge for 5 minutes at 300g, carefully remove the supernatant. Gently resuspend the cell pellet by tapping the tube base. Add 0.3 ml PBS.
18. Acquire at least 200 basophils on the flow cytometer.

Processed samples must be stored at +4°C, protected from light, pending analysis.

1.3 Flow cytometer analysis

The flow cytometer must be equipped to detect forward scatter (FSC), side scatter (SSC) and with at least two fluorescence channels allowing the analysis of FITC- and PE-conjugated antibodies.

Ensure that the flow cytometer is properly aligned and standardized for fluorescence intensity (according to the manufacturer recommendations). Also, ensure that the flow cytometer is properly adjusted for fluorescence compensation.

The basophil population is selected using the CD203c-PE versus SSC plot. A region is created around the CD203c+/SSC^{low} cell population. After gating of the basophil population the proportion of cells expressing CD63-FITC can be estimated. The percentage of CD63-positive basophils in the control samples was below 1.5% and the cut-off determining a positive test was set to 5%.

1.4 Calculation of CD63-sensitivity (CD-sens)

Percent CD63-positive basophils is plotted in a graph versus allergen concentration (logarithmic scale). Choose the allergen concentration giving the maximal CD63 up-regulation and two more allergen concentrations on the up-going dose-response curve. Plot the percentage of CD63 positive basophils against the corresponding allergen concentration in an equation for calculation of a straight line. The computer will calculate the allergen concentration giving 50% of maximal CD63 up-regulation. The term CD63-sensitivity (CD-sens) is defined as the inverted value for the lowest allergen concentration giving a 50% (LC50) of maximum CD63% up-regulation multiplied by 100 and used to describe a patient's allergen sensitivity.

1.5 Definition of non- and low responders

Individuals with basophil, which after anti-FcεRI stimulation, i.e. the positive control, responded with 0-5% CD63-upregulation were regarded as non-responders. For individuals with a response between 5-16% (low-responders) the results should be interpreted with caution. A positive result from allergen stimulation of a low-responder can only be regarded as positive but no further CD-sens calculation should be made. If the allergen stimulation becomes negative no conclusion can be made and further testing with other methods should be performed. The cut off 16% (mean 76% -3SD) was calculated from the positive controls of an in-house reference material of 264 allergic children and adults.

1.6 Materials

Source of human basophils:

Sodium-heparinized blood, (0-24 hours old), kept at +4°C, until use.

Plastic test tubes (12 x 75 mm), used for staining.

Vortex mixer

Waterbath, +37°C

Centrifuge

Timer

Calibrated pipets (20 µl, 100 µl, 1000 µl) and disposable tips

Reagents:

- Fluorescein isothiocyanate (FITC) anti-human anti-CD63 (Beckman Coulter, #PN IM1165U)
- Phycoerythrin (PE) anti-CD203c, (Beckman Coulter, #PN IM3575)
- Anti-FcεRI, positive control, (Flowcast, # B-BSTCON, Bühlmann laboratories, Allschwil, Switzerland)

1.7 Solutions

RPMI 1640 with 25 mM hepes and Glutamax (Hyclone, #SH30255.01)

RPMI is used as negative control and diluent.

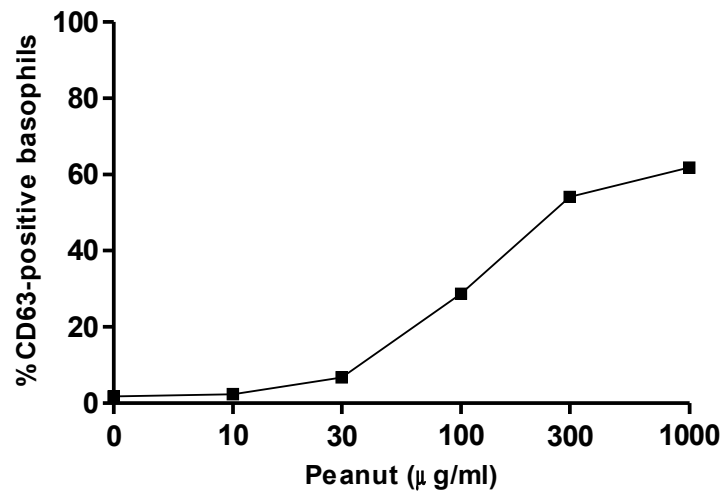
Lysing solution

NH ₄ Cl	(Merck, 1145)	mw 53,49	4,13g
KHCO ₃	(Merck, 533 A 4054)	mw 100	120,50g
EDTA (4xNa)	(Sigma ED4SS)	mw 380,20	0,019g
Water			500 ml

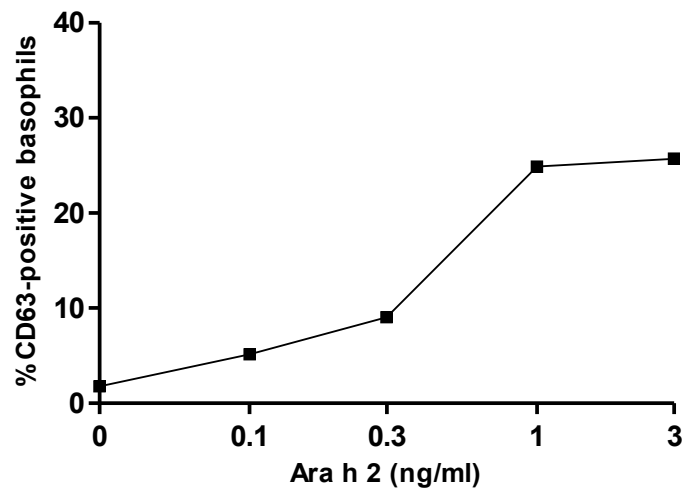
PBS

Phosphate buffered physiological saline, pH 7.4

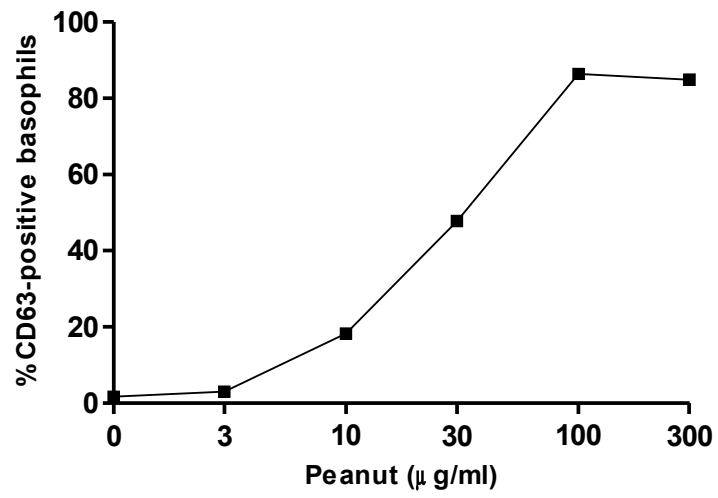
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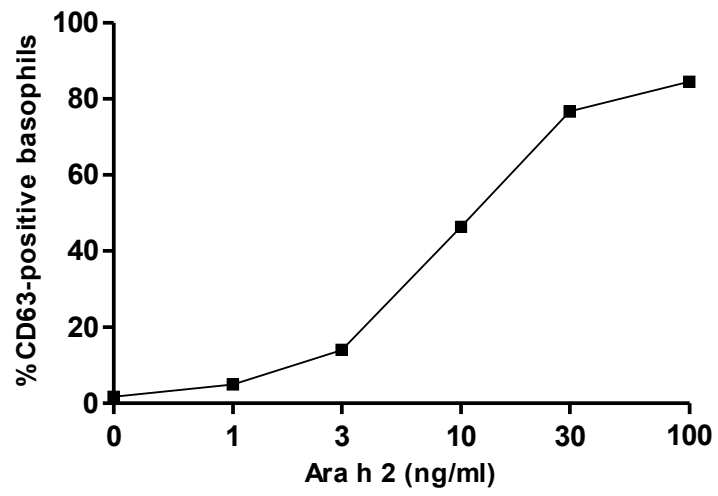
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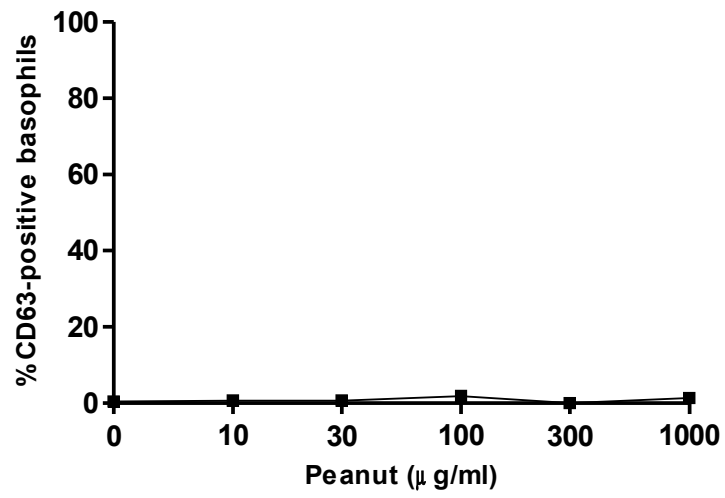
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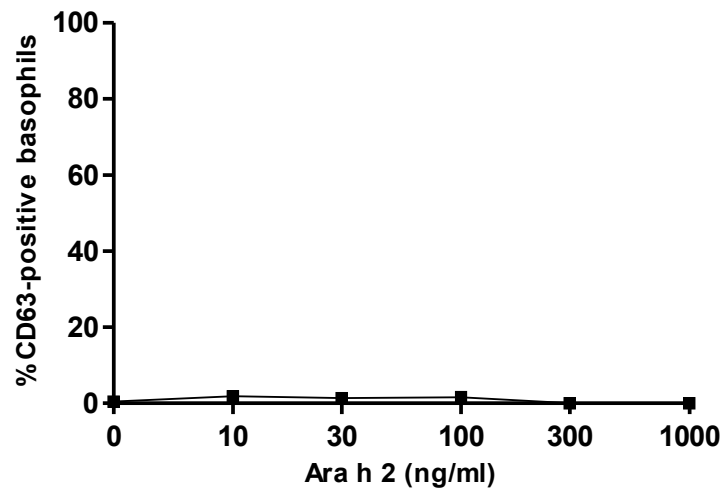
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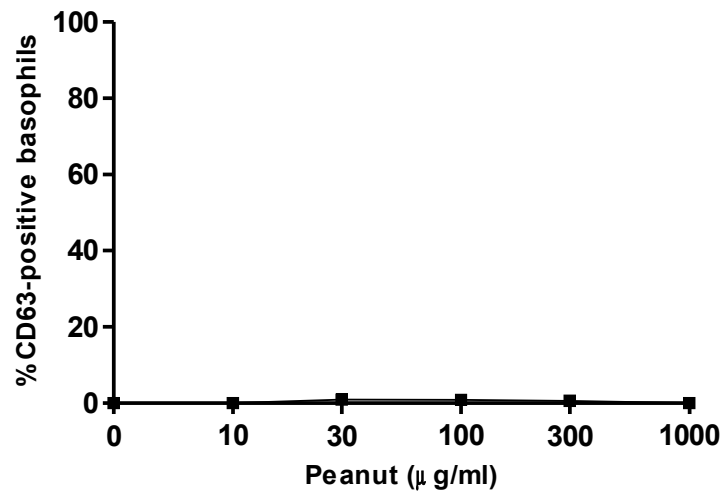
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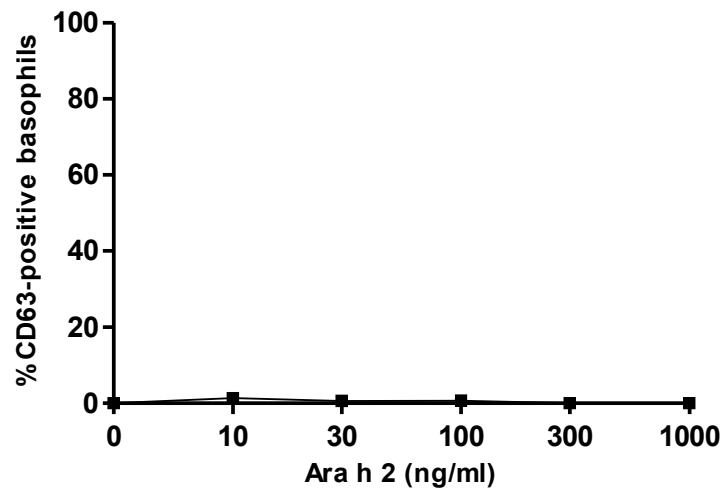
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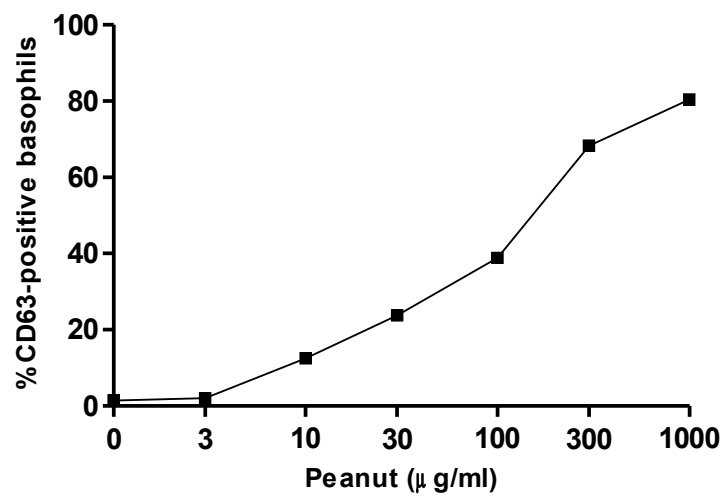
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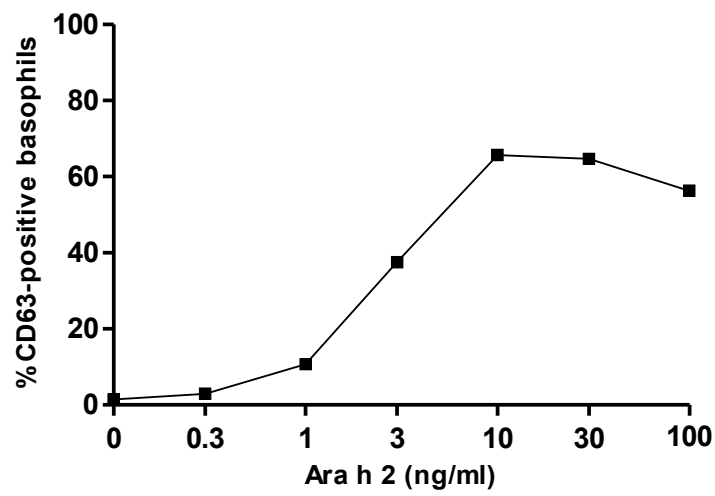
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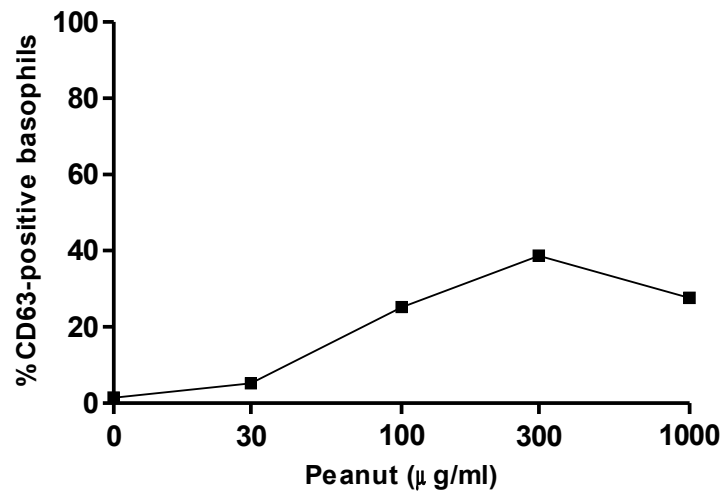
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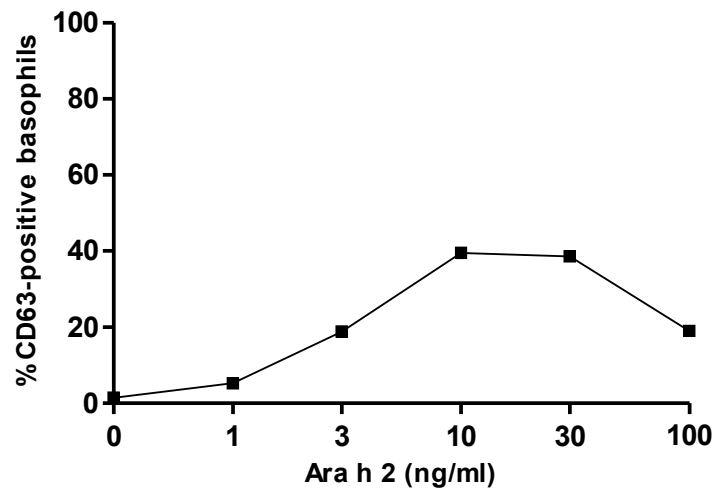
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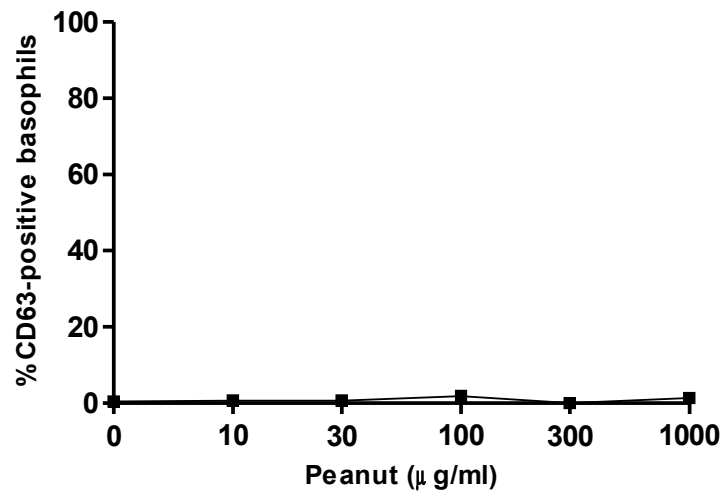
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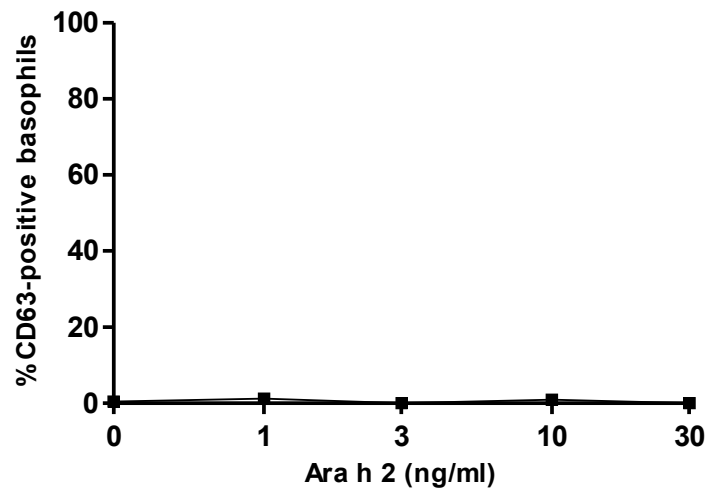
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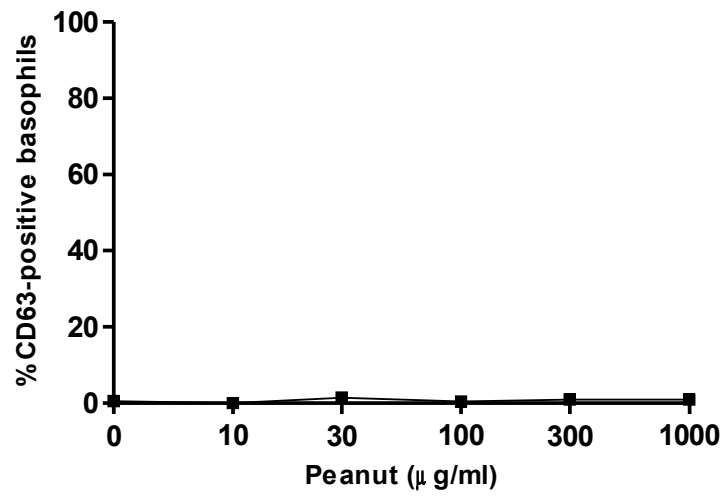
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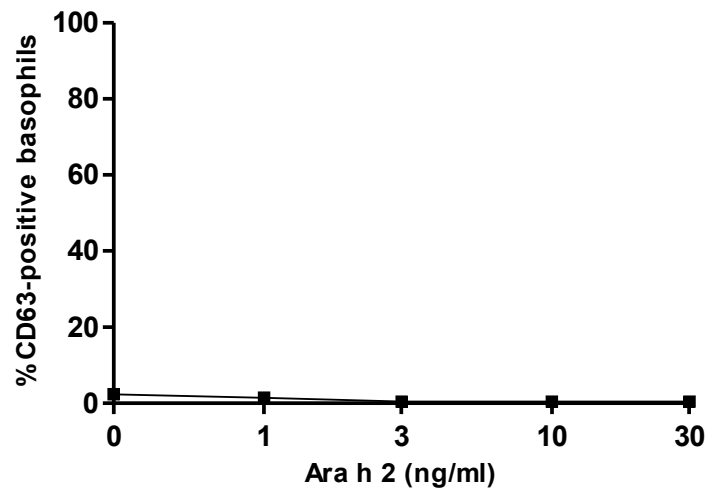
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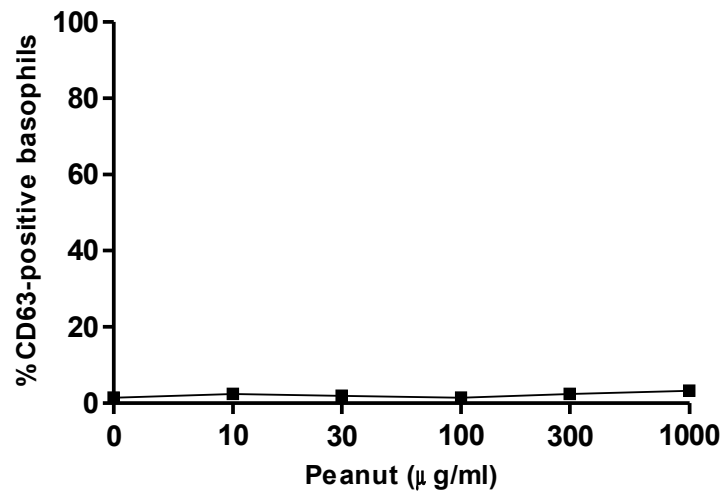
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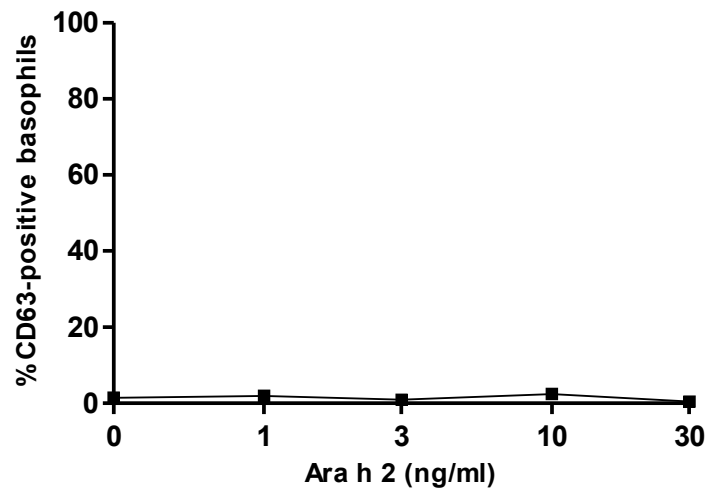
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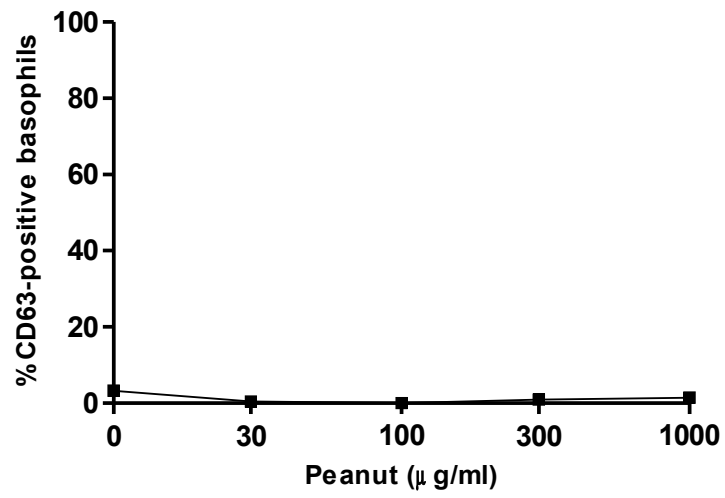
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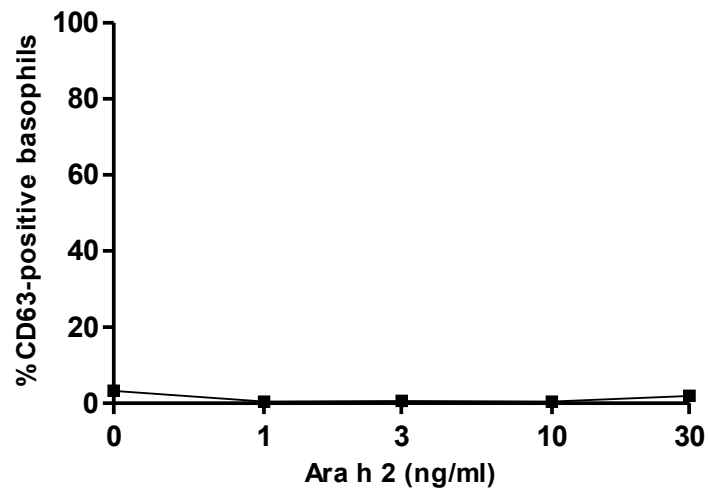
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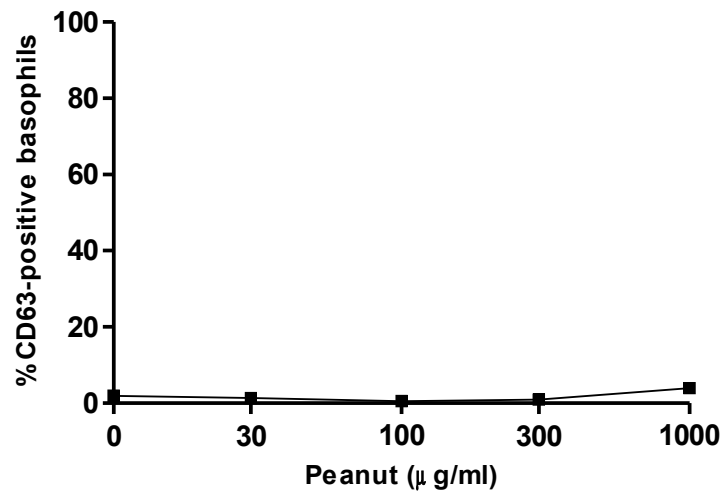
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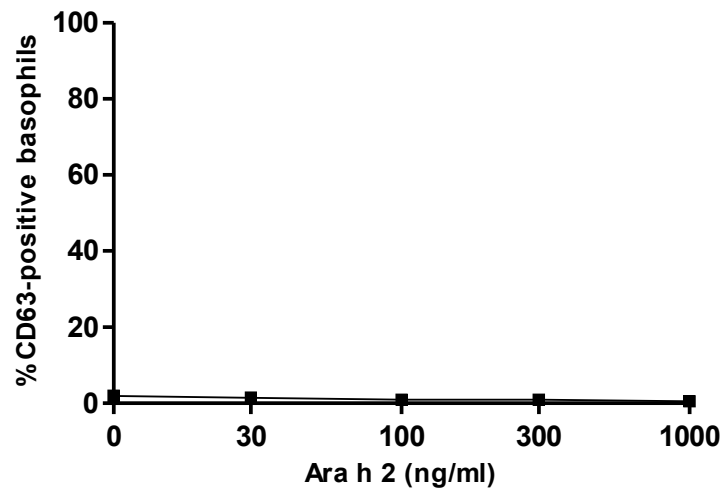
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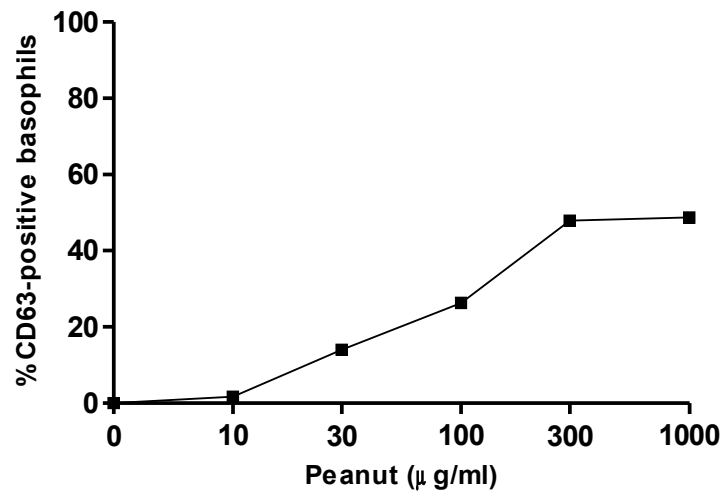
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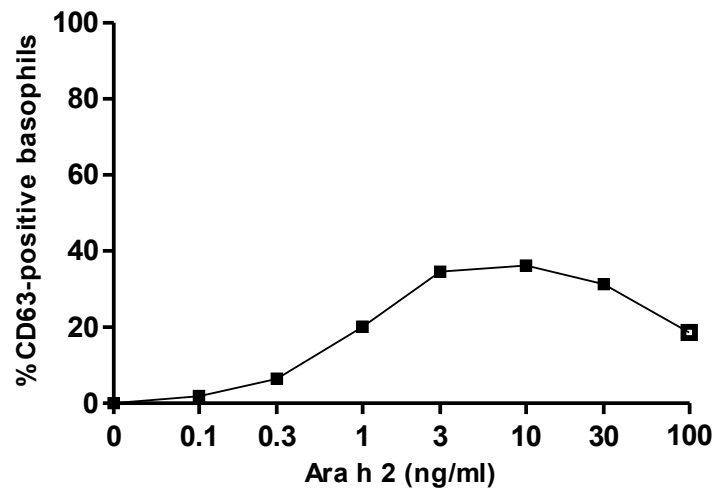
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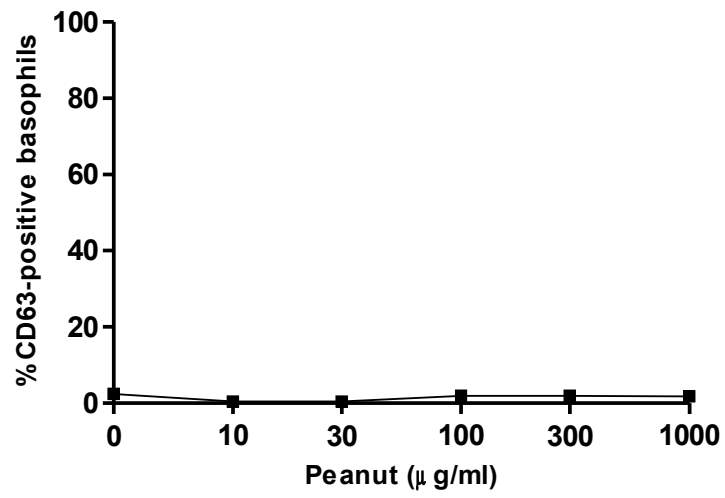
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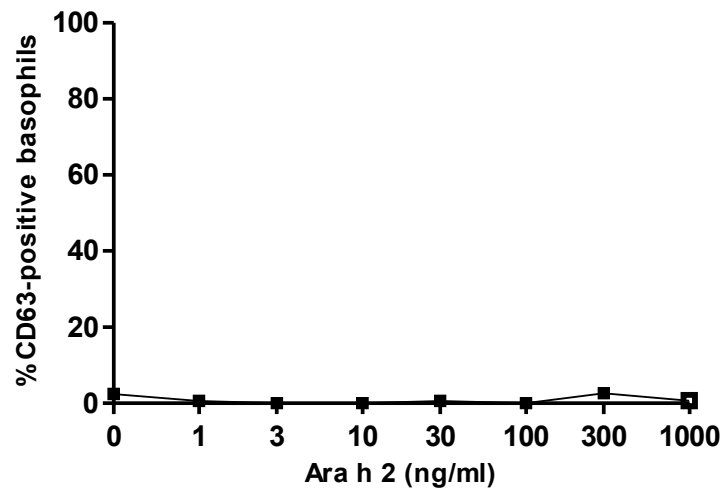
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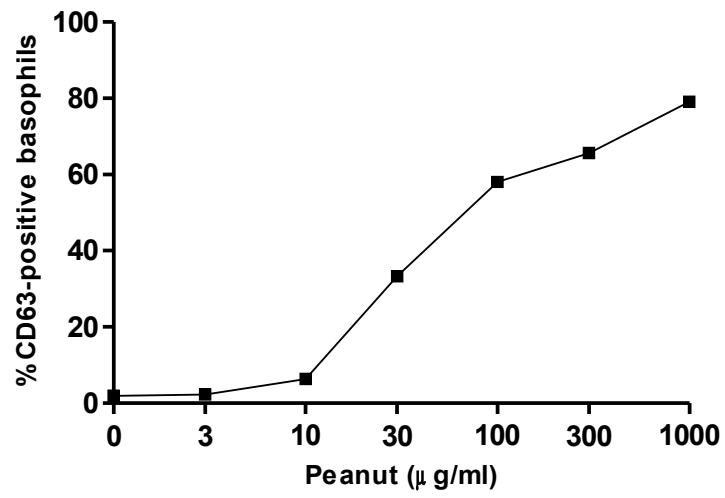
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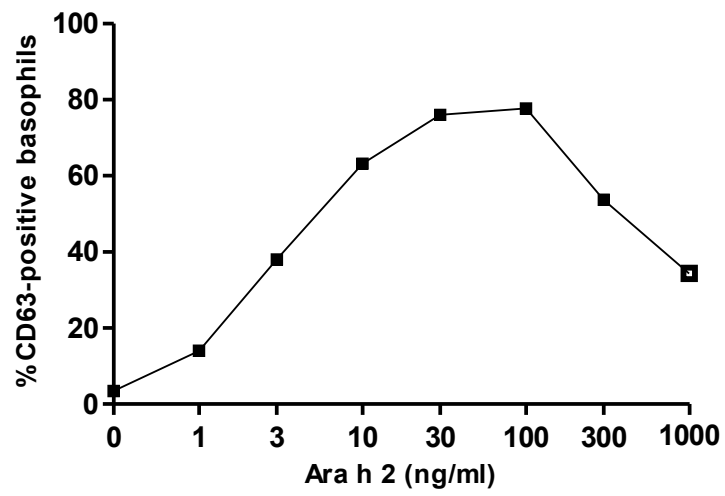
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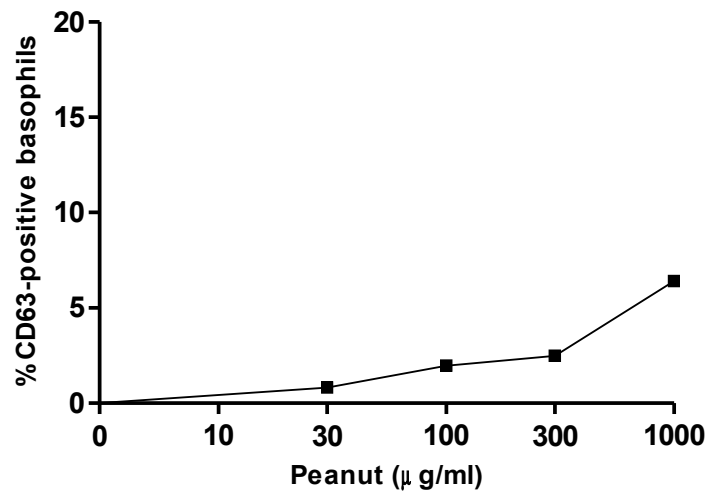
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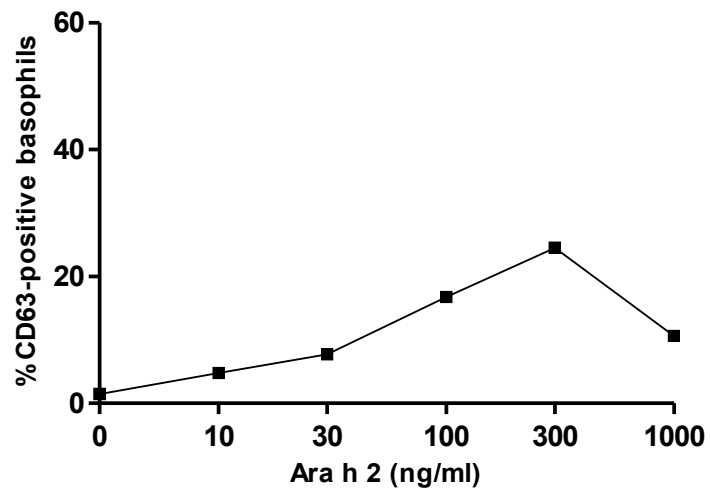
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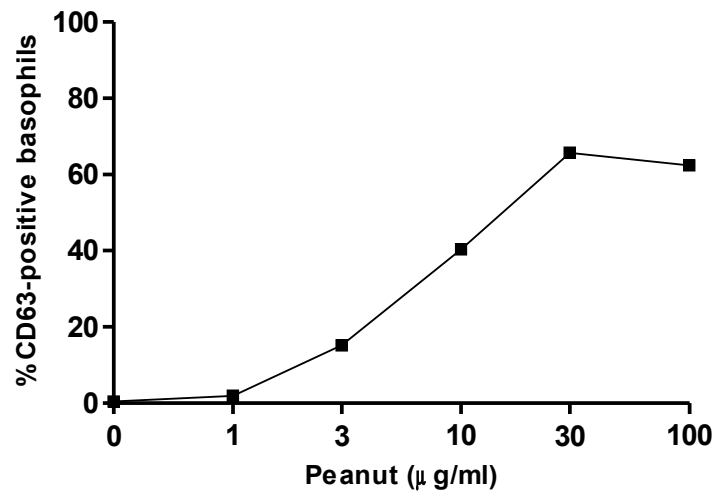
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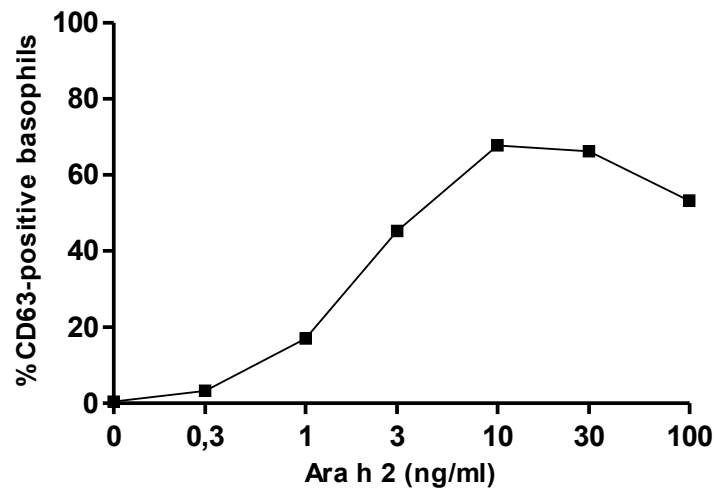
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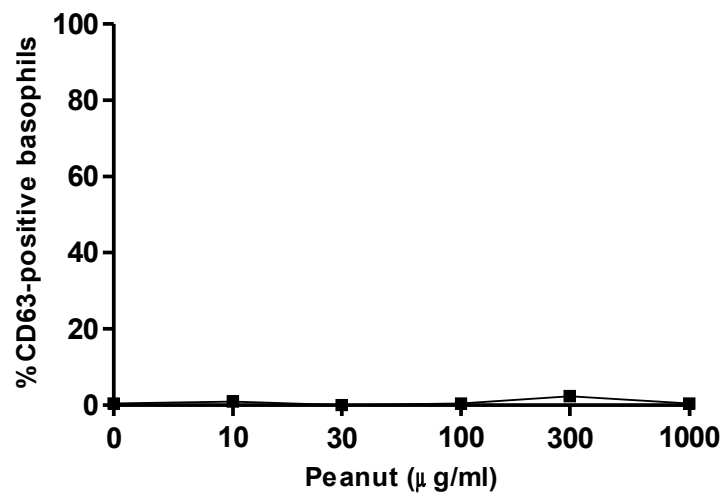
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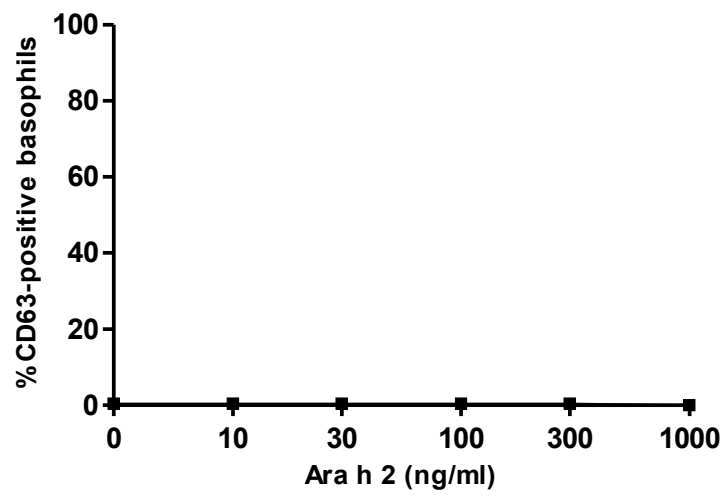
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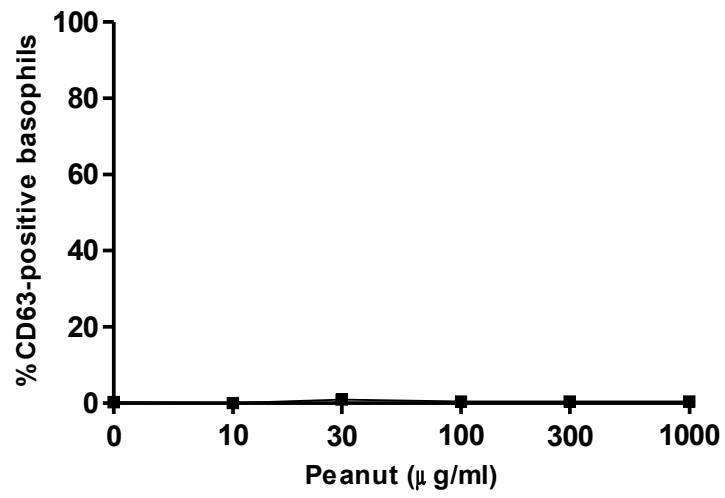
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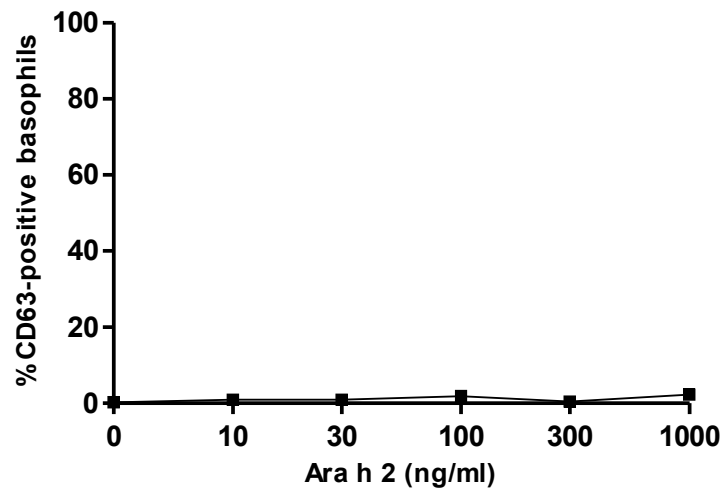
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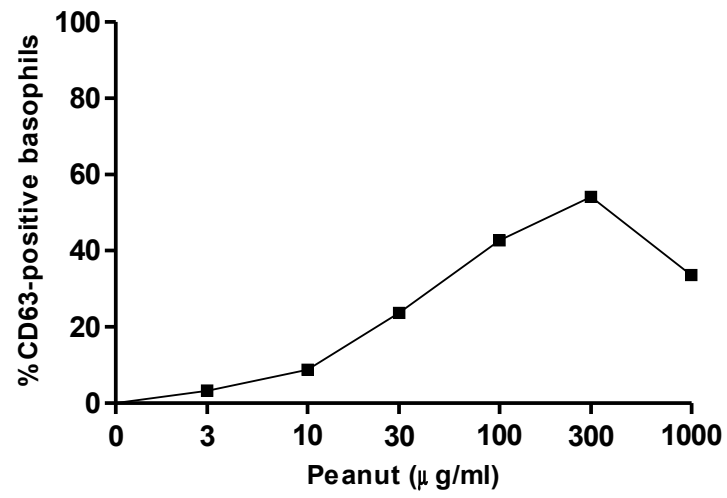
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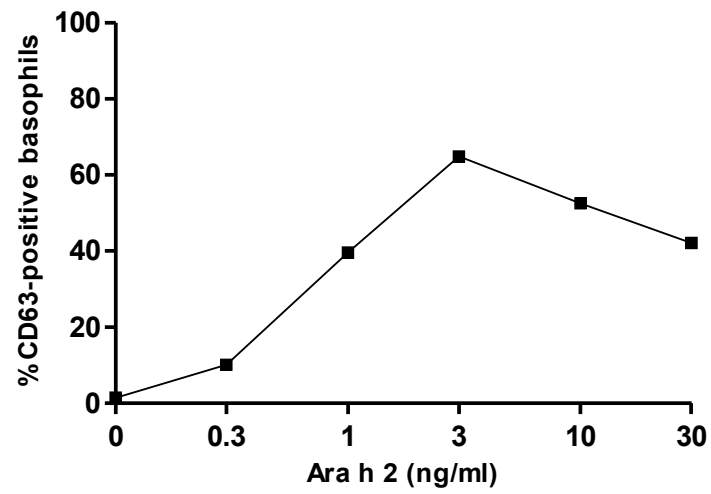
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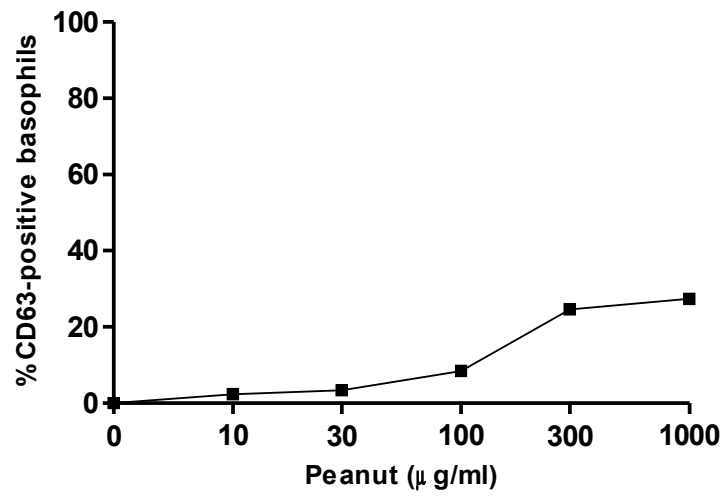
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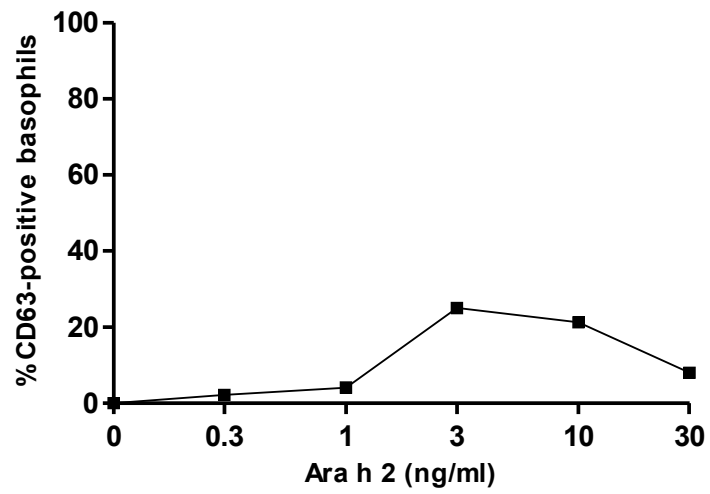
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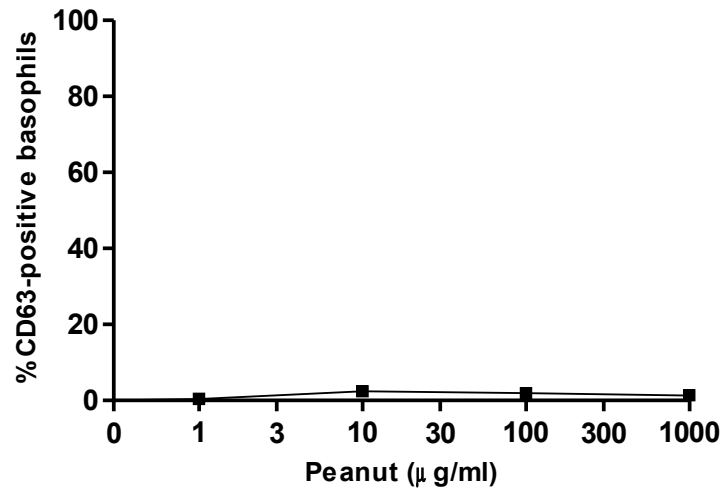
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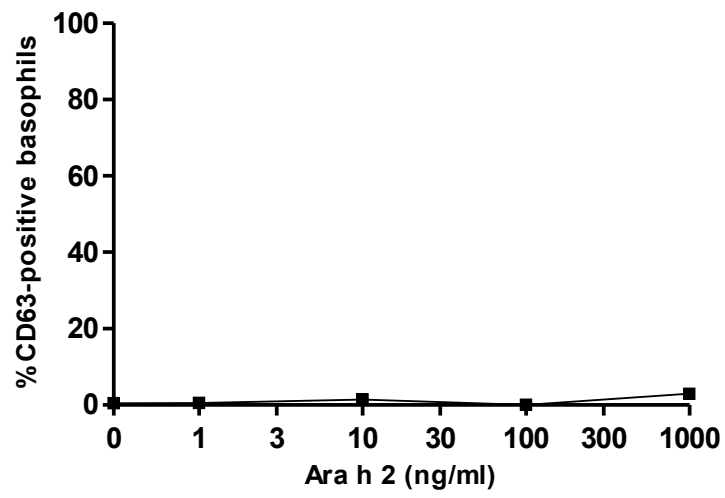
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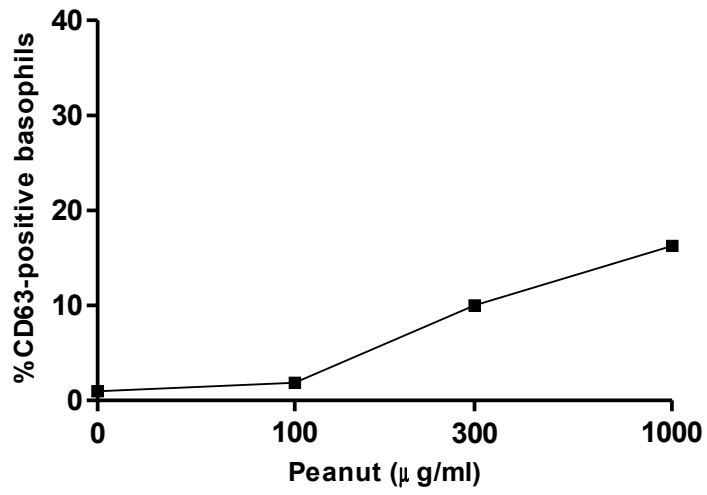
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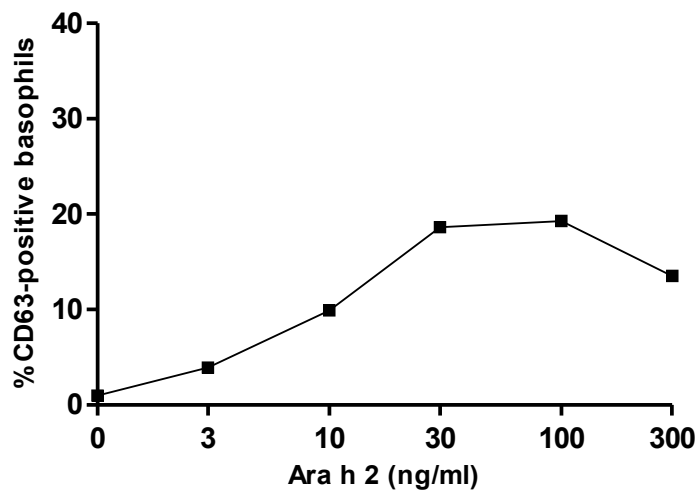
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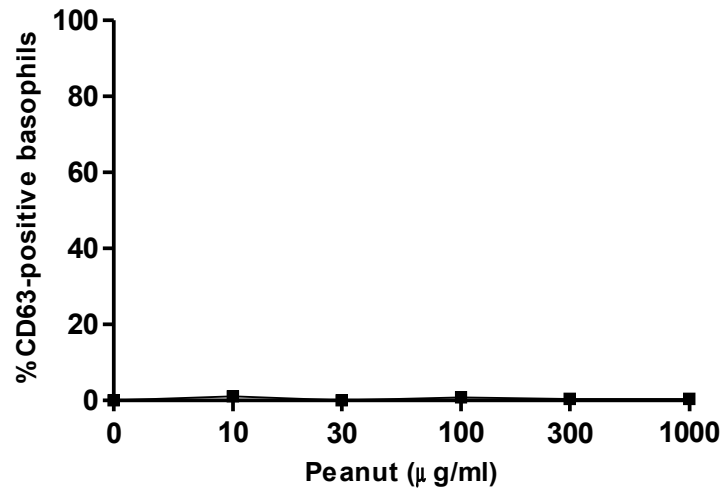
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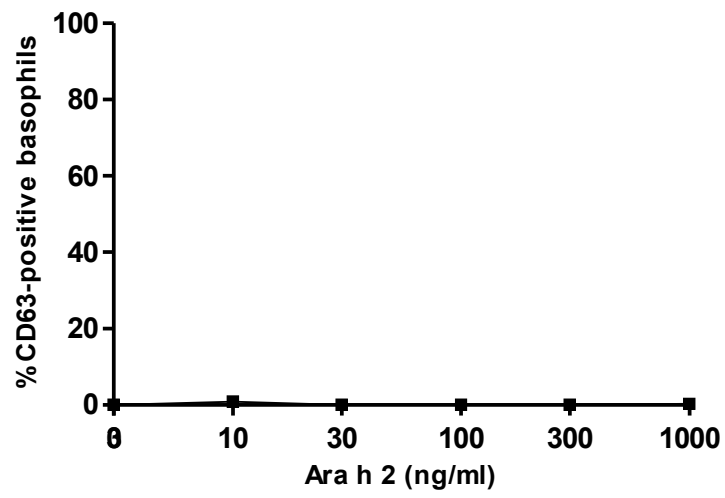
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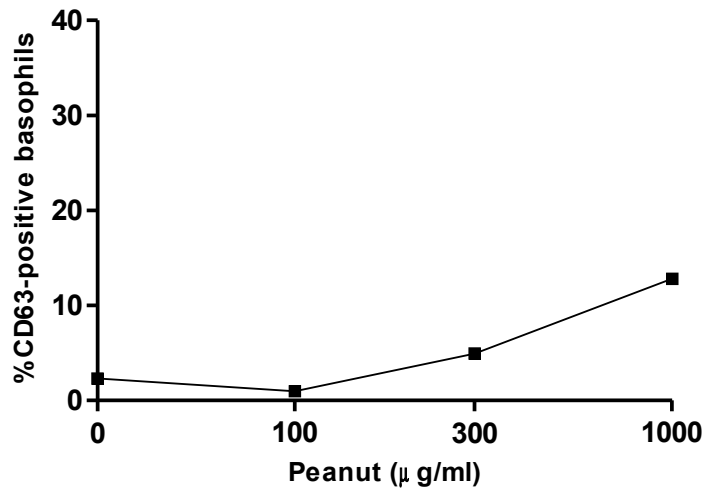
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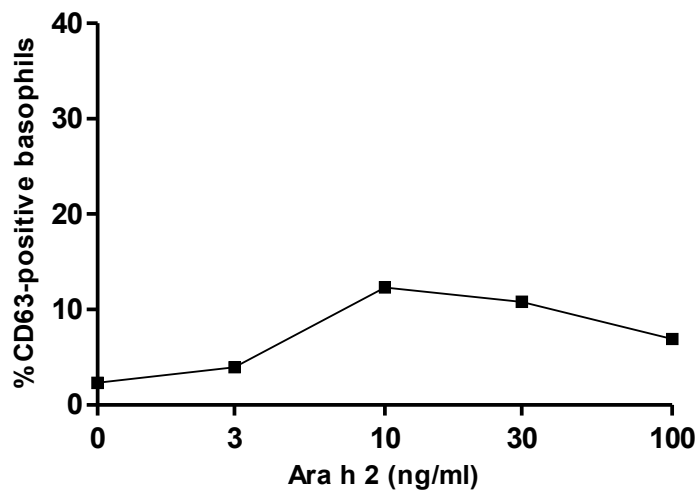
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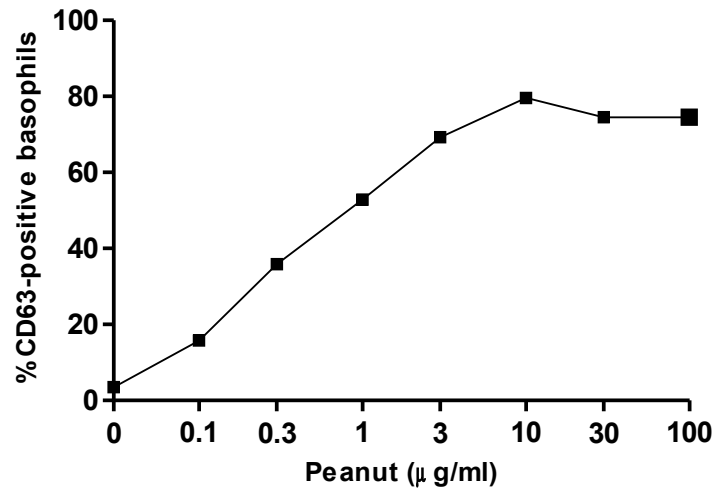
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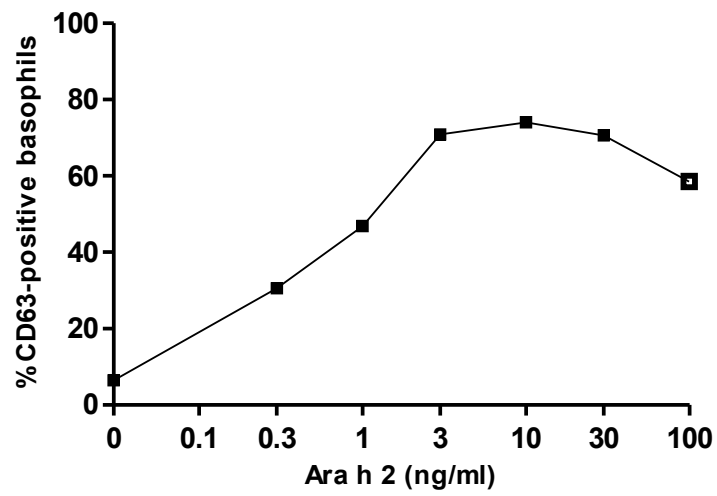
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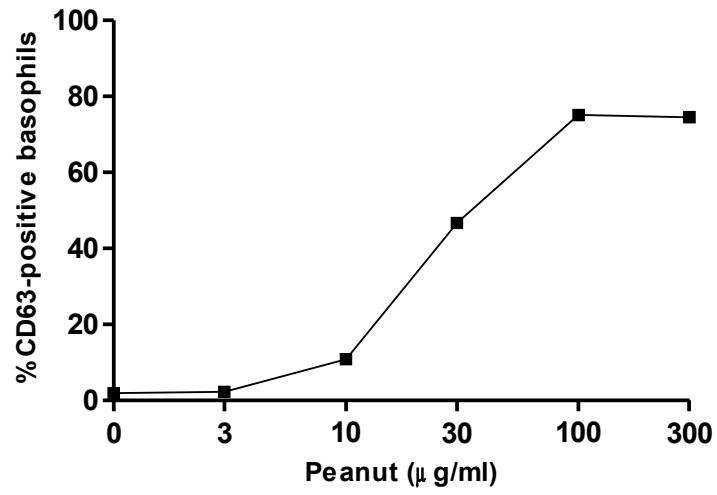
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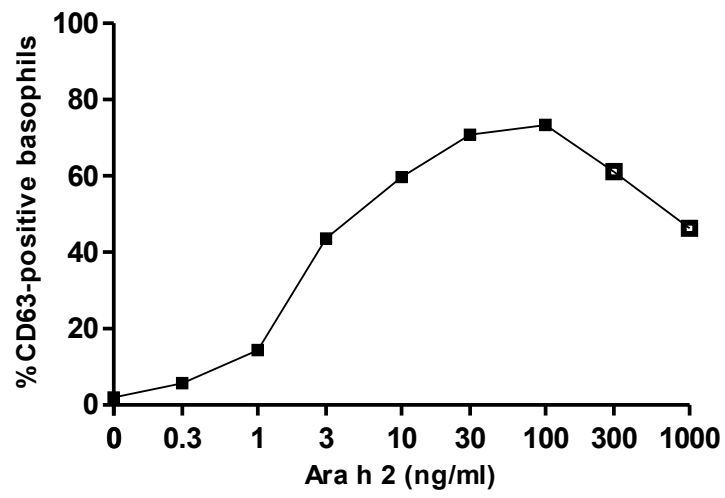
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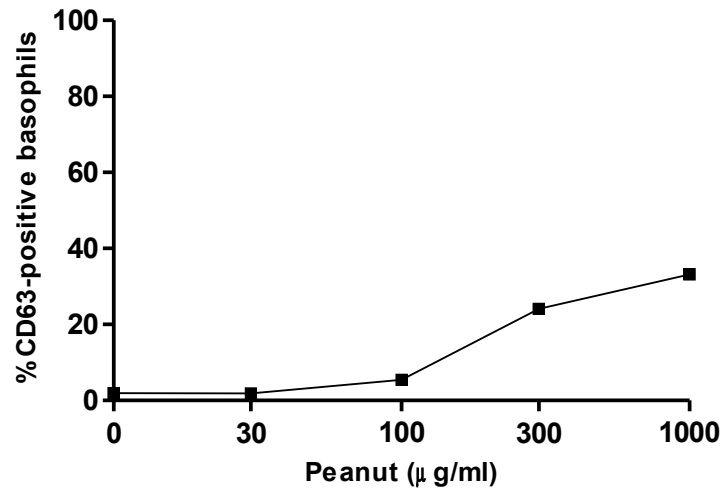
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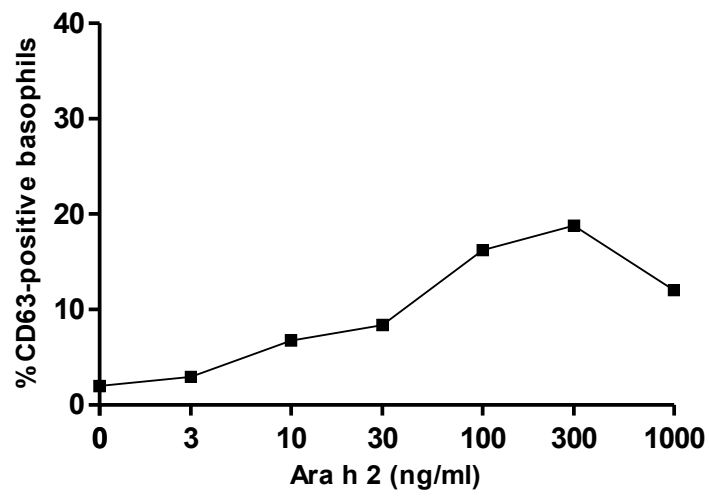
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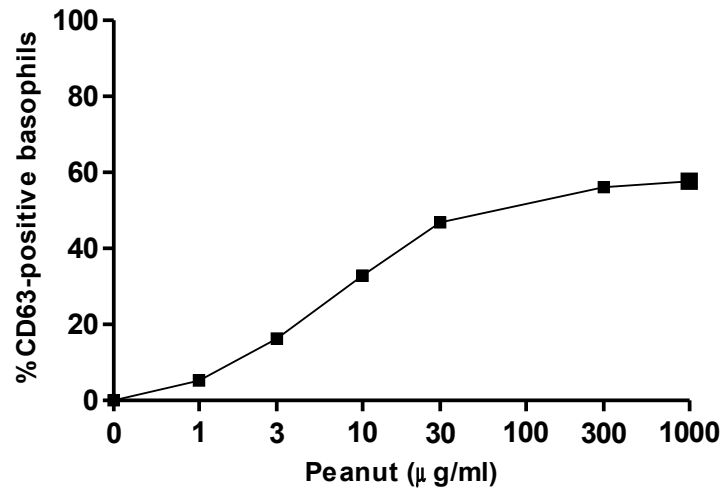
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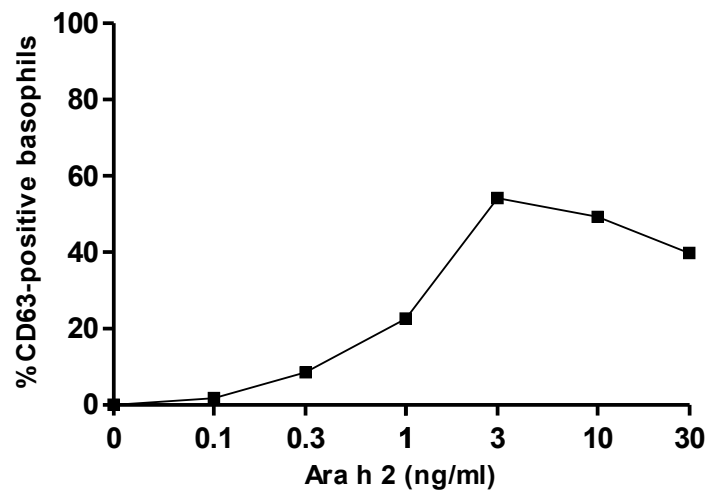
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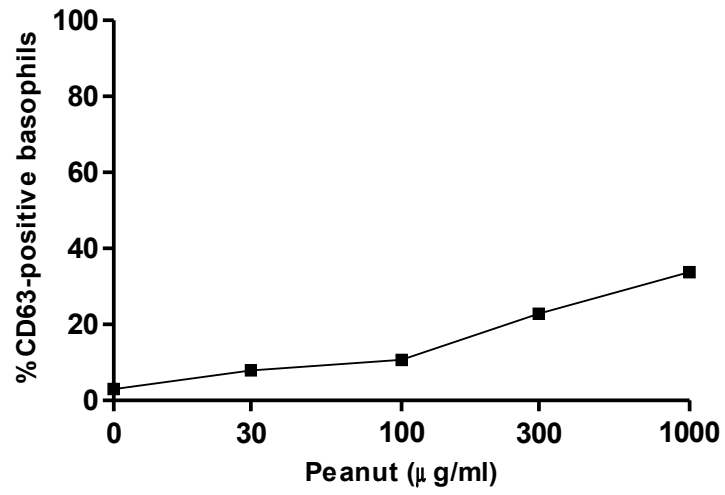
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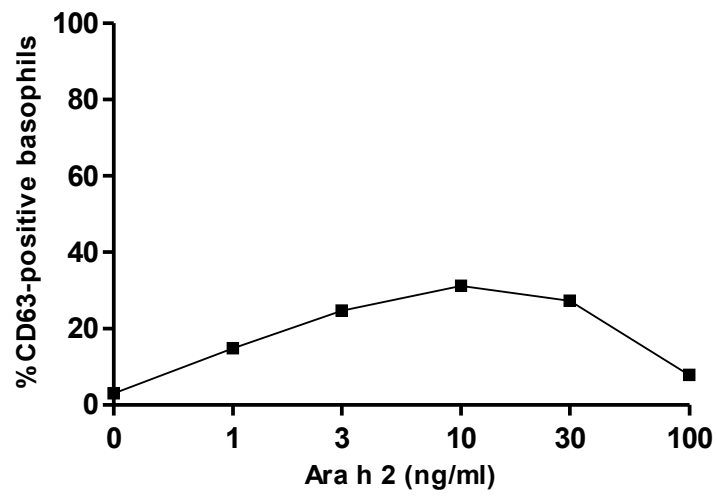
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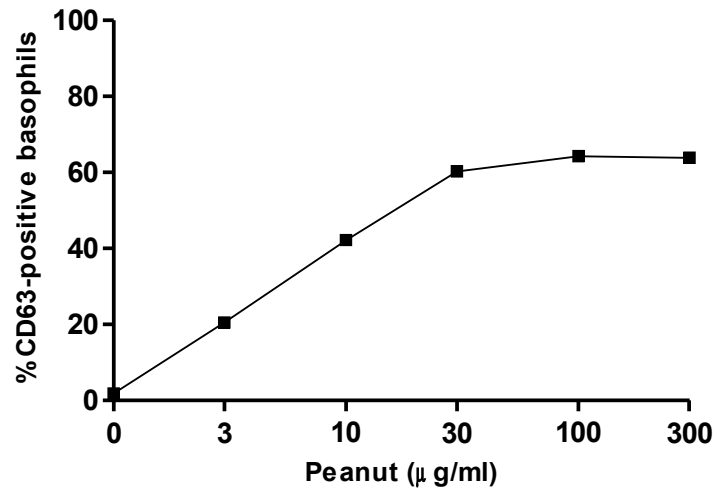
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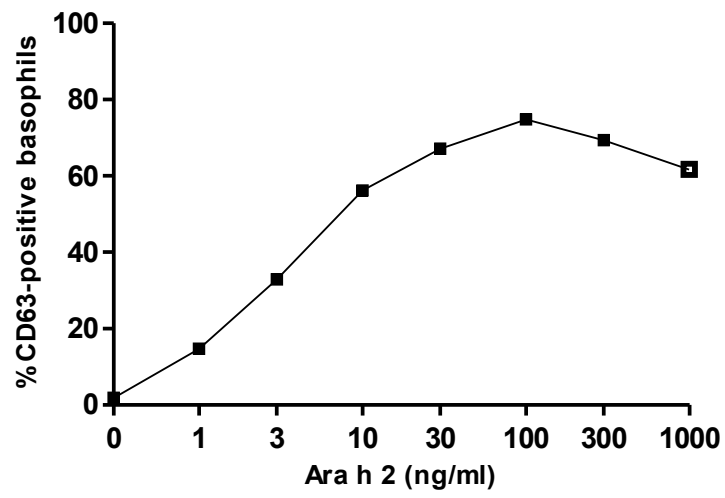
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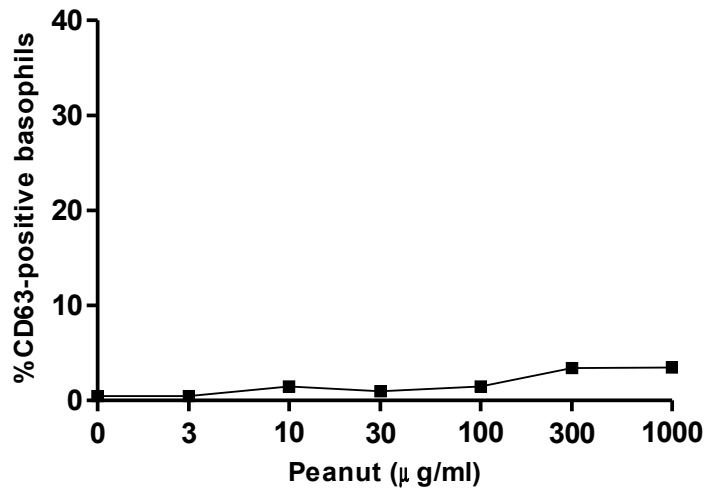
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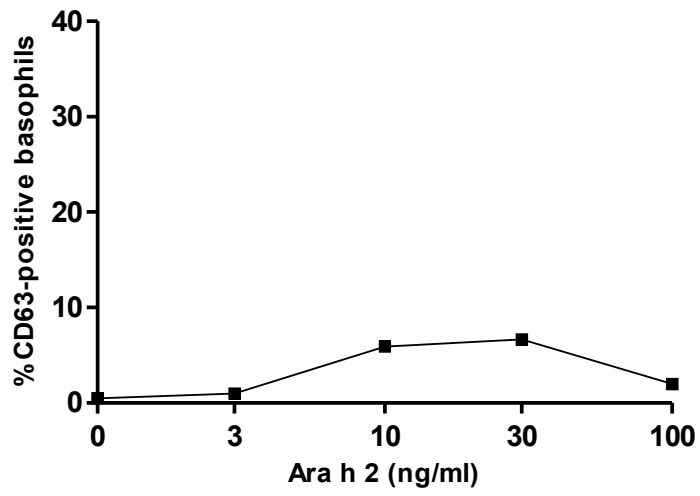
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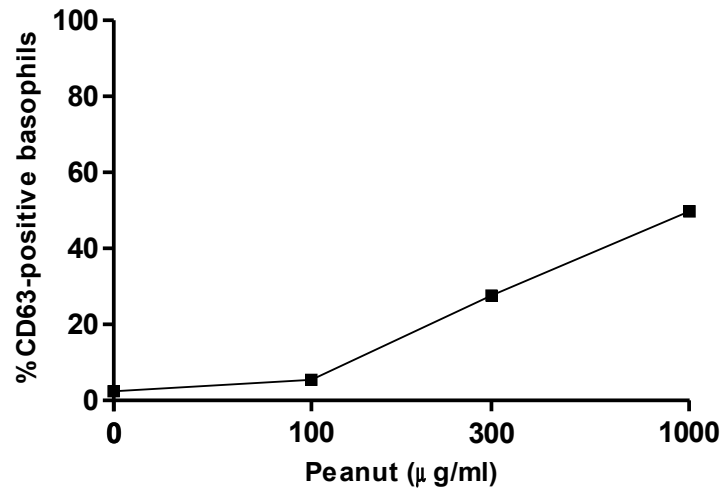
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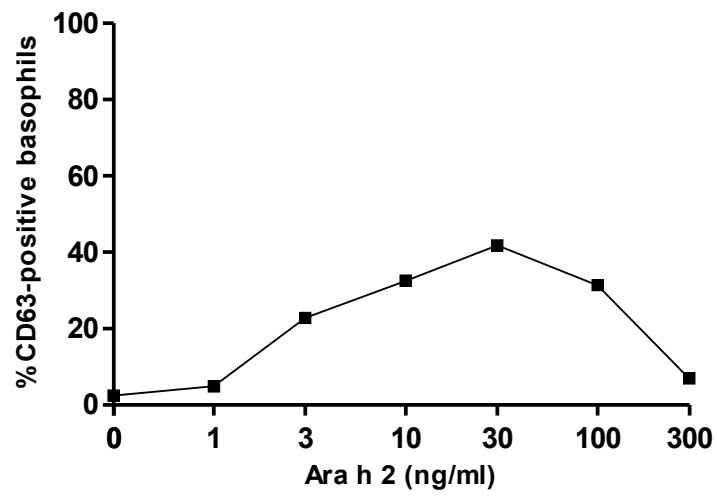
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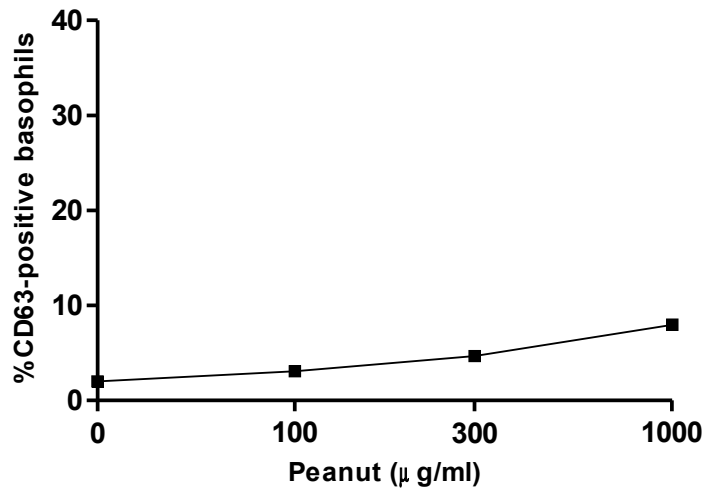
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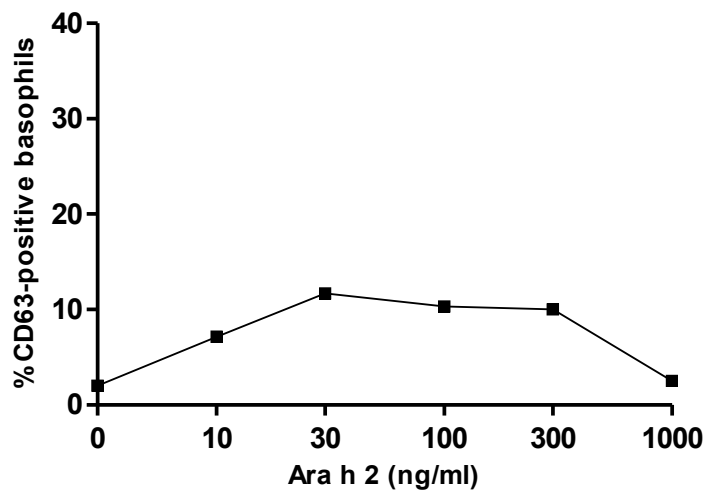
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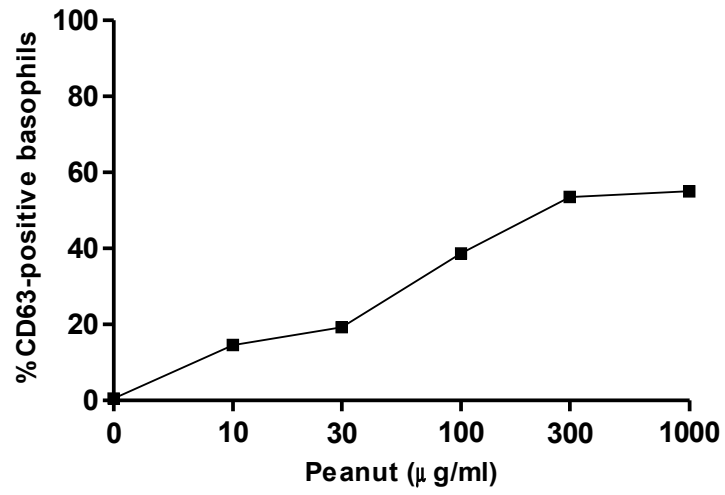
Pat 39



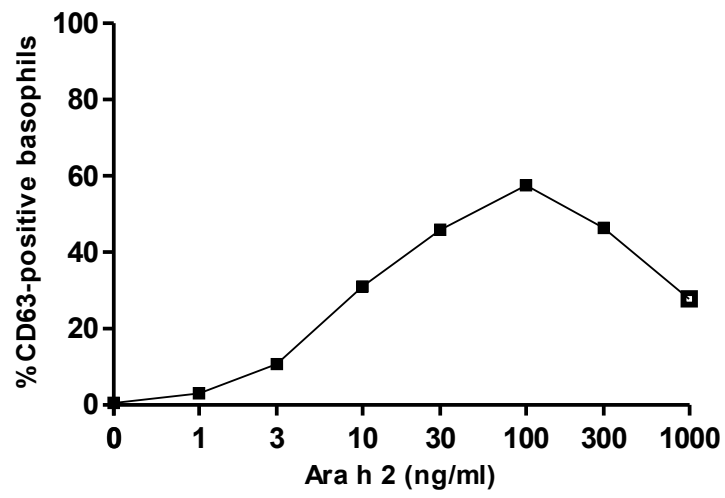
Pat 39



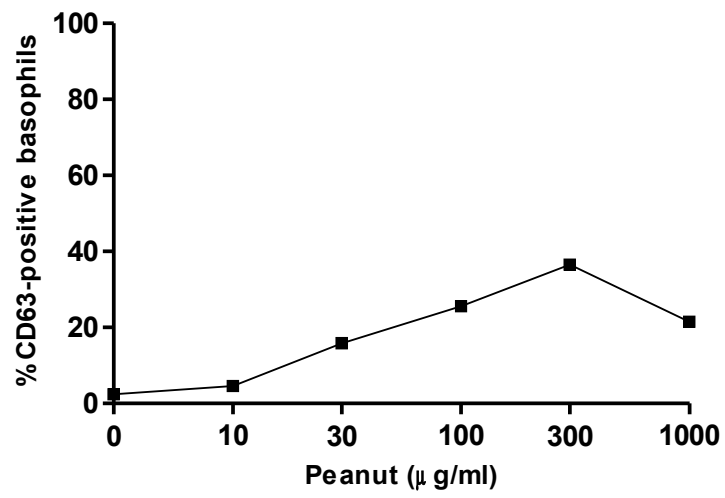
Pat 40



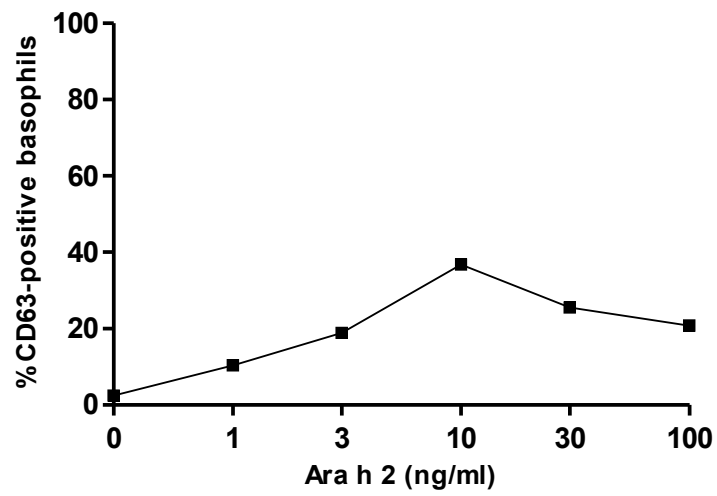
Pat 40



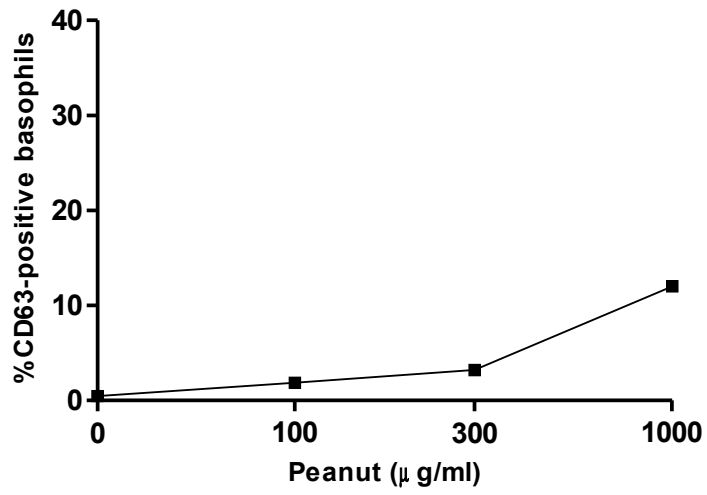
Pat 41



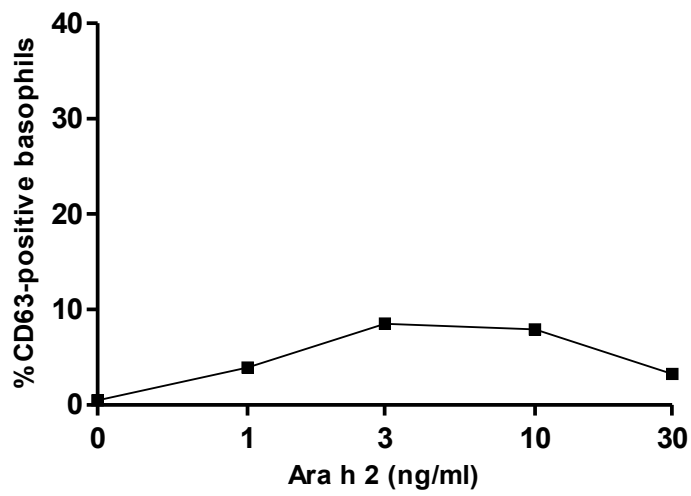
Pat 41



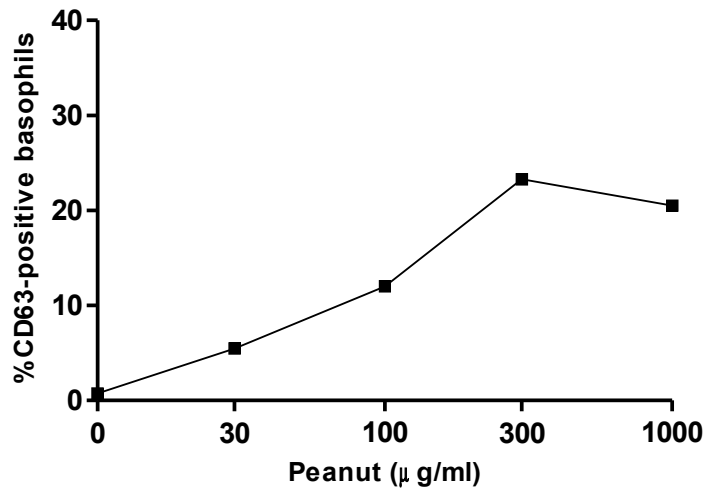
Pat 42



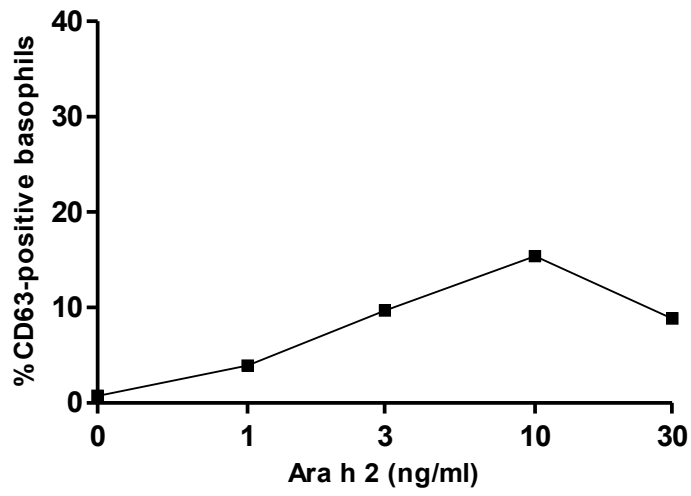
Pat 42



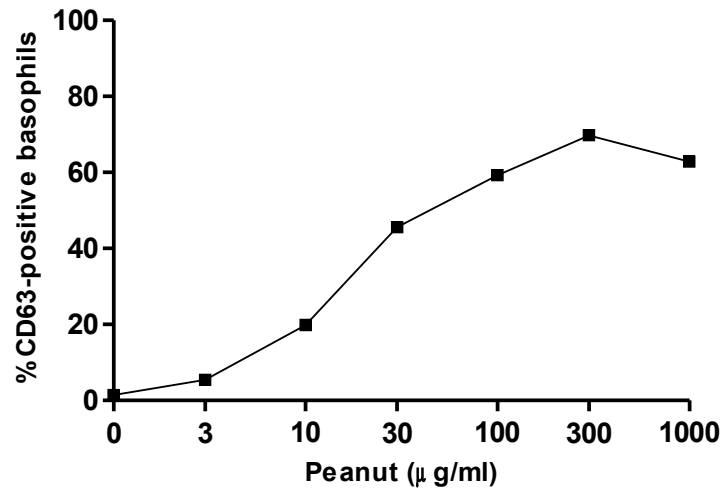
Pat 43



Pat 43



Pat 44



Pat 44

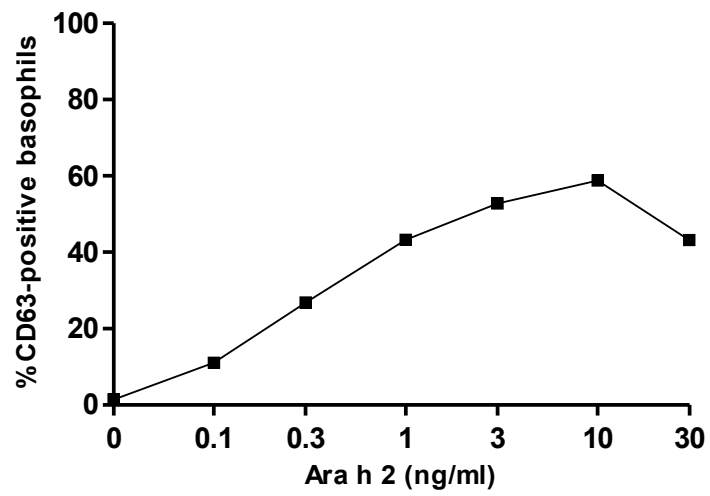
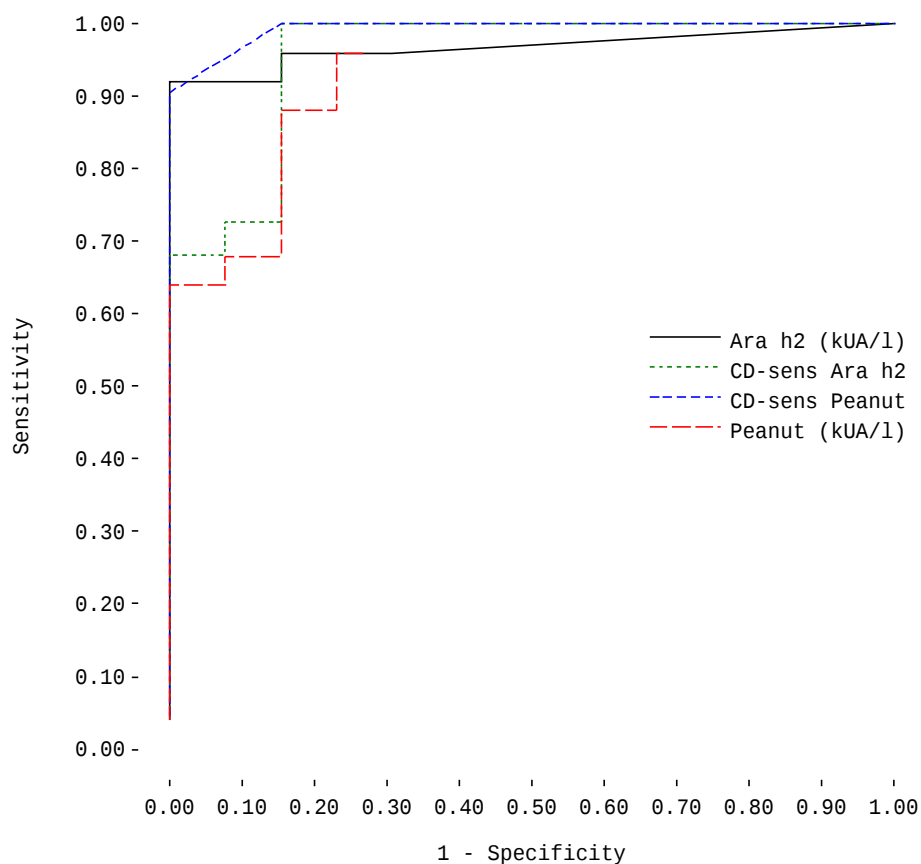


Figure 1. ROC curves for CD-sens to peanut, CD-sens to ara h 2, IgE-ab to peanut and IgE-ab to ara h 2 in relation to DBPCFC. Patients with any quantifiable test result are included*.



* CD-sens to peanut: DBPCFC positive n=21, DBPCFC negative n=13.

Patient 22 and 28 are missing result because of being low responders. Patient 31 is not included because of missing CD-sens. Patient 37 is not included because of a positive but not quantifiable CD-sens.

CD-sens to ara h 2: DBPCFC positive n=22, DBPCFC negative n=13.

Patient 22 and 28 are missing result because of being low responders. Patient 37 is not included because of a positive but not quantifiable CD-sens.

IgE-ab to peanut: DBPCFC positive n=25, DBPCFC negative n=13.

IgE-ab to ara h 2: DBPCFC positive n=25, DBPCFC negative n=13.

Table 1a. Results connected to Figure 1. CD-sens to peanut in relation to DBPCFC.

Cut Off Level	A Pos/Pos (Ref/Test)	B Pos/Neg (Ref/Test)	C Neg/Pos (Ref/Test)	D Neg/Neg (Ref/Test)	Sensi- tivity	Speci- ficity	Predictive Value Positive	Predictive Value Negative
29.3	1	20	0	13	0.0476	1.0000	1.0000	0.3939
12.0	2	19	0	13	0.0952	1.0000	1.0000	0.4063
10.6	3	18	0	13	0.1429	1.0000	1.0000	0.4194
4.5	4	17	0	13	0.1905	1.0000	1.0000	0.4333
4.2	5	16	0	13	0.2381	1.0000	1.0000	0.4483
3.9	6	15	0	13	0.2857	1.0000	1.0000	0.4643
3.4	7	14	0	13	0.3333	1.0000	1.0000	0.4815
3.2	8	13	0	13	0.3810	1.0000	1.0000	0.5000
2.6	9	12	0	13	0.4286	1.0000	1.0000	0.5200
1.4	10	11	0	13	0.4762	1.0000	1.0000	0.5417
1.3	12	9	0	13	0.5714	1.0000	1.0000	0.5909
1.0	13	8	0	13	0.6190	1.0000	1.0000	0.6190
0.9	14	7	0	13	0.6667	1.0000	1.0000	0.6500
0.8	15	6	0	13	0.7143	1.0000	1.0000	0.6842
0.7	16	5	0	13	0.7619	1.0000	1.0000	0.7222
0.6	19	2	0	13	0.9048	1.0000	1.0000	0.8667
0.5	20	1	1	12	0.9524	0.9231	0.9524	0.9231
0.4	21	0	2	11	1.0000	0.8462	0.9130	1.0000
0.3	21	0	3	10	1.0000	0.7692	0.8750	1.0000
0.0	21	0	13	0	1.0000	0.0000	0.6176	.

Table 1b. Results connected to Figure 1. CD-sens to ara h 2 in relation to DBPCFC.

Cut Off Level	A Pos/Pos (Ref/Test)	B Pos/Neg (Ref/Test)	C Neg/Pos (Ref/Test)	D Neg/Neg (Ref/Test)	Sensi- tivity	Speci- ficity	Predictive Value Positive	Predictive Value Negative
385.0	1	21	0	13	0.0455	1.0000	1.0000	0.3824
370.0	2	20	0	13	0.0909	1.0000	1.0000	0.3939
222.0	3	19	0	13	0.1364	1.0000	1.0000	0.4063
196.0	4	18	0	13	0.1818	1.0000	1.0000	0.4194
170.0	5	17	0	13	0.2273	1.0000	1.0000	0.4333
130.0	8	14	0	13	0.3636	1.0000	1.0000	0.4815
109.0	9	13	0	13	0.4091	1.0000	1.0000	0.5000
106.0	10	12	0	13	0.4545	1.0000	1.0000	0.5200
105.0	11	11	0	13	0.5000	1.0000	1.0000	0.5417
64.0	12	10	0	13	0.5455	1.0000	1.0000	0.5652
37.9	13	9	0	13	0.5909	1.0000	1.0000	0.5909
37.0	14	8	0	13	0.6364	1.0000	1.0000	0.6190
34.1	15	7	0	13	0.6818	1.0000	1.0000	0.6500
33.2	15	7	1	12	0.6818	0.9231	0.9375	0.6316
26.5	16	6	1	12	0.7273	0.9231	0.9412	0.6667
25.6	16	6	2	11	0.7273	0.8462	0.8889	0.6471
20.0	17	5	2	11	0.7727	0.8462	0.8947	0.6875
17.0	18	4	2	11	0.8182	0.8462	0.9000	0.7333
15.8	19	3	2	11	0.8636	0.8462	0.9048	0.7857
15.7	20	2	2	11	0.9091	0.8462	0.9091	0.8462
14.0	21	1	2	11	0.9545	0.8462	0.9130	0.9167
9.0	22	0	2	11	1.0000	0.8462	0.9167	1.0000
3.7	22	0	3	10	1.0000	0.7692	0.8800	1.0000
0.0	22	0	13	0	1.0000	0.0000	0.6286	.

Table 1c. Results connected to Figure 1. IgE-ab to peanut in relation to DBPCFC.

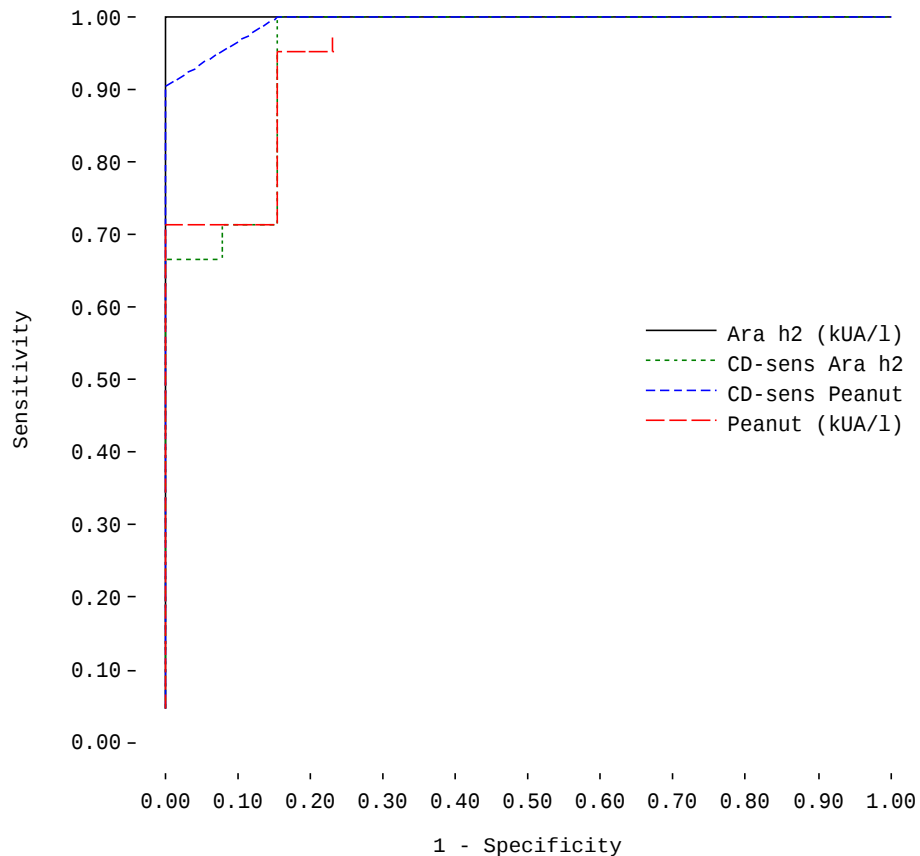
Cut Off Level	A Pos/Pos (Ref/Test)	B Pos/Neg (Ref/Test)	C Neg/Pos (Ref/Test)	D Neg/Neg (Ref/Test)	Sensi- tivity	Speci- ficity	Predictive Value Positive	Predictive Value Negative
1022.00	1	24	0	13	0.0400	1.0000	1.0000	0.3514
388.10	2	23	0	13	0.0800	1.0000	1.0000	0.3611
293.41	3	22	0	13	0.1200	1.0000	1.0000	0.3714
264.45	4	21	0	13	0.1600	1.0000	1.0000	0.3824
240.37	5	20	0	13	0.2000	1.0000	1.0000	0.3939
214.85	6	19	0	13	0.2400	1.0000	1.0000	0.4063
214.00	7	18	0	13	0.2800	1.0000	1.0000	0.4194
194.00	8	17	0	13	0.3200	1.0000	1.0000	0.4333
186.78	9	16	0	13	0.3600	1.0000	1.0000	0.4483
135.97	10	15	0	13	0.4000	1.0000	1.0000	0.4643
134.31	11	14	0	13	0.4400	1.0000	1.0000	0.4815
126.11	12	13	0	13	0.4800	1.0000	1.0000	0.5000
113.59	13	12	0	13	0.5200	1.0000	1.0000	0.5200
90.78	14	11	0	13	0.5600	1.0000	1.0000	0.5417
85.43	15	10	0	13	0.6000	1.0000	1.0000	0.5652
78.05	16	9	0	13	0.6400	1.0000	1.0000	0.5909
46.89	16	9	1	12	0.6400	0.9231	0.9412	0.5714
40.19	17	8	1	12	0.6800	0.9231	0.9444	0.6000
36.59	17	8	2	11	0.6800	0.8462	0.8947	0.5789
36.46	18	7	2	11	0.7200	0.8462	0.9000	0.6111
34.90	19	6	2	11	0.7600	0.8462	0.9048	0.6471
26.40	20	5	2	11	0.8000	0.8462	0.9091	0.6875
23.49	21	4	2	11	0.8400	0.8462	0.9130	0.7333
17.62	22	3	2	11	0.8800	0.8462	0.9167	0.7857
9.15	22	3	3	10	0.8800	0.7692	0.8800	0.7692
8.32	23	2	3	10	0.9200	0.7692	0.8846	0.8333
5.75	24	1	3	10	0.9600	0.7692	0.8889	0.9091
4.40	24	1	4	9	0.9600	0.6923	0.8571	0.9000
1.30	24	1	5	8	0.9600	0.6154	0.8276	0.8889
1.05	24	1	6	7	0.9600	0.5385	0.8000	0.8750
1.01	24	1	7	6	0.9600	0.4615	0.7742	0.8571
1.00	24	1	8	5	0.9600	0.3846	0.7500	0.8333
0.99	25	0	8	5	1.0000	0.3846	0.7576	1.0000
0.92	25	0	9	4	1.0000	0.3077	0.7353	1.0000
0.25	25	0	10	3	1.0000	0.2308	0.7143	1.0000

Cut Off Level	A Pos/Pos (Ref/Test)	B Pos/Neg (Ref/Test)	C Neg/Pos (Ref/Test)	D Neg/Neg (Ref/Test)	Sensi- tivity	Speci- ficity	Predictive Value Positive	Predictive Value Negative
0.24	25	0	11	2	1.0000	0.1538	0.6944	1.0000
0.22	25	0	12	1	1.0000	0.0769	0.6757	1.0000
0.11	25	0	13	0	1.0000	0.0000	0.6579	.

Table 1d. Results connected to Figure 1. IgE-ab to ara h 2 in relation to DBPCFC.

Cut Off Level	A Pos/Pos (Ref/Test)	B Pos/Neg (Ref/Test)	C Neg/Pos (Ref/Test)	D Neg/Neg (Ref/Test)	Sensi- tivity	Speci- ficity	Predictive Value Positive	Predictive Value Negative
918.000	1	24	0	13	0.0400	1.0000	1.0000	0.3514
202.952	2	23	0	13	0.0800	1.0000	1.0000	0.3611
197.400	3	22	0	13	0.1200	1.0000	1.0000	0.3714
141.755	4	21	0	13	0.1600	1.0000	1.0000	0.3824
119.229	5	20	0	13	0.2000	1.0000	1.0000	0.3939
95.467	6	19	0	13	0.2400	1.0000	1.0000	0.4063
90.569	7	18	0	13	0.2800	1.0000	1.0000	0.4194
89.640	8	17	0	13	0.3200	1.0000	1.0000	0.4333
74.364	9	16	0	13	0.3600	1.0000	1.0000	0.4483
67.008	10	15	0	13	0.4000	1.0000	1.0000	0.4643
64.443	11	14	0	13	0.4400	1.0000	1.0000	0.4815
59.680	12	13	0	13	0.4800	1.0000	1.0000	0.5000
54.018	13	12	0	13	0.5200	1.0000	1.0000	0.5200
50.858	14	11	0	13	0.5600	1.0000	1.0000	0.5417
46.780	15	10	0	13	0.6000	1.0000	1.0000	0.5652
44.768	16	9	0	13	0.6400	1.0000	1.0000	0.5909
17.550	17	8	0	13	0.6800	1.0000	1.0000	0.6190
13.380	18	7	0	13	0.7200	1.0000	1.0000	0.6500
13.300	19	6	0	13	0.7600	1.0000	1.0000	0.6842
12.700	20	5	0	13	0.8000	1.0000	1.0000	0.7222
11.964	21	4	0	13	0.8400	1.0000	1.0000	0.7647
9.730	22	3	0	13	0.8800	1.0000	1.0000	0.8125
2.284	23	2	0	13	0.9200	1.0000	1.0000	0.8667
1.922	23	2	1	12	0.9200	0.9231	0.9583	0.8571
0.890	23	2	2	11	0.9200	0.8462	0.9200	0.8462
0.570	24	1	2	11	0.9600	0.8462	0.9231	0.9167
0.249	24	1	3	10	0.9600	0.7692	0.8889	0.9091
0.112	24	1	4	9	0.9600	0.6923	0.8571	0.9000
<0.01	25	0	13	0	1.0000	0.0000	0.6579	.

Figure 2. ROC curves for CD-sens to peanut, CD-sens to ara h 2, IgE-ab to peanut and IgE-ab to ara h 2 in relation to DBPCFC. Only patients with complete data for all tests are included*.



* DBPCFC positive n=21, DBPCFC negative n=13.

Patient 22 and 28 are not included because of missing CD-sens to peanut and ara h 2 (low responders). Patient 31 is not included because of a missing CD-sens to peanut. Patient 37 is not included because of a positive but not quantifiable CD-sens to peanut and ara h 2.

Table 2a. Results connected to Figure 2. CD-sens to peanut in relation to DBPCFC.

Cut Off Level	A Pos/Pos (Ref/Test)	B Pos/Neg (Ref/Test)	C Neg/Pos (Ref/Test)	D Neg/Neg (Ref/Test)	Sensi- tivity	Speci- ficity	Predictive Value Positive	Predictive Value Negative
29.3	1	20	0	13	0.0476	1.0000	1.0000	0.3939
12.0	2	19	0	13	0.0952	1.0000	1.0000	0.4063
10.6	3	18	0	13	0.1429	1.0000	1.0000	0.4194
4.5	4	17	0	13	0.1905	1.0000	1.0000	0.4333
4.2	5	16	0	13	0.2381	1.0000	1.0000	0.4483
3.9	6	15	0	13	0.2857	1.0000	1.0000	0.4643
3.4	7	14	0	13	0.3333	1.0000	1.0000	0.4815
3.2	8	13	0	13	0.3810	1.0000	1.0000	0.5000
2.6	9	12	0	13	0.4286	1.0000	1.0000	0.5200
1.4	10	11	0	13	0.4762	1.0000	1.0000	0.5417
1.3	12	9	0	13	0.5714	1.0000	1.0000	0.5909
1.0	13	8	0	13	0.6190	1.0000	1.0000	0.6190
0.9	14	7	0	13	0.6667	1.0000	1.0000	0.6500
0.8	15	6	0	13	0.7143	1.0000	1.0000	0.6842
0.7	16	5	0	13	0.7619	1.0000	1.0000	0.7222
0.6	19	2	0	13	0.9048	1.0000	1.0000	0.8667
0.5	20	1	1	12	0.9524	0.9231	0.9524	0.9231
0.4	21	0	2	11	1.0000	0.8462	0.9130	1.0000
0.3	21	0	3	10	1.0000	0.7692	0.8750	1.0000
0.0	21	0	13	0	1.0000	0.0000	0.6176	.

Table 2b. Results connected to Figure 2. CD-sens to ara h 2 in relation to DBPCFC.

Cut Off Level	A Pos/Pos (Ref/Test)	B Pos/Neg (Ref/Test)	C Neg/Pos (Ref/Test)	D Neg/Neg (Ref/Test)	Sensi- tivity	Speci- ficity	Predictive Value Positive	Predictive Value Negative
385.0	1	20	0	13	0.0476	1.0000	1.0000	0.3939
370.0	2	19	0	13	0.0952	1.0000	1.0000	0.4063
222.0	3	18	0	13	0.1429	1.0000	1.0000	0.4194
196.0	4	17	0	13	0.1905	1.0000	1.0000	0.4333
130.0	7	14	0	13	0.3333	1.0000	1.0000	0.4815
109.0	8	13	0	13	0.3810	1.0000	1.0000	0.5000
106.0	9	12	0	13	0.4286	1.0000	1.0000	0.5200
105.0	10	11	0	13	0.4762	1.0000	1.0000	0.5417
64.0	11	10	0	13	0.5238	1.0000	1.0000	0.5652
37.9	12	9	0	13	0.5714	1.0000	1.0000	0.5909
37.0	13	8	0	13	0.6190	1.0000	1.0000	0.6190
34.1	14	7	0	13	0.6667	1.0000	1.0000	0.6500
33.2	14	7	1	12	0.6667	0.9231	0.9333	0.6316
26.5	15	6	1	12	0.7143	0.9231	0.9375	0.6667
25.6	15	6	2	11	0.7143	0.8462	0.8824	0.6471
20.0	16	5	2	11	0.7619	0.8462	0.8889	0.6875
17.0	17	4	2	11	0.8095	0.8462	0.8947	0.7333
15.8	18	3	2	11	0.8571	0.8462	0.9000	0.7857
15.7	19	2	2	11	0.9048	0.8462	0.9048	0.8462
14.0	20	1	2	11	0.9524	0.8462	0.9091	0.9167
9.0	21	0	2	11	1.0000	0.8462	0.9130	1.0000
3.7	21	0	3	10	1.0000	0.7692	0.8750	1.0000
0.0	21	0	13	0	1.0000	0.0000	0.6176	.

Table 2c. Results connected to Figure 2. IgE-ab to peanut in relation to DBPCFC.

Cut Off Level	A Pos/Pos (Ref/Test)	B Pos/Neg (Ref/Test)	C Neg/Pos (Ref/Test)	D Neg/Neg (Ref/Test)	Sensi- tivity	Speci- ficity	Predictive Value Positive	Predictive Value Negative
1022.00	1	20	0	13	0.0476	1.0000	1.0000	0.3939
293.41	2	19	0	13	0.0952	1.0000	1.0000	0.4063
264.45	3	18	0	13	0.1429	1.0000	1.0000	0.4194
240.37	4	17	0	13	0.1905	1.0000	1.0000	0.4333
214.85	5	16	0	13	0.2381	1.0000	1.0000	0.4483
214.00	6	15	0	13	0.2857	1.0000	1.0000	0.4643
194.00	7	14	0	13	0.3333	1.0000	1.0000	0.4815
186.78	8	13	0	13	0.3810	1.0000	1.0000	0.5000
135.97	9	12	0	13	0.4286	1.0000	1.0000	0.5200
134.31	10	11	0	13	0.4762	1.0000	1.0000	0.5417
126.11	11	10	0	13	0.5238	1.0000	1.0000	0.5652
113.59	12	9	0	13	0.5714	1.0000	1.0000	0.5909
90.78	13	8	0	13	0.6190	1.0000	1.0000	0.6190
85.43	14	7	0	13	0.6667	1.0000	1.0000	0.6500
78.05	15	6	0	13	0.7143	1.0000	1.0000	0.6842
46.89	15	6	1	12	0.7143	0.9231	0.9375	0.6667
36.59	15	6	2	11	0.7143	0.8462	0.8824	0.6471
36.46	16	5	2	11	0.7619	0.8462	0.8889	0.6875
34.90	17	4	2	11	0.8095	0.8462	0.8947	0.7333
26.40	18	3	2	11	0.8571	0.8462	0.9000	0.7857
23.49	19	2	2	11	0.9048	0.8462	0.9048	0.8462
17.62	20	1	2	11	0.9524	0.8462	0.9091	0.9167
9.15	20	1	3	10	0.9524	0.7692	0.8696	0.9091
5.75	21	0	3	10	1.0000	0.7692	0.8750	1.0000
4.40	21	0	4	9	1.0000	0.6923	0.8400	1.0000
1.30	21	0	5	8	1.0000	0.6154	0.8077	1.0000
1.05	21	0	6	7	1.0000	0.5385	0.7778	1.0000
1.01	21	0	7	6	1.0000	0.4615	0.7500	1.0000
1.00	21	0	8	5	1.0000	0.3846	0.7241	1.0000
0.92	21	0	9	4	1.0000	0.3077	0.7000	1.0000
0.25	21	0	10	3	1.0000	0.2308	0.6774	1.0000
0.24	21	0	11	2	1.0000	0.1538	0.6563	1.0000
0.22	21	0	12	1	1.0000	0.0769	0.6364	1.0000
0.11	21	0	13	0	1.0000	0.0000	0.6176	.

Table 2d. Results connected to Figure 2. IgE-ab to ara h 2 in relation to DBPCFC.

Cut Off Level	A Pos/Pos (Ref/Test)	B Pos/Neg (Ref/Test)	C Neg/Pos (Ref/Test)	D Neg/Neg (Ref/Test)	Sensi- tivity	Speci- ficity	Predictive Value Positive	Predictive Value Negative
918.000	1	20	0	13	0.0476	1.0000	1.0000	0.3939
202.952	2	19	0	13	0.0952	1.0000	1.0000	0.4063
197.400	3	18	0	13	0.1429	1.0000	1.0000	0.4194
141.755	4	17	0	13	0.1905	1.0000	1.0000	0.4333
119.229	5	16	0	13	0.2381	1.0000	1.0000	0.4483
90.569	6	15	0	13	0.2857	1.0000	1.0000	0.4643
89.640	7	14	0	13	0.3333	1.0000	1.0000	0.4815
74.364	8	13	0	13	0.3810	1.0000	1.0000	0.5000
67.008	9	12	0	13	0.4286	1.0000	1.0000	0.5200
64.443	10	11	0	13	0.4762	1.0000	1.0000	0.5417
59.680	11	10	0	13	0.5238	1.0000	1.0000	0.5652
54.018	12	9	0	13	0.5714	1.0000	1.0000	0.5909
50.858	13	8	0	13	0.6190	1.0000	1.0000	0.6190
44.768	14	7	0	13	0.6667	1.0000	1.0000	0.6500
17.550	15	6	0	13	0.7143	1.0000	1.0000	0.6842
13.380	16	5	0	13	0.7619	1.0000	1.0000	0.7222
13.300	17	4	0	13	0.8095	1.0000	1.0000	0.7647
12.700	18	3	0	13	0.8571	1.0000	1.0000	0.8125
11.964	19	2	0	13	0.9048	1.0000	1.0000	0.8667
9.730	20	1	0	13	0.9524	1.0000	1.0000	0.9286
2.284	21	0	0	13	1.0000	1.0000	1.0000	1.0000
1.922	21	0	1	12	1.0000	0.9231	0.9545	1.0000
0.890	21	0	2	11	1.0000	0.8462	0.9130	1.0000
0.249	21	0	3	10	1.0000	0.7692	0.8750	1.0000
0.112	21	0	4	9	1.0000	0.6923	0.8400	1.0000
<0.1	21	0	13	0	1.0000	0.0000	0.6176	.