

A rapid lateral flow immunoassay for the detection of fungal alpha-amylase at the workplace

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Fungal alpha-amylase is a flour supplement which is added to improve the quality of bakery products. Various studies have shown that exposure to this enzyme is an important risk factor for the development of bakers' allergy and this allergy is reported to be one of the most frequent causes of occupational asthma. A rapid assay was developed to monitor exposure to occupational allergens directly at the workplace. The sensitivity of the developed assay is 0.32 ng amylase mL⁻¹ in a buffer system with the commercially available alpha-amylase preparation Fungamyl 1600S as the standard. Initial validation tests ($n = 33$) were performed with airborne and settled dust from an industrial bakery. The new lateral flow immunoassay detected amylase in 22 of the 26 samples regarded as positive in an enzyme immunoassay, and was negative for all seven enzyme immunoassay-negative samples, while the four lateral flow immunoassay-negative/enzyme immunoassay-positive samples all had levels below 2 ng mL⁻¹. The sensitivity of 2 ng mL⁻¹ of the amylase lateral flow immunoassay is sufficient for first screening purposes and, therefore, this simple and rapid assay may allow direct on-site demonstration of work-related hazards of bio-allergen exposure. This would be particularly useful in occupational hygiene practice, especially in traditional or small-scale bakeries which lack the technological skills for testing the exposure to respiratory allergens.

Introduction

Fungal alpha-amylase (1,4- α -D-glucan glucanohydrolase) is used as a flour supplement in the baking industry; it converts starch into polysaccharides and this results in an improvement of the quality of bread. Usually the enzyme is derived from *Aspergillus oryzae* and is routinely added to the flour at the mill or bakery.

Fungal alpha-amylase is an important risk factor for the development of bakers' allergy and asthma.^{1,2} Exposure-response studies show that even very low levels of this enzyme can cause sensitisation.³⁻⁵ In several industrialised countries bakers' allergy is reported to be one of the most frequent causes of occupational asthma.^{6,7}

Houba *et al.*⁴ and Sander *et al.*⁸ developed enzyme immunoassays (EIAs) for the detection of fungal alpha-amylase in extracts of personal dust samples. However, these assays require the skilled personnel and equipment of a laboratory, thus often cannot be assessed at the place where allergen-containing materials like enzyme-containing flours are handled. Moreover, since sampling and measurement often take considerable time no immediate actions can be taken to

diminish exposure and therefore sensitisation to the allergen. To overcome these drawbacks, the specific antibodies developed for these EIAs were used to set up a simple one-step immunochromatography assay, based on the principle of a lateral flow immunoassay (LFIA). In these LFIA's carbon nanoparticles were applied as the signal.^{9,10} LFIA's are especially suited for low-facility laboratory and field settings; the tests are simple and straightforward, the results are easy interpretable, expensive equipment is not necessary and specific skills are not required.

Here, we describe the development and performance of a rapid assay in tube format for the detection of fungal alpha-amylase in settled dust extracts. Wipe samples were prepared by a simple and short extraction procedure and directly applied to the device. LFIA's allow direct on-site demonstration of work-related hazards of bio-allergen exposure, which would be particularly useful in occupational hygiene practice.

Experimental

Reagents

For development of LFIA's, previously described antibody reagents against the fungal α -amylase allergen were used.^{4,8} All other immune reagents were obtained from Jackson ImmunoResearch (Soham, UK). Chemicals used in the development of the LFIA's were analytical pure and obtained from Merck (Darmstadt, Germany). Bovine Serum Albumin (BSA) was purchased from Sigma (Zwijndrecht, The Netherlands). To determine sensitivity and specificity of the occupational

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allergen LFIA, Fungamyl 1600s (Novozymes, Bagsvaerd, Denmark), was used as a standard.

Purification of antibodies

Polyclonal rabbit antibodies were affinity-purified on an alpha-amylase column.⁴ The monoclonal anti-alpha-amylase antibody, IX3c4,⁸ was isolated from hybridoma culture supernatants by affinity-purification on an anti-mouse IgG agarose column (Rockland, Philadelphia, USA). The culture supernatant was filtered with 0.45 µm filters, applied on the column, washed with phosphatebuffer (20 mM, pH 7) and eluted with glycine buffer (0.1 M, pH 2.7). Eluted fractions were neutralised with Tris-HCl buffer (1 M, pH 9) followed by dialyses against borate buffer 5 mM, pH 8.8 to replace the buffer with a buffer with a low ionic strength. Centrifugal filter devices (50 000 NMWL, Millipore, Billerica, USA) were used to desalt and concentrate the affinity purified antibody batches. Protein yields of all the different antibody fractions were estimated by the Bicinchoninic Acid Assay (BCA) kit as described by the manufacturer (Pierce, Rockford, USA).

Preparation of carbon nanoparticles—antibody detector ligand

The monoclonal anti-amylase antibody, IX3c4, was conjugated to colloidal carbon nanoparticles by the following method. A colloidal carbon (Spezial Schwartz 4, Degussa AG, Frankfurt, Germany) suspension was prepared as a stock at 1% (w/v) in demineralised water. The colloidal carbon stock suspension was diluted four times with coupling buffer (borate buffer, 5 mM, pH 8.8) to give a concentration of 0.25% (w/v). The suspension was sonicated for 5–10 min on ice using a Branson Model 250 Sonifier (output control 3 f 27 W, 20 kHz). The antibody dissolved in the coupling buffer was added to the diluted colloidal carbon suspension to a concentration of 100 µg of protein mL⁻¹. The pH was readjusted to pH 8.8 and the suspension was incubated by end-over-end mixing at 4 °C overnight. Subsequently, 1/10 volume of blocking solution (1% (w/v) BSA in conjugation buffer) was added and incubation was prolonged for four hours at 4 °C by end-over-end mixing. The suspension was washed with stabilisation buffer (borate 100 mM, 0.1% (w/v) BSA, 3% (w/v) trehalose, 0.05% (w/v) polyethylene glycol (MW 20 000)) three times by centrifugation at 13 636 × *g* for 15 min. Following the last centrifugation step, the pellet of colloidal carbon nanoparticles with the detection ligand adsorbed (carbon conjugate) was resuspended in the stabilisation buffer as a 0.25% (w/v) suspension and stored in the dark at 4 °C.

Immobilisation of the capture antibody

Unbacked nitro-cellulose membranes (AE99, Whatman Schleicher and Schuell, Middlesex, UK; 35 mm × 35 cm) were fixed to a vinyl backing (Mylar AD Back 79373, Whatman Schleicher and Schuell). The polyclonal anti-amylase antibodies were applied to these membranes in a line format using a Linomat IV (CAMAG, Muttentz, Switzerland) in 1 µL/5 mm at various concentrations, ranging between 100 and 1000 ng of capture antibody in 5 mM borate buffer (pH 8.8). Finally, 500 ng antibody/5 mm was used in the tube assay. As a positive control, a line of goat anti-mouse IgG was applied

at a concentration of 100 ng/5 mm. The lines were immobilised at a distance of 13 mm for the specific line and 18 mm for the control line to the beginning of the strip. The membranes were dried for at least 2 h at 37 °C and cut into individual strips of 5 mm width by a Cutter 4000 (BioDot, Chichester, UK).

Assay format and assemblage

Preliminary characterisation of the antibodies in an experimental LFIA setup was performed as described by Aldus *et al.*¹⁰ Sample pads (Cytosep, Pall, New York, USA; 12 × 5 mm) were blocked with running buffer (0.1 M borate buffer, pH 8.8; 1% (w/v) BSA; 0.05% (w/v) Tween 20 and 0.02% (w/v) NaN₃) and dried at 37 °C. This step was repeated twice. Absorption pads (filter paper 2886, Schleicher & Schuell “300”, 20 × 17 mm) were used untreated. Tubes (Maxisorp, 75 × 12 mm, Nunc, Roskilde, Denmark) were blocked with running buffer (300 µL per tube), followed by incubation at 37 °C for 2 hours. Carbon conjugate, 25 times diluted in storage buffer (0.1 M borate buffer, pH 8.8 + 1% (w/v) BSA + 0.02% (w/v) NaN₃ + 0.05% (w/v) trehalose), was added to the tube (25 µL) and allowed to dry overnight.

The format of the LFIA was according to the schematic overview (Fig. 1) and composed on a vinyl template (0.010" White, Matte Vinyl GL-187, G&L, San Jose, USA) for use as tube assay. The individual assays were sealed in aluminium pouches with a silica desiccant pellet. After incubation for 2 days at 37 °C the packages were stored at room temperature.

Eluates or extracts of samples (100 µL) were applied in running buffer (0.1 M borate buffer, pH 8.8; 1% (w/v) BSA; 0.1% (w/v) Tween 20 and 0.02% (w/v) NaN₃), directly on top of the dried carbon conjugate at the bottom of the tube. Subsequently, the membrane with attached absorbent pad was placed in the tube to start the assay. The simple tube format used has a maximum result reading time of 30 minutes, but above certain concentrations results are visible within approximately 5 min after starting the assay.

Sensitivity analysis of LFIA

To determine the sensitivity of the assay, Fungamyl 1600s was used in a series of concentrations ranging from 0.02 to 20 ng mL⁻¹. Protein concentration was determined according to the Bradford assay with bovine serum albumin as reference.¹¹ Various extracts of flours and baking additives containing

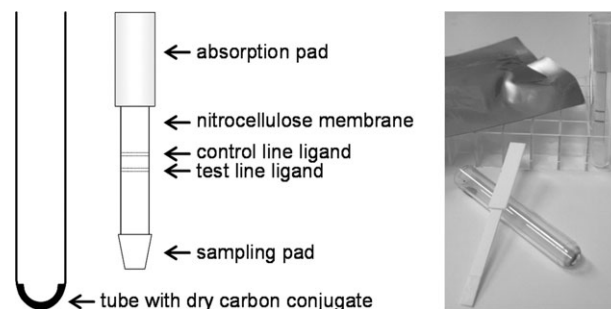


Fig. 1 An example is shown of a one-step tube assay. Carbon conjugate detector ligand is dried on the bottom of the tube. Sample is applied on top of the carbon conjugate and the strip placed into the tube. If allergen is present, a black line will appear.

alpha-amylase were diluted in assay buffer and applied to the tubes and the amount of alpha-amylase present was measured with a monoclonal antibody based sandwich EIA (mab-EIA).⁸

Test lines were scanned with a flatbed scanner (Epson Perfection 3200 Photo) and the line intensities were analysed with TotalLab (nonlinear dynamics). Curve fitting was done with SlideWrite (Advanced Graphics Software).

Lab trial

Samples from relevant workplaces were collected, extracted and applied to the LFIA. LFIA results were compared with the data obtained by conventional immunoassays.^{4,8} Both airborne dust samples already available from former studies^{12,13} and newly collected wipe samples of settled dust in bakeries were used.

1. Airborne dust samples

Airborne dust samples were collected with a parallel sampler in bakeries in various countries and at several locations. After sampling filters were extracted in one laboratory.¹³ Subsequently, sample extract aliquots were distributed among two laboratories for analyses of wheat and amylase allergens by EIA. Additionally, full-shift personal air samples were taken in a German bakery where airborne alpha-amylase had been detected before,¹⁴ using Gesamtstaubprobenahme (GSP) sampling heads equipped with Teflon filters of 3.7 cm diameter, and an air flow of 3.5 L min⁻¹. Extracts with known amylase allergen content were used in the rapid assay to compare LFIA performance with the EIA results. For LFIA, filter extracts were mixed with an equal volume of double-strength running buffer and 100 µl of this diluted sample was added to the assay.

2. Settled dust (wipe) samples

Wipe dust samples were collected in industrial bakeries in The Netherlands. Filter paper (Whatman Schleicher and Schuell, Middlesex, UK, no. 4 with 5.5 cm diameter) was used to wipe an area as large as a regular-sized paper (A4-size, simple comparison size). Samples were collected both with a dry filter which was used to wipe the surface, and (from an adjacent parallel surface) also with a filter that had been pre-wetted with Phosphate Buffered Saline (PBS) containing 0.05% Tween 20. A disposable 3 mL pipette was used to apply 8 droplets of solution to the filter (approx. 0.2–0.3 mL). Filters were placed in a 15 mL tube and eluted with 6 mL running buffer, by just shaking it by hand a couple of times followed by vortexing for 10 seconds at maximum speed. Some of the samples were used directly without centrifugation, and the others were centrifuged at 1000 g. Samples/supernatants were tested directly in the LFIA assay.

Results

A rapid one-step immunoassay was developed for the detection of fungal alpha-amylase, using monoclonal⁸ and polyclonal⁴ antibodies as detection and capture antibodies, respectively. The LFIA was adapted to a tube-format (Fig. 1) to enable easy handling while reducing the overall costs of the assay. To determine the limit of detection of the tube-LFIA dilution series of the alpha-amylase standard (Funga-

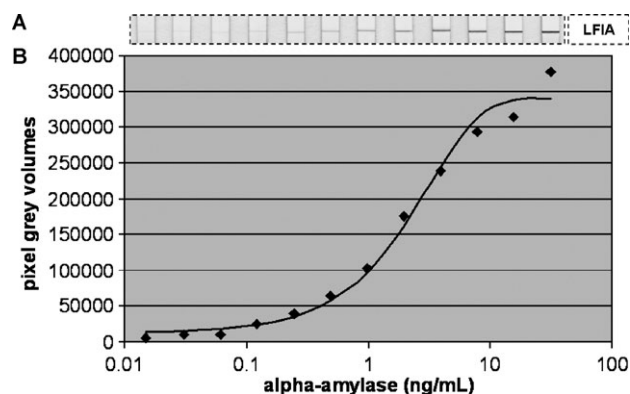


Fig. 2 Limit of detection of the amylase tube-LFIA. Panel A shows a dilution series (from 0.015 to 31 ng mL⁻¹) of the standard alpha-amylase in running buffer applied to the tube-LFIA. The strips correspond to the points depicted in the figure. Panel B shows the calculated pixel grey volumes after flatbed scanning and image analysis.

myl 1600S) in buffer were applied to the assay. The line intensities were analysed by flatbed scanning, and pixel grey volumes were calculated with TotalLab analysis software. This resulted in an LFIA with a detection limit of 0.32 ng allergen mL⁻¹ (Fig. 2).

Sensitivity and preliminary specificity data of the assay were tested using extracts of various flours and baking additives with different amylase content. Extracts of the powder samples were diluted directly into the assay buffer and applied to the tube. Up to 1 ng allergen mL⁻¹ could be detected with the one-step tube assay (tested range: 0.9–40 ng amylase mL⁻¹, Fig. 3). No non-specific signal was visible when control samples (*i.e.*, flours without amylase) were run.

A preliminary two-laboratory trial was performed both on airborne and settled dust samples. From various places in an industrial bakery wipe samples were collected. Extracts of these filters were applied to the tubes (Fig. 4, a selection of strips is shown). LFIAs showed positive assay results at places where high allergen levels were expected, *e.g.* near the dough mixer. In the office where the flour level is supposed to be minimal, the LFIAs scored negative. This was in concordance with the results of the EIA which has been used to assess the amylase levels in the samples. Furthermore, 16 airborne samples were tested, where a lower amylase yield is to be

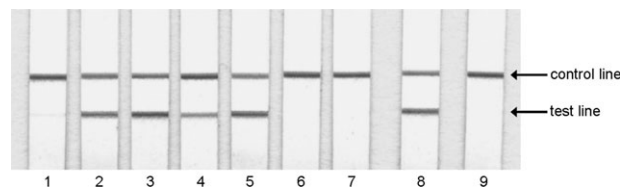


Fig. 3 Extracts of flour and baking additives (fungal alpha-amylase content of the extracts as determined with mab-EIA⁸ in brackets) were tested in the alpha-amylase tube LFIA. 100 times diluted in assay buffer: (1) Hefe-Quarkteig-Fertigmehl (90 ng mL⁻¹), (2) Stabilo (4000 ng mL⁻¹), (3) Rubina Mega 2000 (2500 ng mL⁻¹), (4) Rubina Berliner Royal (200 ng mL⁻¹), (5) Rubina Eiszeit (3500 ng mL⁻¹), (6) Weizenmehl (wheat flour; negative control), (7) Roggenmehl (rye flour; negative control), (8) Fungamyl 1600S (Novozymes, positive control 50 ng mL⁻¹) and (9) buffer control.

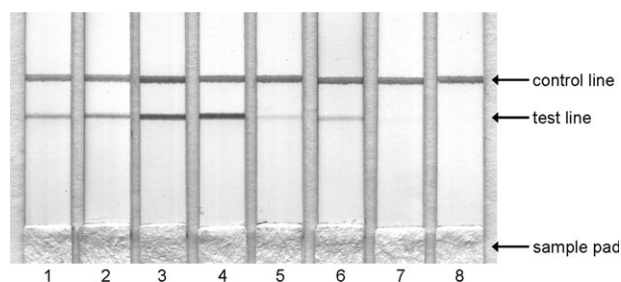


Fig. 4 Results of wiped dust samples taken at various places in a bakery, and directly applied to the amylase tube assay. Amylase content of the extracts was determined with EIA:⁴ (1) top of the dough mixer (27 ng mL⁻¹), (2) top of the dough mixer (81 ng mL⁻¹), (3) top of the dough mixer (1184 ng mL⁻¹), (4) trolley with a computer close to the dough mixer (485 ng mL⁻¹), (5) surface below dough transport-track (7 ng mL⁻¹), (6) floor in the area where dough transport-track is located (11 ng mL⁻¹), (7) cupboard with books in the bakery office (<limit of detection EIA) and (8) blank filter (<limit of detection EIA).

expected. It was possible to detect amylase in a concentration range from 4 to 1185 ng amylase mL⁻¹ measured by mab-EIA. As the airborne filter extracts had been diluted 1:2 in the running buffer before testing in the LFIA, the actual detection limit of the assay was 2 ng mL⁻¹. In total 33 samples of various origin were tested with the tube amylase-LFIA. In EIA, 26 samples were tested positive and 22 of these were positive in LFIA as well (Table 1). The four EIA-positive samples tested negative in LFIA all had alpha-amylase levels below 2 ng mL⁻¹, *i.e.*, the cut-off level of the assay (Fig. 3). In conclusion, all EIA-positive samples with an amylase content of > 2 ng mL⁻¹ were correctly identified. Seven samples tested negative in EIA were also negative in LFIA (indicating a good specificity as well).

These test series also showed that the alpha-amylase tube LFIA can be used to analyse extracts in which the amylase concentration differs several orders of magnitude, *i.e.*, from 7 ng mL⁻¹ (Fig. 4, strip 5) to 1185 ng mL⁻¹ (strip 3). At very high amylase concentrations (> 1000 ng mL⁻¹), the assay still gives a positive signal. Samples having amylase concentrations up to 7700 ng mL⁻¹ could be easily scored on the basis of intense lines (result not shown).

The correlation between EIA values and the subjective readings of the LFIA results (done by the same 2 or 3 persons; there was full agreement on positive and negative readings) for the 33 samples is depicted in Fig. 5. It appeared that the good signal-to-noise ratio of the black carbon label enabled easy interpretation of the results and 100% assignment of positive samples with alpha-amylase levels above the detection limit of the LFIA, *i.e.*, 2 ng mL⁻¹.

Table 1 Fungal alpha-amylase results showing statistical data for the detection in tube assays

LFIA result	EIA positive	EIA negative	Total
Positive	22	0	22
Negative	4	7	11
Total	26	7	33

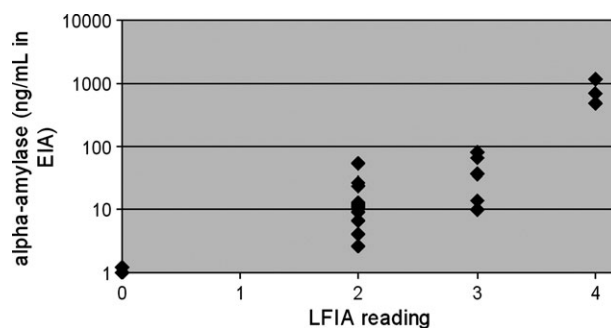


Fig. 5 Correlation between EIA and LFIA results ($n = 33$; depicted as 0 = -, 1 = $\pm$, 2 = +, 3 = ++, 4 = +++), where the line intensity is the average score of the observers' readings and indicated as weak ($\pm$) to very strong (+++). For dust samples the figure shows the wide range of amylase concentrations that can be measured with the amylase-LFIA.

Discussion

Fungal alpha-amylase is an important cause of occupational allergy in bakeries and the flour industry. Exposure assessment is one of the most important parts of every epidemiological study.¹⁵ Especially in the case of airborne allergens it is an advantage to determine the actual allergen levels at the workplace to be able to timely take necessary protective measures. In view of this, a simple one-step immunochromatographic tube assay was developed to detect fungal alpha-amylase allergen in extracts of airborne and settled dust samples in bakeries. The LFIA, set up with antibodies previously used in EIAs,^{4,8} has a sensitivity of 0.32 ng mL⁻¹ in a buffer system with known standards. The simple tube format used has a maximum result reading time of 30 minutes, but above certain concentrations results are visible within approximately 5 min.

Preliminary studies with this LFIA in bakeries showed that detection of allergens in the low ng mL⁻¹ range could be achieved both in settled dust and airborne dust samples. Although the assays were originally meant to be applied for analyses of settled dust samples, expected to contain higher allergen amounts, the sensitivity of the LFIA appeared to be sufficient to measure the alpha-amylase allergen in airborne dust samples as well. In comparison to the traditional assays as EIAs, these LFIAs combine speed, low-cost and simplicity with high sensitivity and accuracy.

LFIAs are semi-quantitative assays with a particular cut-off level. However, as with other immunoassays, LFIA results may be obscured by the so-called hook-effect, *i.e.*, when samples contain very high levels of the specific protein. However, even up to very high levels, *i.e.*, 7700 ng mL⁻¹ a clear test line was still visible. This indicates that the alpha-amylase tube LFIA can be used for samples with amylase concentrations that differ well over three orders of magnitude. For use in a bakery setting and by non-skilled personnel this is a big advantage, meaning that in most cases it will not be necessary to dilute samples to exclude a possible false-negative result due to the hook-effect. The hook-effect may occur at strong antigen excess, when a large proportion of both detection and capture antibodies is complexed to antigen, thereby diminishing the absolute number of detection and

capture antibodies available for tripartite sandwich formation. Consequently, colour formation at the specific precipitation line would be weaker or even absent. Therefore, the broad dynamic range of the alpha-amylase tube LIFA is especially beneficial for a user-friendly application at the workplace.

With respect to the onset of clinical symptoms upon amylase exposure literature data are inconclusive.^{16,17} Levels as low as 1 to 5 ng m⁻³ have been suggested to induce amylase allergy. In principle such levels would be measurable with the amylase-LFIA provided the air sampling and extraction protocols (pump flow, sampling time, extraction volume, *etc.*) would be optimised for this test system. For wipe samples a relationship between clinical symptoms and amylase levels have not been studied. However, the amount of amylase per surface area may be indicative of a potential health hazard upon increased air flows in the room where the wipe sample was taken. Therefore, the amylase-LFIA can be applied as a warning system as well, making workers aware of occupational allergy risks.

Some parameters of the amylase-LFIA still have to be further investigated. The present pilot series should be extended with larger numbers of samples to allow further statistical evaluation of its sensitivity and specificity in field/population studies. Furthermore, while for other already commercialised LFIA's based on carbon nanoparticles a shelf life of two years is guaranteed,[†] it is as yet unknown what the shelf-life of the alpha-amylase assay is. In addition, although preliminary experiments with the LFIA's are promising with respect to the levels of sensitivity, the method of sampling may be further optimised. The sampling method has to be easy and as quick as possible to make the LFIA a real rapid one-step methodology. The optimal sampling method used up to now is wiping the surface with a pre-wetted filter and extraction of the dust sample in LFIA running buffer by just shaking, vortexing for 10 seconds at maximum speed and centrifugation at 1000 g for several minutes. According to our more recent field experience the centrifugation step may even be omitted without leading to a different outcome.¹⁸ Further optimisation may be foreseen in the number of handling steps by developing a one-step sampling device. Finally, in these preliminary test series we only assessed extracts made at the laboratory from airborne or settled dust samples collected at the workplace. In a further and more extensive field study in a series of industrial bakeries we recently confirmed the value of the amylase LFIA, showing that it indeed also can be used directly at the workplace itself.¹⁸

Conclusion

Potentially, the alpha-amylase specific LFIA is a very useful and sensitive tool for the rapid and semi-quantitative assessment of allergen levels at the workplace. The assay can be used to monitor amylase exposure directly at the workplace and, therefore, may be instrumental in optimising work processes in bakeries. Knowledge of actual levels of allergen exposure allows one to take timely protective measures to

prevent further contact with the allergen and possible sensitisation to the enzyme. Especially in traditional or small-scale bakeries, which lack the technological skills for testing the exposure to respiratory allergens,¹⁵ such simple and rapid assays could be easily introduced in daily practice. As an additional advantage, the possibility of measuring actual levels of airborne allergens would contribute to a better awareness of the people at risk, *i.e.*, the bakery workers. Moreover, although traditional testing is focussed on personal air samples, the alpha-amylase LFIA can also be used for analysing the ingredients to be used in the bakery process, such as pre-mixes, and for analysing all kinds of surfaces in the working environment.

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† Ptofcare_sales_card_hcg.pdf at the Genzyme Diagnostics website.