



CAST[®]-2000 ELISA

Cellular Antigen Stimulation Test

Order Code: EK-CAST
 192 tests

The BÜHLMANN CAST[®]-2000 ELISA is intended for the quantitative determination of sulfidoleukotrienes produced by isolated leukocytes upon contact with antigens.

For *in vitro* research use only.

研 究 用 試 薬

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I. SUMMARY AND EXPLANATION

Introduction

The sulfidoleukotrienes (sLT) LTC₄, LTD₄ and LTE₄ are products of arachidonic acid transformed by 5-lipoxygenase and glutathione-S-transferase. In the past, they were collectively termed "Slow Reacting Substances of Anaphylaxis (SRS-A)". They are synthesized by many cell types such as mast cells, basophils, macrophages, eosinophils and kidney mesangial cells and play an important role in various pathological events, particularly IgE-mediated allergic reactions (3).

Blood basophils generate sLT and histamine in response to allergens in an IgE-dependent manner (4), particularly when pretreated (= primed) with the cytokines IL-3, IL-5 or GM-CSF (5). However, the generation of *de novo* sLT by challenged basophils or mast cells is not always paralleled by the release of histamine (6).

Since sLT may also be generated by other inflammatory cells than blood basophils and mast cells (which are the almost exclusive sources of histamine in tissue inflammation), it can be expected that other inflammatory mechanisms than IgE-mediated reactions may cause or contribute to the production of sLT. This may be put to diagnostic advantage, since several pseudo-allergic reactions which are not IgE-dependent, such as hypersensitivity reactions to some drugs (e.g. aspirin), may lead to the generation of sLT (7-9).

While LTC₄ is the sulfidoleukotriene produced by cells upon challenge, it is rapidly metabolized in biological fluids to LTD₄ and further to LTE₄ which is the relatively stable metabolite forming the bulk of sLT in urine (10,11). The present CAST[®]-2000 ELISA, which is based on a monoclonal antibody recognizing LTC₄, LTD₄ and LTE₄ is aimed for applications in clinical diagnosis and research, as all relevant sLT species are determined in a single convenient assay.

Basically, the CAST[®]-ELISA may be used in the following way: Measurement of the sLT production following isolation of tissue cells (e.g. blood leukocytes) and challenge *in vitro* by various sLT producing agents such as allergens, anti-IgE antibodies, anti-IgE receptor antibodies, anaphylatoxins, complement C5a, drugs, etc. (for review see 12,13). More specifically, the CAST[®]-ELISA has been successfully used for the following applications:

Inhalation Allergies

CAST has been shown to give comparable results to other allergy diagnosis test when a qualitative answer to common inhalation allergens is expected. The advantage of the test becomes apparent, when the results are analyzed in a quantitative manner. Here the CAST correlates much better to skin tests than measurement of serum IgE. This finding has been anticipated, since measuring the mediators of an allergic reaction should not only show a sensitization but also its intensity. There is evidence that results of the CAST[®]-ELISA correlate with allergen provocation *in vivo*, and it is likely, that such costly and cumbersome provocations can be abandoned in many cases, when CAST results are positive. Nevertheless, there are a certain number of patients with inhalation allergies, with whom skin tests cannot be performed or whose IgE determinations do not give clear-cut results (14,15). In such cases the CAST[®]-ELISA can be very useful. In addition, CAST can help to determine whether a sensitization diagnosed by means of skin tests or IgE determination is of clinical relevance (16).

Immunotherapy

The decision whether or not to initiate a specific immunotherapy is not easy. Because of the high costs, the long duration and the possible risks for the patient, such a decision has to remain in the responsibility of the allergist. When patient history, skin test and IgE correlate entirely, it is advised to test in addition the clinical relevance of the diagnosed sensitization. This is especially important for patients with perennial symptoms and patients with multiple sensitizations. To do this, allergists often perform an *in vivo* provocation test. The results so far obtained with the CAST[®]-ELISA are promising in the sense that this test might be used to replace such potentially risky and expensive *in vivo* provocations in many cases. Of particular interest was of course the question whether the CAST[®]-ELISA can be used to monitor specific

immunotherapy. In four studies with 98 patients, it could be shown that the releasability of blood cells from patients undergoing immunotherapy decreases (14). In a double blind, placebo controlled study, it could be shown that house dust mite-induced production of sLT decreased by 50% or more in 50% of the *verum*-treated patients after one year of treatment, whereas no reduction was observed in the *placebo*-treated group (17).

Allergy Diagnosis in Infants

Allergy diagnosis in small children is often more difficult than in adults. Anamnesis is missing or incomplete and IgE tests are not sensitive enough. Additionally, skin tests can have a traumatic effect. In children over the age of four years, CAST results showed a correlation to skin tests or specific IgE test that was similar to that found in adults. However, a positive CAST result was often the only objective confirmation of a suspected allergy in younger children in the absence of positive IgE tests or skin tests. TOP CAST, a screening panel with the most common allergens (*cf.* CAST® Allergen Kit Insert), could be used as an indicator for the early detection of the existence or development of an atopy (16,17).

Insect Venom Allergies

Classical diagnostic tools often are not reliable for the detection of bee and wasp venom allergies. Skin tests can bear a high risk of anaphylaxis in patients with a high reactivity to these allergens. Skin tests as well as IgE tests sometimes are negative, also in patient with positive *in vivo* provocation tests. Here the CAST®-ELISA has been shown to be of particular value and its introduction for routine diagnosis of insect venom allergies has brought a clear progress and additional security. With respect to the difficulties concerning the decision whether or not to start a specific immunotherapy, and the risk related with it for the patient, as well as the risk that an *in vivo* provocation still represents, CAST results serve as a valuable additional help to make such decisions (18,19).

Food Allergies

Diagnosis of food allergies often represents a special problem. Not all clinical manifestations of a food allergy are IgE mediated and, therefore, skin tests or specific IgE tests are negative. On the other hand skin tests may be falsely positive since many food extracts contain histamine or other reagents which may act as mediators themselves. So far there are only a few studies where CAST has been used for food allergy testing. Nevertheless, the results are very promising. For example, in 10 of 14 children with atopic dermatitis and *in vivo* allergen provocation proved allergy to egg, CAST showed a clearly positive result and a very good correlation with skin test and IgE tests. It is well possible that a clearly positive CAST result may, as an *in vitro* provocation test, replace risky *in vivo* provocations in such cases.

Allergic and Pseudo-allergic Reactions to Drugs

The diagnosis of allergies and pseudo-allergies to drugs represent, besides food allergy diagnosis, one of the most frustrating problems for the allergist (20). Skin tests often do not work with drug allergens, IgE tests are often still missing, and cellular proliferation tests are complicated and very time consuming. Therefore, tests for the detection of drug allergies are rarely offered in the routine laboratory, in spite of contributing ~10% of cases that are examined by allergists, and about 3-5% of the population being affected by such allergies (20).

Preliminary, promising results obtained with betalactam-type antibiotics and aspirin (acetylsalicylic acid, ASA) with the CAST®-ELISA have encouraged several groups to initiate further studies concerning this topic. An interesting finding was reported by Bircher *et al.* (21,22). The authors found that only these patients with an anaphylactic reaction against betalactam-type antibiotics were clearly positive in the CAST assay, whereas patients with milder reactions, such as urticaria were negative. Provided these results can be confirmed, they would have be of great practical importance, since it is one of the major objectives of drug allergy diagnosis to identify those individuals who are at risk of anaphylactic reactions. It is obvious that this goal cannot be reached with skin tests or serum IgE testing.

The diagnosis of pseudo-allergic reactions have ever been of special interest since the introduction of the CAST[®]-ELISA. It is well known that sulfidoleukotrienes contribute substantially to the symptoms of such reactions, since increased levels of LTE₄ are found in the urine of patients after provocation with aspirin (7). On the other hand, there is certainly no IgE involved in the vast majority of ASA-induced reactions. To date, there is no other *in vitro* test, which would allow the diagnosis of this relatively common and dangerous sensitization. Therefore, *in vivo* provocation tests are commonly used for diagnosis of ASA pseudo-allergies. The CAST[®]-ELISA is the first diagnostic *in vitro* test that gave promising results in the context of diagnosis of pseudo-allergies against ASA and non-steroidal anti-inflammatory drugs (NSAIDs).

It has been found by several independent researchers (9,23-25) that patients suffering from ASA intolerance had highly reactive basophils which reacted much stronger than normal controls or atopic patients, when stimulated unspecifically with reagents such as the complement factor C5a (in presence and absence of aspirin, respectively). There are currently a number of studies going on using C5a to stimulate patient cells. Preliminary results of these studies are very promising. Nevertheless, it is clear that the measurement of sLT production after stimulation with ASA or with C5a or with both combined is of clinical interest for the diagnosis of patients with aspirin intolerances. Virtually all clearly CAST-positive patients have been followed by a positive *in vitro* provocation test, whereas patients with ASA-negative provocation also showed a negative CAST result.

Here again, a positive CAST[®]-ELISA result in patients with suspicion for aspirin intolerance could replace the potentially harmful, risky and costly *in vivo* provocation. **On the other hand, a negative CAST result does not allow to absolutely exclude the existence of an allergy or a pseudo-allergy.** However, as no other *in vitro* test exists for the diagnosis of pseudo-allergies, the CAST[®]-ELISA, when applied correctly, could be of valuable help in such cases. This has also been shown in patients with suspicion for food additive and analgesic allergies (23).

II. PRINCIPAL OF THE ASSAY AND RESULT INTERPRETATION

In the **C**ellular **A**ntigen **S**timulation **T**est (CAST[®]), sedimented leukocytes from patient blood are simultaneously primed with the cytokine IL-3 and stimulated with allergens (1). Basophilic cells among others generate the allergic mediator, sulfidoleukotriene LTC₄, and its metabolites LTD₄ and LTE₄. *De novo* formation of LTC₄ can be both IgE dependent or non-IgE dependent. The latter event is usually described as pseudo-allergy (2). These freshly synthesized sulfidoleukotrienes (sLT) are subsequently measured in an ELISA test (Enzyme Linked ImmunoSorbent Assay).

The CAST[®]-ELISA can be separated into three procedural parts: 1) leukocyte isolation, 2) cell stimulation and 3) leukotriene determination in the cell supernatant. The principle of this CAST[®]-ELISA is covered by a patented technology (Patent No. US5,487,977;).

1. Isolation of Leukocytes

Dextran is added to patient blood in order to increase the blood viscosity. After 90 minutes at ambient temperature, the erythrocytes are sedimented, whereas leukocytes and thrombocytes stay in the plasma fraction. Subsequently, the blood supernatant is being carefully transferred into separate tubes and the leukocytes are sedimented in a brief centrifugation step. The plasma supernatant containing >90% of the thrombocytes is discarded and the leukocyte pellet is resuspended in the Stimulation Buffer containing IL-3.

2. Cell Stimulation

Each patient's cells are tested for the basal level release (= background) and for the release after stimulation with an anti-IgE receptor antibody (= stimulation control).

The cells are stimulated during a 40-minutes incubation at 37°C. Finally, the cells are centrifuged and the cell supernatant is either frozen for subsequent storage or immediately tested for sLT concentration in the ELISA.

3. Leukotriene Determination

The ELISA is performed using precoated microtiter plates (2x96 wells). 16 wells per assay are used for the standard curve and controls. Two wells per patient are used for the background, two wells per patient for the stimulation control and two wells for each allergen.

Enzyme Label and Antibody Solution are added to the cell supernatants as well as to the standards and controls and incubated for 2 hours at 18-28°C (alternative procedure: 16-20 hours at 2-8°C). After a washing step, ready to use Substrate Solution is added to each well and incubated for another 30 minutes at 18-28°C. Finally, Stop Solution is added to each well and the color absorbance is measured at 405 nm in a microtiter plate reader.

4. Interpretation of Results

The difference of the sLT concentration in the sample stimulated by allergen compared to the background sample (stimulated with Stimulation Buffer only) shows the releasability of the patient's basophils. The background concentration determined in more than 700 blood donors from the Swiss Red Cross Center, Basle, was 20-140 pg/ml (2.5-97.5% percentile). However, patients exposed to the allergen *in vivo* or atopic patients particularly patients sensitive to latex, may exhibit much higher background sLT values. Nevertheless, a significant difference between stimulated and non-stimulated samples was still observed in most cases. As a **general guideline**, we recommend that individuals with net stimulation yields (difference between allergen stimulation and background stimulation) **below 100 pg/ml** should be regarded as negative for the allergen tested.

As a positive control that proves the viability and functionality of the cells, a third blood sample of the patient is stimulated with an antibody directed against the high affinity IgE receptor (FcεRI). Similar to an allergen, this antibody leads to the cross-linking of the Fcε receptor I and, therefore, to the stimulation of the cells. This anti-IgE receptor antibody binds to domain 1 on the α-subunit of the high affinity IgE receptor and is a non-inhibiting antibody. Thus, it will bind to the receptor irrespective of the receptor being free from or loaded with IgE. In allergic as well as in non-allergic individuals, a net stimulation control yield (after subtraction of the patient's background) of at least 200 pg/ml should be expected.

III. WARNINGS AND PRECAUTIONS

FOR RESEARCH AND *IN VITRO* USE ONLY. Not for internal and external use in humans or animals.

General Precautions

Concerning the proper precautions for the handling and disposal of kit reagents and patient specimens, respectively, we highly recommend to first consult the special local regulations of your country. Some of these basic safety rules are listed below:

- Cover working area with disposable absorbent paper.
- No food, beverages, cosmetics or tobacco products should be admitted in areas where patient specimens or kit reagents are handled.
- Wear disposable gloves while handling patient specimens and kit reagents, wash hands afterwards.
- Do not pipet any reagent by mouth.

Reagents Containing Human Source Material

The Stimulation Buffer, Stimulation Control, Microtiter Plate, Standard, Controls, Blanking Reagent, ELISA Buffer, Antiserum and Enzyme Label of this kit contain human serum albumin. Each plasma donor unit used in the preparation of the above kit components was tested by a FDA approved method and found negative for hepatitis B surface antigen (HBsAg) or Human Immunodeficiency Virus (HIV) antibody. While these methods are highly accurate, there is no guarantee that this material cannot transmit hepatitis or AIDS. **Therefore, all patient specimens and kit components should be handled as if capable of transmitting infections.** Those products should be handled in accordance with good laboratory practices using appropriate

precautions as described in the Centers for Disease Control and Prevention/National Institutes of Health Manual, "Biosafety in Microbiological and Biomedical Laboratories", 3rd ed., 1993. HHS Publication No. (CDC) 93-8395.

Substrate and Stop Solution

The Substrate Solution contains para-Nitrophenyl-Phosphate (pNPP). The Stop Solution contains Sodium Hydroxide. Each of those reagents is irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothing. Wear suitable protective clothing, gloves and eye protection. After contact with eyes or skin, wash immediately with plenty of water.

Reagents Containing Sodium Azide and Thimerosal (Merthiolate)

This kit does **not** include reagents containing Sodium Azide and Thimerosal, respectively, as a preservative.

IV. REAGENTS SUPPLIED AND PREPARATION

1. Reagents for Cell Isolation and Cell Stimulation

Reagents	Quantity	Code	Reconstitution
Dextran Solution Sterile, without preservatives	1 vial 20 ml	B-SLT-DS	Ready to use
Stimulation Buffer Without preservatives	1 vial lyophilized	B-SLT-STB	Add 50 ml of ultra-pure, apyrogenic water
Stimulation Control Without preservatives	1 vial lyophilized	B-CAST-STCON	Add 3.5 ml of ultra-pure, apyrogenic water

2. ELISA Reagents

Reagents	Quantity	Code	Reconstitution
Microtiter Plate 2 x 96 wells precoated with rabbit anti-mouse Ig	2 pieces: 12 x 8-well strips with holder	B-CAST-MP	Wash 1x before use
Plate Sealer	6 pieces		
Wash Buffer Concentrate (20X) With preservatives	1 bottle 50 ml	B-CAST-WB	Dilute with 950 ml of deionized water to prepare 1000 ml of Wash Buffer
ELISA Buffer With preservatives	1 bottle 30 ml	B-CAST-EB	Ready to use
Standard*) Leukotriene D ₄ in a buffer matrix	5 x 1 vial lyophilized	B-CAST-CA5	Add 1 ml of deionized water
Blanking Reagent*) Leukotriene D ₄ in a buffer matrix	1 vial lyophilized	B-CAST-BR	Add 2 ml of deionized water
Enzyme Label Leukotriene D ₄ conjugated to alkaline phosphatase with preservatives	1 vial lyophilized	B-CAST-EL	Add 11 ml of ELISA Buffer (yellow solution after reconstitution)
Antibody Mouse anti-leuko-triene C ₄ /D ₄ /E ₄ in a buffer matrix with preservatives	1 vial 11 ml	B-CAST-AS	Ready to use (blue solution)
Substrate Solution para-Nitrophenyl-Phosphate (pNPP)	1 vial 42 ml	B-CAST-PNPP	Ready to use (colourless)
Stop Solution 2 N sodium hydroxide	1 vial 11 ml	B-CAST-NAOH	Ready to use Corrosive agent

*) After reconstitution, the Standard contains 3200 pg/ml of leukotriene D₄ and the Blanking Reagent contains 32'000 pg/ml of leukotriene D₄, respectively.

V. STORAGE AND SHELF LIFE OF REAGENTS

Unopened Kit	Store at 2-8°C, except Standards and Blanking Reagent which must be stored at -20°C or below . Do not use past kit expiration date. All unopened kit components are stable at 2-8°C (Standard and Blanking Reagent at -20°C) until the expiration date printed on the labels.	
Opened / Reconstituted Reagents	Dextran Solution	Store at 2-8°C until expiration date printed on the label.
	Stimulation Buffer	Store at -20°C for up to 2 months. Aliquote if repeated use is expected.
	Stimulation Control	
	Microtiter Plate	Remove the unused strips from the holder and make sure that every well is at least half way filled with storage buffer. Add Tris-Buffered Saline (TBS) if too much storage buffer is lost. Cover these extra strips with a plate sealer, return them to the foil pouch and reseal along the entire edge of zip-seal. Store for up to 6 months at 2-8°C.
	Diluted Wash Buffer	Store for up to 2 months at 2-8°C.
	Standard	Do not store.
	Blanking Reagent	Store at -20°C or below for up to 2 months. Aliquote if repeated use is expected.
	Enzyme Label	Store for up to 2 months at 2-8°C.
	ELISA Buffer	
	Antibody	Store at 2-8°C until expiration date printed on the label.
Substrate Solution		
Stop Solution	Store at ambient temperature or at 2-8°C until expiration date printed on the label	

VI. ALLERGENS AND REAGENTS SUPPLIED UPON REQUEST

Reagents	Quantity	Code	Reconstitution and Storage
Venipuncture Tubes contain special anti-coagulant for CAST	1 box with 100 pieces	B- SLT-VPT	Store protected from direct light at 18-28°C.
Ionomycin Release Control	1 vial	B-SLT-IOCON	Store at 2-8°C. Add 3.5 ml of Stimulation Buffer. After reconstitution store at -20°C for up to 2 months. Aliquote if repeated use is expected.
Low and High Control*) Leukotriene D ₄ in a buffer matrix	2 x 5 vials lyophilized	B-CAST-CONSET	Store at -20°C or below. Add 1 ml of deionized water. Do not store after reconstitution.
Allergens**)	1 vial lyophilized or ready to dilute	see kit insert: CAST®-Allergens	Store at 2-8°C. Add 250 µl of Stimulation Buffer. Do not store after reconstitution.

*)The Controls contain lot-specific amounts of leukotriene D₄. Refer to the additional Data Sheet for exact concentrations.

) **Allergen Reagents from other Sources: Allergens from other sources may be used in the CAST®-ELISA with the following limitations:

- No matrix-bound allergens (solid or liquid phase).
- No allergen preparations containing leukotrienes.
- No allergen preparations containing cytotoxic compounds (stabilizers, preservatives) such as glycerol, phenol, sodium azide or merthiolate (thimerosal).

The optimum allergen concentration should be determined as described in the allergen booklet (page 8-9).

VII. MATERIALS REQUIRED BUT NOT PROVIDED

- Adjustable precision pipettes with disposable tips: 10-100 µl, 200-1000 µl, 1-5 ml.
- Adjustable multishot pipette with disposable tips: 25-1000 µl.
- Disposable polypropylene tubes for the cell separation.
- Disposable polypropylene or polystyrene tubes and tissue culture grade microtiter plates for the cell stimulation, respectively.
- Laminar flow for cell preparation and stimulation (optional).
- Refrigerated centrifuge for centrifugation at 130 - 1000 x g.
- **Ultrapure, apyrogenic water for the cell stimulation reagents.**
- Incubator or water bath set at 37°C.
- 50 ml cylinder for the preparation of the Stimulation Buffer.
- 1000 ml cylinder for the dilution of the Wash Buffer Concentrate.
- Microtiter plate washing/aspiration device or squeeze bottle for Wash Buffer.
- Distilled or deionized water.
- Blotting paper.
- Microtiter plate mixer.
- Microtiter plate reader for measuring absorbance at 405 nm.

VIII. SPECIMEN COLLECTION AND STORAGE

It is recommended that patients should avoid systemically administered antiallergenic drugs such as corticosteroids, chromoglycic acid (DSCG) or indomethacin for at least 24 hours prior to blood sampling.

Collect sufficient blood into EDTA venipuncture tubes or preferably into CAST venipuncture tubes. **Do not centrifuge the blood.** Determine the minimum amount of blood needed according to the following table:

No. of allergens to be tested	Required amount of blood (ml)
1-5	2.0
6-10	3.0
11-15	4.0
16-20	5.0

EDTA venipuncture tubes: Perform the cell stimulation within 3 hours after blood collection. Do not refrigerate or freeze blood.

CAST venipuncture tubes: Proceed as with EDTA tubes or store blood sample refrigerated for up to 24 hours. Do not freeze blood.

IX. PROCEDURAL NOTES

1. General Notes

- A thorough understanding of this package insert is necessary for the successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following this package insert.
- Do not mix various lots of any kit component within an individual assay. Do not mix strips from different microtiter plates. Do not use any kit component beyond the expiration date indicated on its label.
- Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubations.
- Avoid microbial contamination of reagents, particularly of the Dextran Solution, Stimulation Buffer, Stimulation Control and the diluted Wash Buffer, respectively.

- To avoid cross-contamination, change disposable pipette tips between additions of each Standard and Control level, between sample additions and between reagent additions. Also, use separate reservoirs for each reagent.
- Avoid contamination of the pNPP Substrate Solution with the Enzyme Label.
- When mixing or reconstituting protein solutions and solutions containing surfactants, respectively, always avoid foaming.
- It is recommended to assay each Standard, Control and specimen in duplicate each time a test is performed. Since conditions vary from assay to assay, a completely new standard curve must be generated each time a new assay is performed. Background and Stimulation Control must be assayed for each patient sample.
- Incubation times or temperatures other than those specified could cause erroneous results. Perform the assay continuously, according to the recommended procedure and without interruption.
- Incomplete washing will adversely affect outcome and precision of an assay.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- To minimize potential assay drifts due to the variation in the substrate incubation time, take care to add the Stop Solution into the wells in the same order and speed used to add the pNPP Substrate Solution.
- The pNPP Substrate Solution should remain colorless until added to the microtiter wells. Avoid direct sunlight on the microtiter plate during incubation with pNPP Substrate Solution. The pNPP Substrate Solution should change from colorless to gradations of yellow colors.
- If the initial concentration of an unknown sample is greater than the highest standard, the cell supernatant should be diluted with ELISA Buffer and assayed again according to the assay procedure. The additional dilution must be considered when calculating the actual concentration of sulfidoleukotrienes present in the unknown sample.

2. Recommended Water Quality for the CAST®-ELISA

Cell stimulation: The use of **ultrapure, apyrogenic water** for reconstituting cell stimulation reagents (Stimulation Buffer and Stimulation Control) is essential for a good and reproducible leukocyte stimulation. The following sources of water may be used: Cell culture grade water, infusion grade water or deionized, double distilled water that is ultrafiltrated in a periodically sanitized 10 kDa ultrafilter.

ELISA: All ELISA reagents must be reconstituted with deionized, double distilled water or the same water quality that is used for the cell stimulation reagents.

3. Precautions to avoid Allergen Contamination during Cell Stimulation

Aeroallergens in the laboratory may contaminate open blood samples and cell suspensions from patients potentially causing an elevated background release or a falsely positive stimulation. Therefore, care must be taken to cover blood samples and cell stimulation tubes. Avoid dust mites, pollinating plants and open windows in the laboratory where the cell stimulation is performed. **We recommend to carry out the cell preparation and stimulation steps in a laminar flow hood.**

4. Standard Dilution

Reconstitute a new standard vial and prepare a fresh standard curve each time a new assay is performed. Reconstituted and diluted Standards (and Controls) are not stable and must be used in the ELISA without delay.

In order to obtain an entire standard curve, serial dilutions of the Standard are prepared as follows:

1. Label three tubes S2 through S4 and pipet 300 µl of ELISA Buffer into tubes S2 through S4.
2. Pipet 100 µl of reconstituted Standard (S1, 3200 pg/ml) into tube S2 and vortex.
3. Transfer 100 µl from S2 to S3, vortex. Transfer 100 µl from S3 to S4, vortex.

The corresponding sLT concentrations will be:

- S1 3200 pg/ml
- S2 800 pg/ml
- S3 200 pg/ml
- S4 50 pg/ml
- S0 Zero standard consisting of ELISA Buffer only.

X. ASSAY PROCEDURE

A. Cell Stimulation

The reagent volumes in steps 1. to 4. depend on the sample volume and must be determined according to the table below:

Volume Table for the Cell Stimulation Reagents		
Blood Sample	Dextran Solution	Stimulation Buffer
1 ml	0.25 ml	1 ml
2 ml	0.50 ml	2 ml
3 ml	0.75 ml	3 ml

The volumes in steps 1. to 8. are calculated from **2 ml blood sample volume from a single patient tested for a single allergen as an example:**

1. Pipet 2 ml of blood into a polypropylene tube, add 0.5 ml of Dextran Solution and vortex gently at low speed.
2. Incubate for 90 minutes at 18-28°C.
3. Transfer the upper phase into a second tube and centrifuge it for 15 minutes at 130 x g and 18-28°C.
4. Discard the supernatant and resuspend the cells in 2 ml of Stimulation Buffer. **Proceed to step 5 without interruption.**

Notes: The incubation in step 5. may be carried out in small polypropylene or polystyrene tubes and in non-activated polystyrene microtiter plates, respectively. **The following procedure uses polypropylene tubes in step 5. as an example.** If sufficient cell suspension from step 4. is available, the volumes in step 5. may be doubled. This allows aliquoting and freezing of the supernatants for eventually assaying the samples a second time.

- 5a. Label tubes for each patient: PB (patient background), PC (patient control), A1 (allergen 1), and so on.
- 5b. Pipet 50 µl of **Stimulation Buffer (background)** into the PB tube of each patient.
- 5c. Pipet 50 µl of **Stimulation Control** into the PC tube of each patient.
- 5d. Pipet 50 µl of **Allergen** into the corresponding patient tubes.
6. Pipet 200 µl of each patient's cell suspension into the corresponding tubes.
7. Vortex gently, cover the tubes and incubate for 40 minutes at 37°C.
8. Vortex to dissolve agglutinates. Centrifuge for 3 minutes at 1000 x g and 2-8°C. Refrigeration is recommended for obtaining a firm pellet and to prevent sulfidoleukotriene degradation. Pipet carefully 2 x 100 µl of cell supernatant from each tube for use in step 3e. of the ELISA procedure.

Important: Proceed immediately to the ELISA procedure or store the cell supernatants for up to 4 months at -20°C or below.

B. ELISA Procedure

1. Determine the number of capture antibody-coated microtiter plate strips required to test the desired number of patients and allergens plus 16 wells needed for running Blanking Reagent, Standards and Controls.

If you do not use all of the strips at once, remove the remaining strips from the holder and make sure that every well is at least half way filled with Storage Buffer. Add Tris-Buffered Saline solution (TBS) if too much storage buffer is lost. Cover extra strips with the Plate Sealer and store refrigerated.

2. Empty the coated wells filled with Storage Buffer. Wash the wells once using at least $300\ \mu\text{l}$ of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
- 3a. Pipet $100\ \mu\text{l}$ of Blanking Reagent in duplicate into wells A1+A2.
- 3b. Pipet $100\ \mu\text{l}$ of ELISA Buffer (Zero Standard, S0) in duplicate into wells B1+B2.
- 3c. Pipet $100\ \mu\text{l}$ of Standard S4 (50 pg/ml) in duplicate into wells C1+C2.
Pipet $100\ \mu\text{l}$ of Standard S3 (200 pg/ml) in duplicate into wells D1+D2.
Pipet $100\ \mu\text{l}$ of Standard S2 (800 pg/ml) in duplicate into wells E1+E2.
Pipet $100\ \mu\text{l}$ of Standard S1 (3200 pg/ml) in duplicate into wells F1+F2.
- 3d. Pipet $100\ \mu\text{l}$ of the Low Control in duplicate into wells G1+G2.
Pipet $100\ \mu\text{l}$ of the High Control in duplicate into wells H1+H2.
- 3e. Pipet $100\ \mu\text{l}$ of each cell supernatant in duplicate into the subsequent wells.
4. Add $50\ \mu\text{l}$ of Enzyme Label to all wells.
5. Add $50\ \mu\text{l}$ of Antibody to all wells.
6. Cover the plate with a Plate Sealer, place the plate on a plate rotator set at 800-1000 rpm and incubate for 2 hours at $18-28^{\circ}\text{C}$.

Note: Alternatively, incubate the plate for 16-20 hours at $2-8^{\circ}\text{C}$

7. Remove the Plate Sealer. Empty the wells and wash them three times using at least $300\ \mu\text{l}$ of Wash Buffer per well. Strike the plate firmly onto blotting paper.

Important: Allow the pNPP Substrate Solution to come to $18-28^{\circ}\text{C}$ prior to use.

8. Add $200\ \mu\text{l}$ of pNPP Substrate Solution to all wells.
9. Cover the plate with a Plate Sealer, place the plate on a plate rotator set at 800-1000 rpm, protect the plate from direct light and incubate for 30 minutes at $18-28^{\circ}\text{C}$.
10. Remove the plate sealer. Stop the reaction by adding $50\ \mu\text{l}$ of Stop Solution to all wells. Mix shortly on the microtiter plate rotator.
11. Read the absorbance at 405 nm in a microtiter plate reader.

XI. CALCULATION OF RESULTS

1. Glossary

- **Maximum Binding (MB, S₀):** Technical maximum absorption of the ELISA. This value is used for the calculation of the corresponding percent bound (**B/B₀**) values.
- **Blank (NSB):** Technical, non-specific absorption of the ELISA. This value is subtracted from standard, control and sample absorptions.
- **Background:** Basal sulfidoleukotriene (sLT) release of cells after stimulation with Stimulation Buffer only.
- **Stimulation Control:** sLT release of cells upon stimulation with the anti-IgE receptor antibody (the Stimulation Control reagent).
- **Allergen Stimulation:** sLT release of cells upon stimulation with a single allergen or an allergen mix.

2. Standard Curve

- Record the absorbance at 405 nm for each standard, maximum binding (MB = S₀) and blank (NSB) well.
- Average the duplicate values, subtract the average of the blank wells (NSB) and record the averages (= corrected average absorbance).
- Calculate the binding of each pair of standard wells as a percent of maximum binding, with the NSB-corrected MB absorbance taken as 100%:

$$B/B_0 (\%) = \text{percent bound} = \frac{\text{net absorbance}}{\text{net MB absorbance}} \times 100.$$

- Plot the percent bound (vertical axis) versus the sLT concentration in picograms/ml (pg/ml) of the standards (horizontal axis) using a lin/log graph paper.
- Draw the best fitting curve or calculate the standard curve using a spline smoothed fitting algorithm.

3. Samples and Controls

- Record the absorbance at 405 nm for each sample and control well.
- Average the duplicate values, subtract the average of the blank wells and record the averages (= corrected average absorbance).
- Calculate, as described above, the binding of each pair of sample and control wells as a percent of maximum binding, with the NSB-corrected MB absorbance taken as 100%.
- Locate the B/B₀ value of the samples and controls on the vertical axis, draw a horizontal line intersecting the standard curve and read the sLT concentration (pg/ml) from the horizontal axis.

Note: If the initial concentration of an unknown sample is greater than the highest standard, the cell supernatant should be diluted with ELISA Buffer and assayed again according to the assay procedure. The additional dilution must be considered when calculating the final concentration of sulfidoleukotriene present in the unknown sample.

4. Stimulation Yield

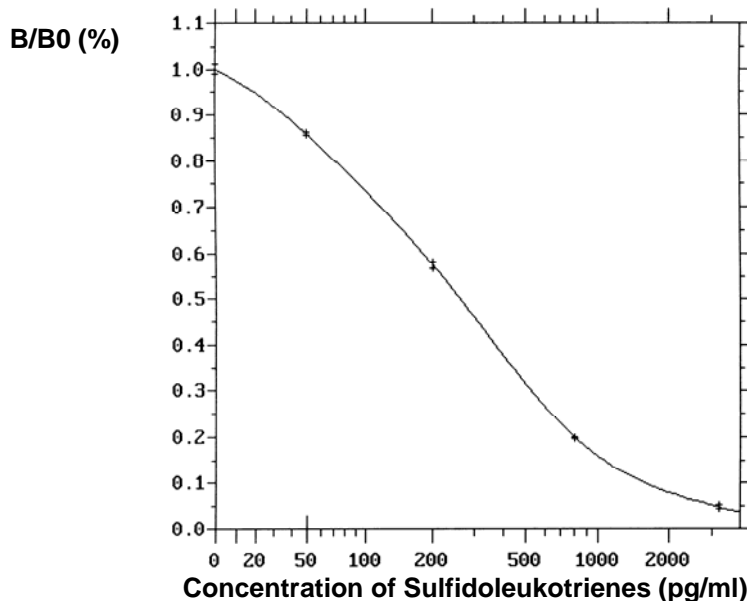
- Yield of Stimulation Control = sLT conc. (Stimulation Control) – sLT conc. (Background).
- Yield of Allergen Stimulation = sLT conc. (Allergen Stimulation) – sLT conc. (Background).

5. Typical Data

These results and standard curve are provided for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.

	Conc. (pg/ml)	Absorbance (OD)	B/B ₀ (%)	Calc. Conc. (pg/ml)	CV Conc. (%)
Blank		0.146			
Blank		0.156			
Blank Avg.		0.151			4.7
Std. S0	0	1.327	100.0		
Std. S0	0	1.303	100.0		
Std. S0 Avg.	0	1.315	100.0		1.3
Std. S4	50	1.146	85.5	51.3	
Std. S4	50	1.155	86.3	48.7	
Std. S4 Avg.	50	1.151	85.9	50.0	3.6
Std. S3	200	0.829	58.2	195	
Std. S3	200	0.812	56.8	205	
Std. S3 Avg.	200	0.821	57.5	200	3.9
Std. S2	800	0.385	20.1	793	
Std. S2	800	0.381	19.8	807	
Std. S2 Avg.	800	0.383	19.9	800	1.2
Std. S1	3200	0.202	4.4	3455	
Std. S1	3200	0.213	5.3	2966	
Std. S1 Avg.	3200	0.208	4.9	3200	10.8
Ctrl. LOW		1.069		76.2	
Ctrl. LOW		1.050		83.2	
Ctrl. L. Avg.		1.059		79.7	6.2
Ctrl. HIGH		0.629		356	
Ctrl. HIGH		0.612		374	
Ctrl. H. Avg.		0.620		365	3.5
Background		1.185		40.2	
Background		1.153		49.3	
Backgr. Avg.		1.169		44.8	14.3
Stim. Control		0.295		1282	
Stim. Control		0.299		1248	
Stim. Ctrl Avg.		0.297		1265	1.9
Allergen		0.474		572	
Allergen		0.490		543	
Allergen Avg.		0.482		557	7.6

ED-20 = 797 pg/ml ED-50 = 262 pg/ml ED-80 = 71 pg/ml



XII. QUALITY CONTROL

Since there are no real controls for sulfidoleukotrienes commercially available, we recommend to use pools of cell supernatants containing different sLT levels for internal quality controls (cell supernatants are stable for up to 4 months at -20°C). All controls should fall within established confidence limits. The confidence limits for the BÜHLMANN synthetic sLT controls are lot-specific and printed on the additional Data Sheet.

It is good laboratory practice to record the following data for each assay:

- Kit lot number
- Opening, reconstitution, dilution and storage dates of all kit components
- Absorbance of blank wells, Standard S0 (maximum binding) and Standard S4, respectively
- Plot of standard curve
- Average absorbance for the High and Low Controls.

The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. If the precision of the assay does not correlate with the established limits and repetition excludes errors in technique, check the following issues:

- Pipetting, temperature controlling and timing devices
- Instrument calibration
- Expiration dates of reagents
- Storage and incubation conditions
- pNPP Substrate Solution should be colorless
- Purity of water.

Note: The BÜHLMANN QC laboratory uses a smoothed spline curve fit.

XIII. LIMITATIONS

A negative CAST[®]-2000 result for a specific allergen can not exclude the potential occurrence of a (even severe) clinical reaction in a patient. Patients with a history of adverse reactions to a drug allergen with a negative CAST[®]-2000 result should therefore be followed up with an additional method, such as an *in vivo* provocation or skin prick test (where appropriate), before any drug is administered.

Best results and reproducibility are obtained when the stimulation part of the CAST[®]-2000 is performed within 3 hours after blood collection. **We strongly recommend to use the CAST venipuncture tubes** (order code: B-SLT-VPT)

Correct and careful washing between the incubation steps of the ELISA part of the assay are a prerequisite for obtaining low background values and reproducible standard, control and patient sample values, respectively.

If the initial concentration of an unknown sample of stimulated cells exceeds that of the highest standard, the sample should be diluted with Incubation Buffer and analyzed again according to the assay procedure.

XIV. EXPECTED VALUES

712 allergic and non-allergic individuals were tested with the **BÜHLMANN CAST®-2000 ELISA** in order to determine **background** and **stimulation control** ranges. The following data were obtained (for calculation details see CALCULATION OF RESULTS on p. 14):

	n	mean [pg/ml]	median [pg/ml]	2.5% Percentile	97.5% Percentile	lowest [pg/ml]	highest [pg/ml]
Background	712	63	56	20	140	<19	788
Stimulation Control	712	1812	1362	199	5429	51	9512

Interpretation of Stimulation Control

In allergic as well as in non-allergic individuals, a net stimulation (after subtraction of background) of at least 200 pg/ml should be expected using the Stimulation Control. The Stimulation Control represents the leukotriene production due to cross-linking of high affinity IgE receptors on basophils by an anti-IgE receptor antibody. The stimulation control may be correlated to the basophil concentration and more important the high affinity IgE receptor density on the patient basophils.

The anti-IgE receptor antibody binds to domain 1 on the alpha subunit of the high affinity IgE receptor (FcεRI) and is a non-inhibiting antibody, i.e. it will bind to the receptor irrespective whether the receptor is free or loaded with IgE.

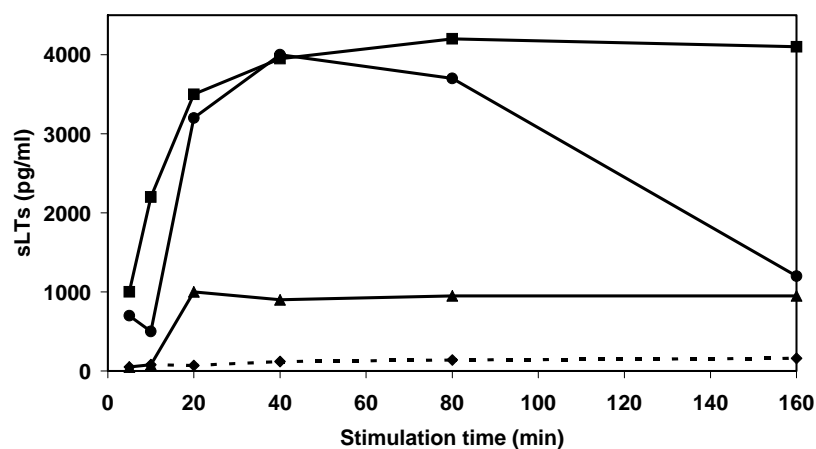
Interpretation of Allergen Stimulation

As a **general guideline**, we propose that individuals with stimulation yields **below 100 pg/ml** should be regarded as negative for the allergen tested.

XV. ASSAY PERFORMANCE

1. Kinetics of Cell Stimulation

An example of the kinetics of sLT production is given in the figure below. The kinetics may differ from one individual to another. However, **an optimum or near optimum is reached**



after 40 minutes of stimulation.

 Background	 Stimulation Control
 D. pteron. (10 ng/ml)	 Timothy grass (10 ng/ml)

2. Intra-Assay Precision ELISA (Within-Run)

The intra-assay precision was calculated from the results of 24 pairs of values from each sample in a single run. The values are presented in pg/ml of sulfidoleukotrienes.

Sample	Mean	SD	% CV
Cell Supernatant 1	54	3.9	7.3
Cell Supernatant 2	221	9.6	4.4
Cell Supernatant 3	467	13.9	3.0
Cell Supernatant 4	592	22.3	3.8
Cell Supernatant 5	1308	44.5	3.4
Cell Supernatant 6	1752	94.9	5.4

3. Intra-Assay Precision of Cell Stimulation and ELISA combined

The statistics were calculated from the results of 10 replicates of cell stimulation from each sample in a single run. The values are presented as pg/ml of sulfidoleukotrienes.

Sample		Mean	SD	% CV
Donor 7	Background	35	5.1	14.7
	Stimulation Control	967	39.4	4.1
Donor 8	Background	46	5.4	11.8
	Stimulation Control	775	71.8	9.3
Donor 9	Background	33	6.5	19.7
	Stimulation Control	711	59.7	8.4

4. Inter-Assay Precision ELISA (Run-to-Run)

The inter-assay precision was calculated for each sample from the results of pairs of values in 15 different runs. The values are presented as pg/ml of sulfidoleukotrienes.

Sample	Mean	SD	% CV
Cell supernatant 10	66	7	10.5
Cell supernatant 11	125	11	8.6
Cell supernatant 12	437	49	11.2
Cell supernatant 13	557	42	7.6
Cell supernatant 14	1121	83	7.4
Cell supernatant 15	1595	148	9.3
Cell supernatant 16	2342	124	5.3

5. Sensitivity

The minimal detectable dose of sulfidoleukotrienes was calculated to be **19 pg/ml** by subtracting two standard deviations from the mean of 20 zero standard replicates (ELISA Buffer, S0) and intersecting this value with the standard curve obtained in the same run.

6. Dilution Linearity

Three patient samples after cell stimulation were assayed either undiluted or diluted with the ELISA Buffer. The values below are presented in pg/ml of sulfidoleukotrienes.

Sample	Dilution Factor	Observed [pg/ml]	Expected [pg/ml]	Recovery O/E [%]
Cell supernatant 17	1:1	1337	--	--
	1:2	749	669	112.0
	1:4	380	334	113.7
	1:8	205	167	122.7
	1:16	91	84	108.9
Cell supernatant 18	1:1	1558	--	--
	1:2	874	779	112.2
	1:4	476	390	122.2
	1:8	252	195	129.4
	1:16	112	97	115.0
Cell supernatant 19	1:1	1421	--	--
	1:2	769	711	108.2
	1:4	408	355	114.8
	1:8	203	178	114.3
	1:16	90	89	101.3

7. Spiking Recovery

Three unstimulated patient samples were assayed before and after spiking with varying amounts of sulfidoleukotriene D₄. The values below are presented in pg/ml of sulfidoleukotrienes.

Sample	Spiked with [pg/ml]	Observed [pg/ml]	Expected [pg/ml]	Recovery O/E [%]
Cell supernatant 20 non spiked: 17 pg/ml	50	75	67	111.9
	100	109	117	93.2
	200	204	217	94.0
	400	444	417	106.5
	800	801	817	98.0
	1600	1488	1617	92.0
	3200	2728	3217	84.8
Cell supernatant 21 non-spiked: 61 pg/ml	50	108	111	97.3
	100	182	161	113.0
	200	269	261	103.1
	400	442	461	95.9
	800	820	861	95.2
	1600	1588	1661	95.6
	3200	3086	3261	94.6
Cell supernatant 22 non-spiked: 40 pg/ml	50	102	90	113.3
	100	116	140	82.9
	200	257	240	107.1
	400	476	440	108.2
	800	862	840	102.6
	1600	1786	1640	108.9
	3200	2941	3240	90.8

8. Specificity

The following cross-reactivities of the monoclonal antibody used in this assay were determined at 50% binding:

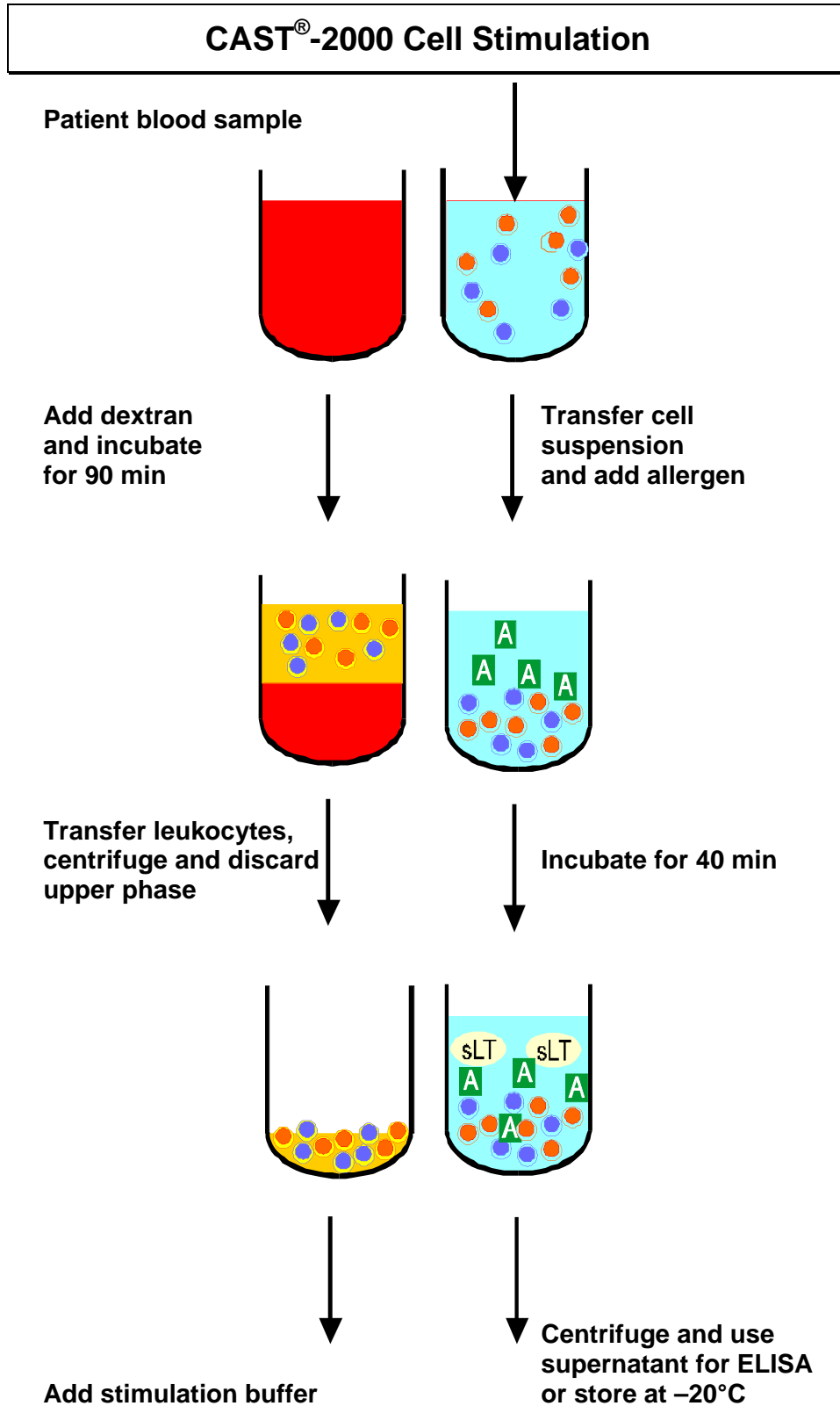
Leukotriene D ₄	100.0%
Leukotriene B ₄	1.1%
Leukotriene C ₄	143.6%
Leukotriene E ₄	63.6%
N-Acetyl-Leukotriene E ₄	84.9%
5-Hydroperoxyeicosatetraenoic acid (HETE)	0.02%
Prostaglandine D ₂	<0.01%
Prostaglandine E ₂	<0.01%
Prostaglandine F _{2α}	<0.01%
Thromboxane B ₂	<0.01%

XVI. REFERENCES

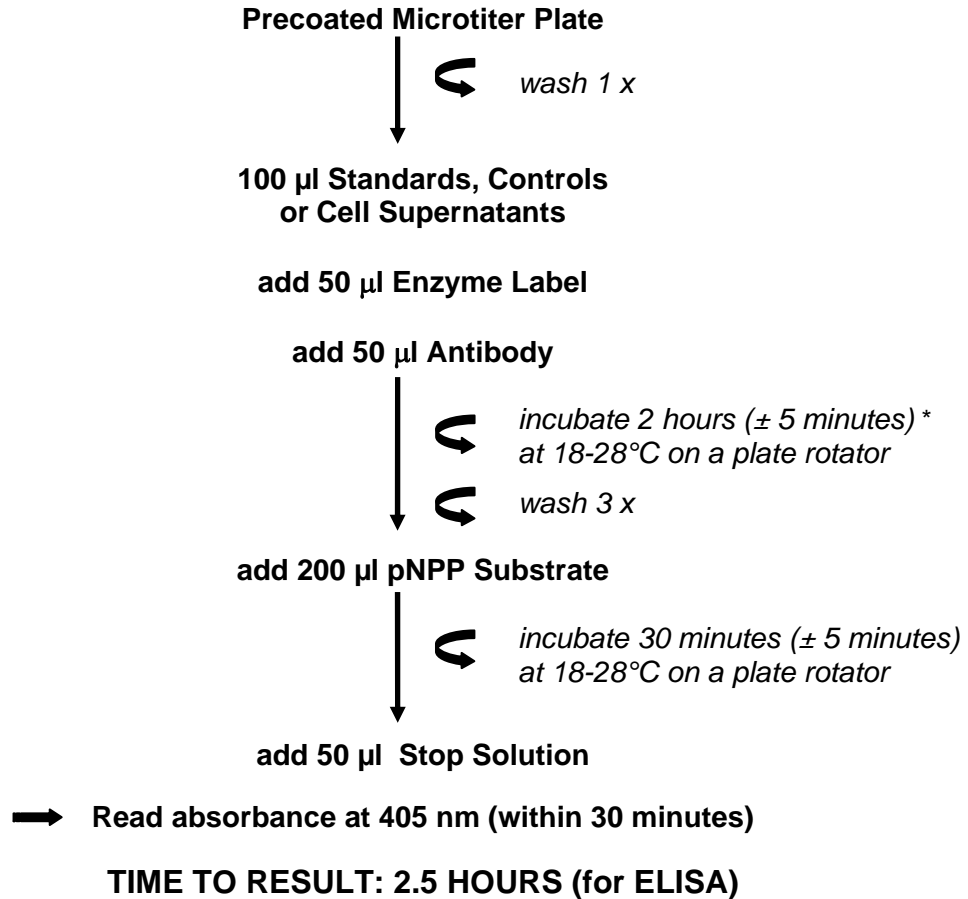
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XVII. PIPETTING PROTOCOLS



TOTAL INCUBATION TIME FOR CELL STIMULATION: 130 MINUTES

CAST[®]-2000 ELISA

**alternative procedure: 16-20 hours at 2-8°C*

XVIII. ORDERING INFORMATION

Two Kit sizes are available: EK-CAST 192 tests
 EK-CAST5 480 tests