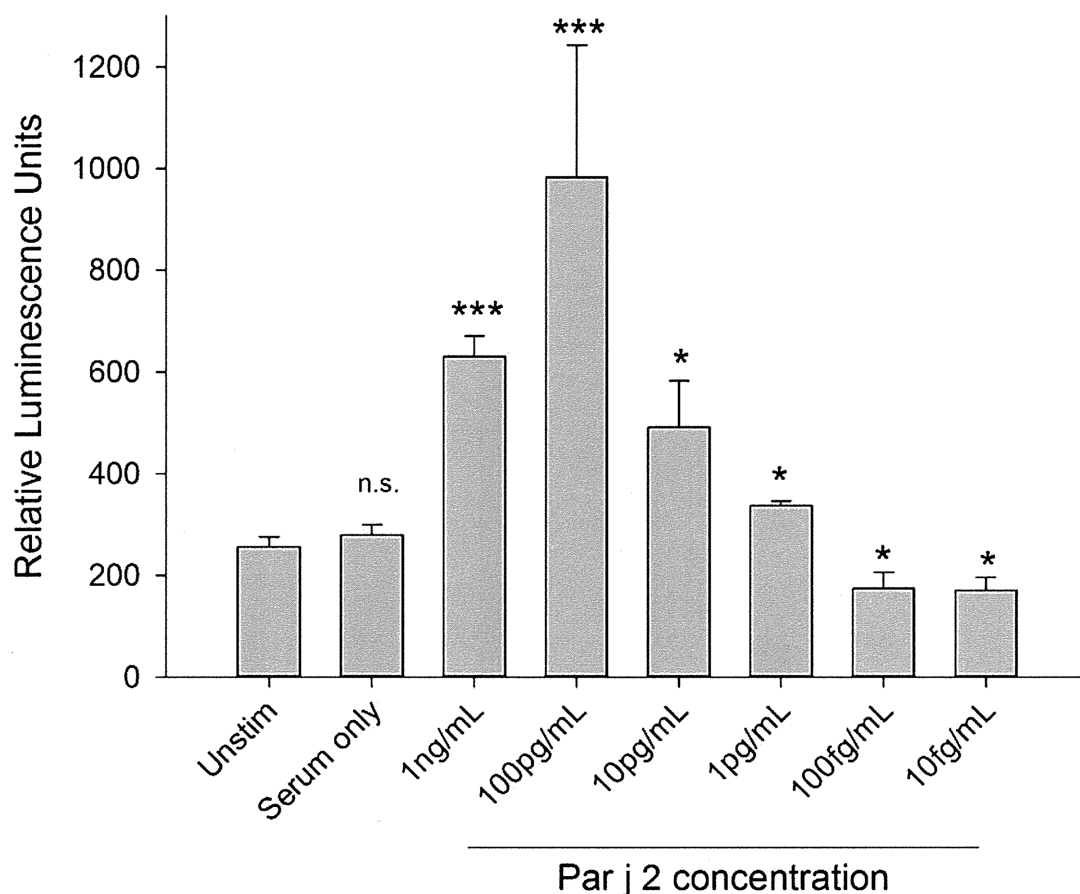



**Figure 3. Optimal concentration and minimal concentration of detectable allergen.** This was determined by sensitising RS-ATL8 cells with the pooled serum (1:50 dilution) from individuals with a specific IgE response to the Par j 2 allergen and challenged with recombinant Par j 2, serially diluted from 1 ng/mL to 10 fg/mL in 1:10 dilution increments. Data are mean ±SD of the readings of three separate wells. Multiplicity adjusted p-values (ANOVA followed by Dunnett's post-hoc test) ***: $p<0.001$, *: $p<0.05$, n.s.: not significant. Representative of three separate experiments performed in triplicates with comparable results.
doi:10.1371/journal.pntd.0003124.g003

displayed a characteristic bell-shaped curve over a wide range of allergen concentrations ranging from 10 µg/mL to 1 pg/mL, with an optimum at 100 pg/mL. An example for such dose-response curve from 1 ng/mL to 10 fg/mL is shown in Figure 3 (see Figure S3 in Supplementary data for higher concentration range).

Higher concentrations of human serum can display strong cytotoxic activities towards RBL cells, requiring the serum to be sufficiently diluted [35]. This dilution however also leads to dilution of the IgE present in the serum, potentially limiting sensitivity of the assay by reducing efficiency of sensitisation, particularly when using the traditional beta-hexosaminidase enzymatic assay for detection of degranulation and sera with low IgE titres [36].

Therefore in order to test the potential cytotoxic activity of the Par j 2-specific serum pool with and without anti-viral treatment the RS-ATL8 cells were sensitized overnight with different serum dilutions, challenging the cells with the previously determined optimal 100 pg/mL Par j 2 concentration and measuring luciferase activity after 4 hours. As shown in Figure 4, the highest concentration of untreated serum (10-fold dilution) reduced the measured luciferase activity by approximately two thirds compared with the optimal 50-fold dilution, which can be ascribed to the well documented cytotoxic effects of some human sera on RBL cells. The anti-viral treatment completely abrogates the luminescent response at higher serum concentrations but can be used efficiently in dilutions higher than 1:100.

We assessed whether it is possible to heat sera in the presence of 2M glucose or 1M $MgSO_4$, conditions which were shown by Binaghi [37] to prevent denaturation of IgE, without affecting successful inactivation of complement. The results are shown in Figure 5. Heating of serum in the presence of 2M glucose resulted in complete protection of IgE as judged by its ability to sensitise RS ATL8 cells and their ability to produce luciferase upon stimulation with anti-IgE or recombinant Par j 2 allergen.

Our next step was to test the ability of the reporter cell line sensitized with a serum pool from *S. mansoni*-infected individuals virally inactivated with Tween-80 to report engagement of the IgE receptor when stimulated with SmTAL-1 and SmTAL-2 produced in wheat germ lysates. A sensitisation to wheat allergens as a source of activation could be categorically ruled out as a source of error as activation only occurred in the presence of translated allergen but not with wheat germ lysate controls.

Figure 6 shows that stimulation of cells sensitized with a serum pool from *S. mansoni*-infected individuals with an optimal dilution of SmTAL-1 results in marked elevation of luciferase production,

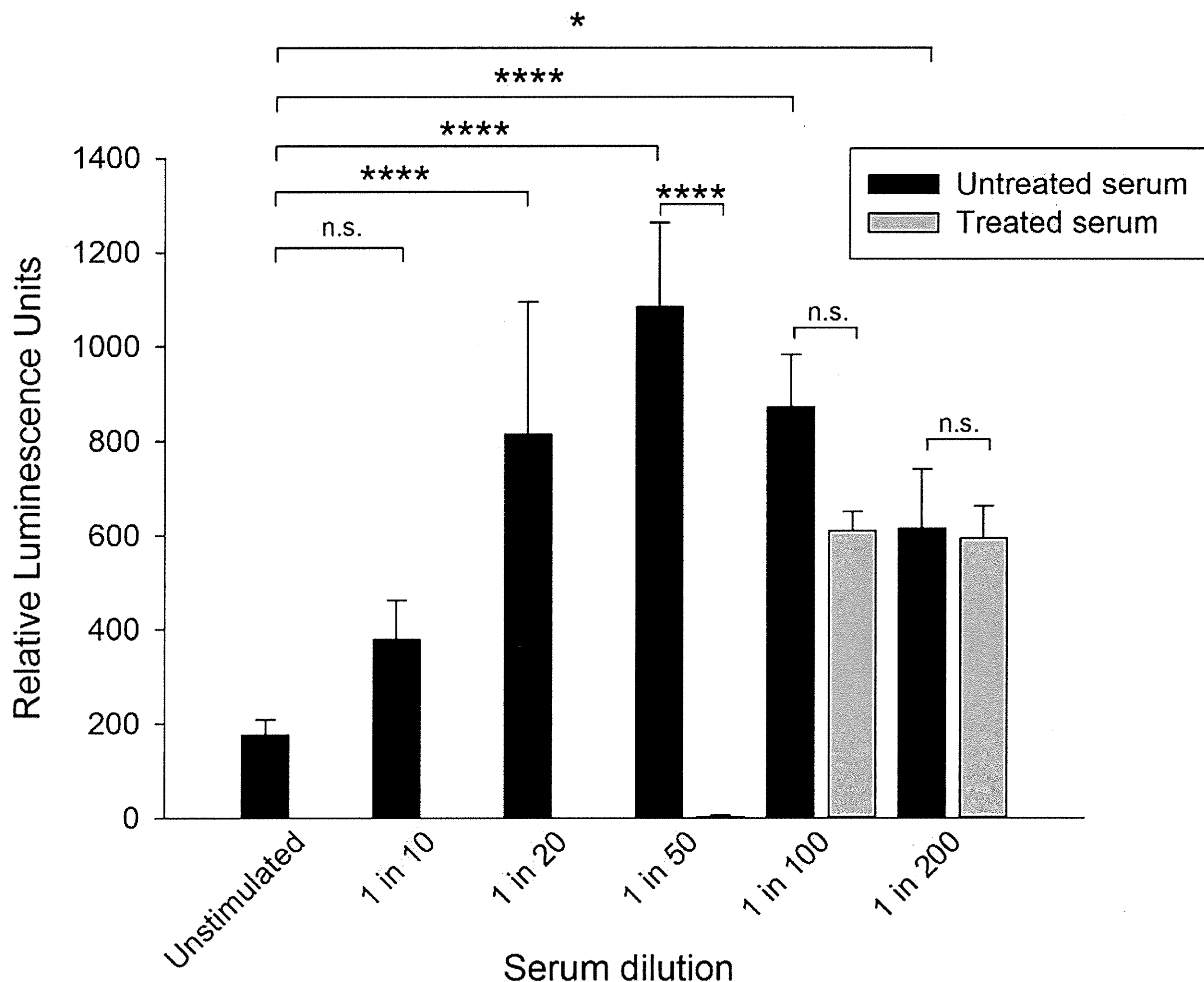



**Figure 4. Luminescence data from the determination of serum-mediated and detergent-mediated cytotoxicity.** The Par j 2 serum pool was incubated with a mixture of 0.3% (v/v) TNBP (Tri-N-butylphosphate) and 1% (v/v) Tween-80 (Polyoxyethyleneorbitan) and incubated under rotation at room temperature for 1 hr. Treated and untreated serum samples were then used in the indicated dilutions (v/v) to sensitise RS ATL8 cells overnight, followed by stimulation with 100 pg/mL Par j 2 recombinant allergen the next day. Data are mean ±SD of the readings of three separate wells. Positive and negative controls included in this experiment (A23187 and PMA: 1840.00±168.02 RLU; IgE+anti-IgE: 4991.00±171.62 RLU) are not shown. N.s.: not significant; n.t.: not tested; *: $p<0.05$; ****: $p<0.0001$ (ANOVA followed by Tukey post-hoc test, all p-values adjusted for multiplicity).
doi:10.1371/journal.pntd.0003124.g004

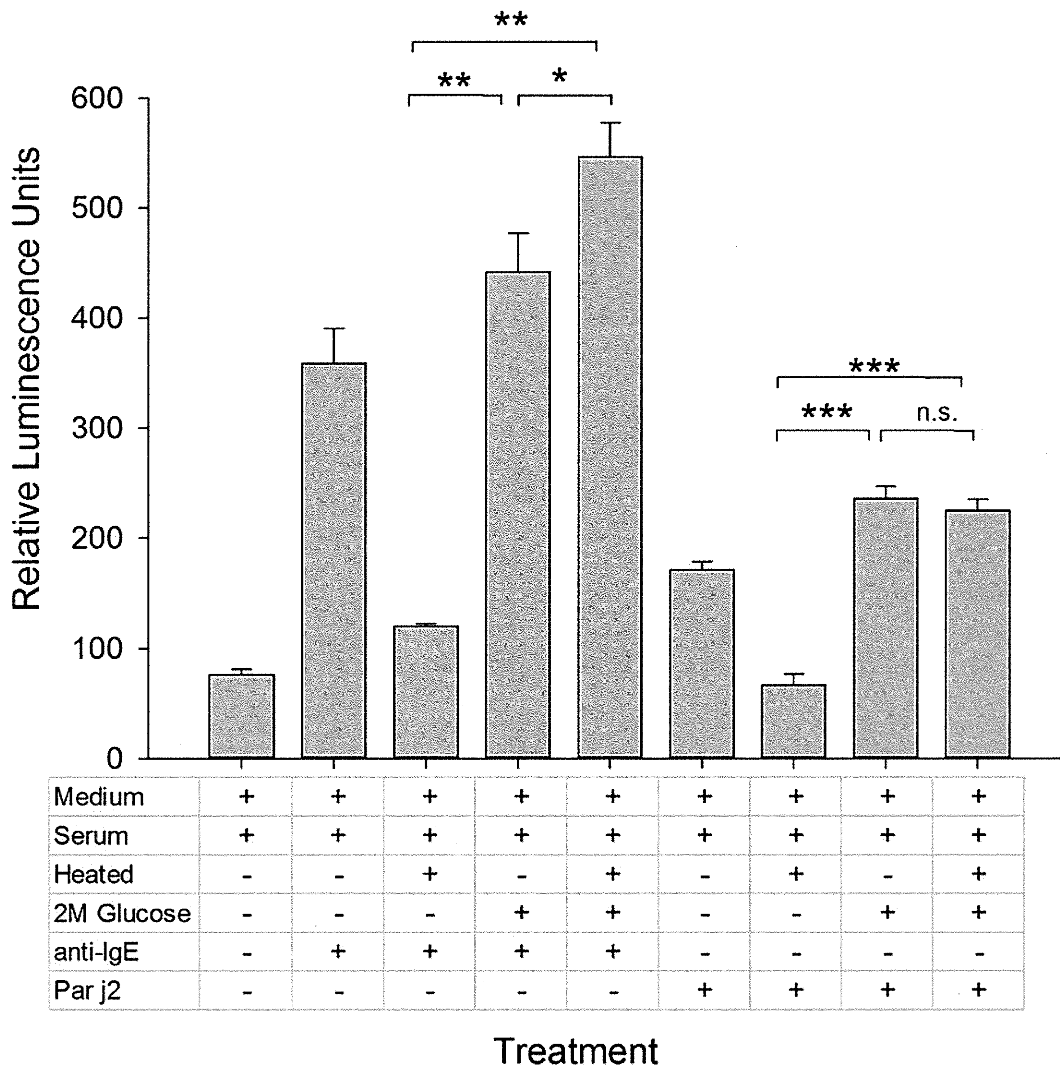
in contrast to stimulation with SmTAL-2. Interestingly, antigen expressed in wheat germ lysate resulted in stronger activation of the reporter system compared with the *E. coli* expressed equivalent, despite using optimal concentration from full dilution curves (Supplementary data Figure S4).

As many secretory proteins will rely on correct folding and formation of intra- or intermolecular disulphide bridges, which may affect their recognition by specific IgE, it is important to assess the ability of the wheat germ expression system to produce correctly folded parasitic antigens.

Assessing the ability of this particular protein to activate RS-ATL8 in this manner would provide further validation of the luciferase system to report IgE dependent activation events, while at the same time informing of the ability of the wheat germ lysate to produce disulphide-bridged homodimers. We therefore assessed the basophil activating properties of IPSE/alpha-1, an IgE-binding factor from *S. mansoni* which is known to rely on dimeric structure for its biological activity [33]. Figure 7 shows a comparison of the ability of wheat germ expressed IPSE/alpha-1 with the same protein expressed and refolded in *E. coli* to induce RS-ATL8 activation.

Both forms of IPSE/alpha-1 were able to dose-dependently induce reporter gene expression. However the effect was much more prominent with the bacterially-expressed refolded recombinant protein, which induced luciferase to an extent similar to the positive controls IgE/anti-IgE and Par j 2-specific serum/Par j 2. The luciferase induction by wheat germ-derived IPSE/alpha-1 was modest, suggesting that it is mainly present in its monomeric form, while the *E. coli* recombinant protein occurs as a mixture of monomers and dimers after refolding [33], and we have previously shown that basophil activation requires IPSE/alpha-1 in its dimeric structure [34].

As specific IgE titres will vary between individuals, we assessed the ability of 11 individual sera from infected individuals (ranging from 3.8 to 15.27 ng/mL SmTAL-1 specific IgE, as determined by ELISA) to sensitise the RS ATL8 cell line. The sera ranged from RAST class 2 (moderate IgE, 07.70–3.49 IU/mL or 1.68–8.39 ng/mL) to RAST class 3 (high IgE, 3.50–17.49 IU/mL or




| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Medium | + | + | + | + | + | + | + | + | + |
| Serum | + | + | + | + | + | + | + | + | + |
| Heated | - | - | + | - | + | - | + | - | + |
| 2M Glucose | - | - | - | + | + | - | - | + | + |
| anti-IgE | - | + | + | + | + | - | - | - | - |
| Par j2 | - | - | - | - | - | + | + | + | + |

**Figure 5. Protective effect of 2M Glucose on the ability of IgE to bind to the high affinity receptor after heating at 56°C for 30 min.** RS ATL8 cells seeded at a density of 50.000 cells per well were sensitised with a 50-fold dilution of Par j 2 serum pool, which had been left unheated or heated either in the presence or in the absence of 2M Glucose. After overnight incubation with the sera, cells were stimulated with optimal concentrations of anti-IgE (1 µg/mL) or Par j 2 recombinant allergen (100 pg/mL) and luciferase production measured after 4 hours. ; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; n.s.: not significant (Student t-test).
doi:10.1371/journal.pntd.0003124.g005

8.40–41.97 ng/mL). As can be seen from Figure 8A, all sera except serum 372-01 gave a positive response upon stimulation with an optimal concentration of SmTAL-1, showing that the RS ATL8 assessment works not only with sera with high levels of allergen-specific IgE, but also with moderate specific IgE levels. All sera gave vigorous responses upon polyclonal stimulation with 1 µg/mL anti-IgE (Fig. 8 B). Non-parametric analysis (Spearman rank correlation test) indicated a statistically significant positive correlation (Fig. 9) between levels of SmTAL-1-specific IgE and the amount of luciferase produced 4 hours after stimulation.

## Discussion

Our aim was to establish a workflow which is suitable for medium- to high-throughput screening of potential allergenicity of *S. mansoni* antigens and includes a set of three steps for stringent quality controls. The first two were implemented at the DNA level (correct size of the PCR amplicon and correct sequence after cloning into expression vector), while the third (successful translation and appropriate protein size by incorporation of fluorescently-tagged lysine) was at the protein level. We chose to incorporate fluorescently labelled Lys in a separate aliquot as this fluorophore may potentially affect the antigenicity or allergenicity of the *in vitro*-translated product.

Wheat germ lysate is a complex biological mixture and as such it has the potential to interfere with the cellular readout. In particular, the lectin wheat germ agglutinin (WGA) may play a critical role in the context of IgE receptor cross-linking. WGA is a 18 kDa lectin contained in wheat germ [38] which naturally occurs as a 36 kDa homodimer in which the two chains are linked with 16 disulfide bonds [39]. WGA is specific for *N*-acetyl-D-glucosamine and the chitin oligomers chitobiose and chitotriose [39]. WGA has been shown to inhibit RBL cell activation by engaging the high affinity IgE receptor FcεRI independently of its

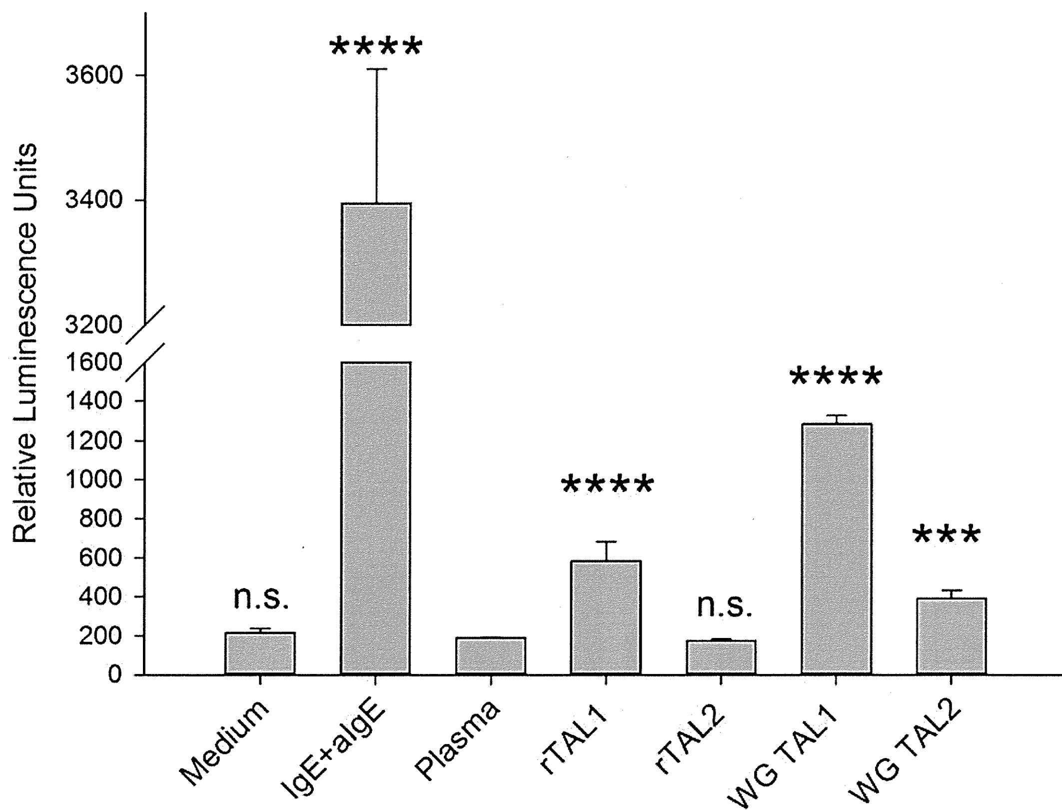



**Figure 6. Luminescence data of RS-ATL8 assay demonstrating the allergenicity of SmTAL-1.** Recombinant SmTAL1 and SmTAL2 were expressed in *E. coli* (rTAL1, rTAL2; both 10 ng/ml) and in WGL (WG TAL1, WG TAL2; diluted 1:10$^{4}$). Cells were sensitized for 16 hours with pooled, virally inactivated plasma from individuals living in a *S. mansoni* endemic area in Uganda (diluted 1:100) and stimulated with the recombinant antigens. The experiment included the negative controls: unsensitized and unstimulated cells (medium), cells incubated only with treated plasma diluted 1:100 v/v (serum) and the positive control (IgE+aIgE, both 1 μg/mL). Data are mean +SD of the readings of three separate wells. Multiplicity adjusted p-values were obtained by ANOVA followed by Dunnett's test for each condition compared with plasma only control. ****: $p<0.0001$; ***: $p<0.001$; n.s.: not significant.

doi:10.1371/journal.pntd.0003124.g006

occupancy by IgE, leading to its down regulation and inhibition of allergen-induced signal transduction [40]. In contrast, in human peripheral blood basophils, WGA can lead to basophil activation and cytokine induction (IL-4 and IL-13) by cross-linking FcεRI-bound IgE via its carbohydrate side chains [41]. In line with this finding, the addition of undiluted wheat germ lysate used in this study resulted in non-specific, high luciferase signals. The WGA can be removed from the lysate by incubation with chitin beads either prior to addition of plasmid, or by the purification of the *in vitro* translated antigen via its incorporated His-Tag and immobilized metal-ion affinity chromatography. However, additional steps are to be avoided in a high-throughput procedure as they increase the overall cost, duration and introduce additional sources of errors. Due to the well-known bell-shaped curve of basophil activation and the high yield of the chosen *in vitro* translation system, the wheat germ lysate containing the translated parasitic protein has to be diluted 1000-fold to reach the concentration range around 100 pg/mL in which stimulation is optimal (as demonstrated by the use of Par j 2 allergen and matching sera in Figure 3). With this dilution, there is no inhibition of IgE receptor crosslinking or basophil activation by WGA. This also means that only small volumes of wheat germ lysate are needed for repeated testing, reducing the overall cost of a high throughput operation.

Thus while the potential disadvantages of choosing wheat germ lysate for translation (such as the lack of glycosylation) are not relevant for this assay when assessing anti-peptide IgE, there are multiple advantages. This system is amenable to high-throughput protein synthesis because it can produce sufficiently large amounts of properly folded proteins, and it bypasses many time-consuming steps of conventional expression systems. It also allows expression of proteins that are toxic to their host organism chosen for expression. Wheat germ lysates, in contrast to e.g. *E. coli* lysates, have very low endogenous mRNA levels, and most of the newly translated protein is thus of parasite (or other target) rather than plant origin. This allows for very efficient incorporation of non-radioactive labels for detection or other purposes. A limitation of the wheat germ system is the inability to provide a non-reducing environment together with all necessary compartmentalised components for disulphide bridge formation. This is demonstrated by our results obtained for IPSE/alpha-1. IPSE/alpha-1 is a secretory protein produced exclusively by *S. mansoni* egg stage [42], which naturally occurs as a homodimer with three intramolecular disulphide bridges and one intermolecular bridge formed by the most C-terminal cysteine in position C132 [33]. We have previously shown that this homodimeric molecule is able to activate human basophils by binding IgE molecules [43], and that IgE-dependent human basophil activation is dependent on its

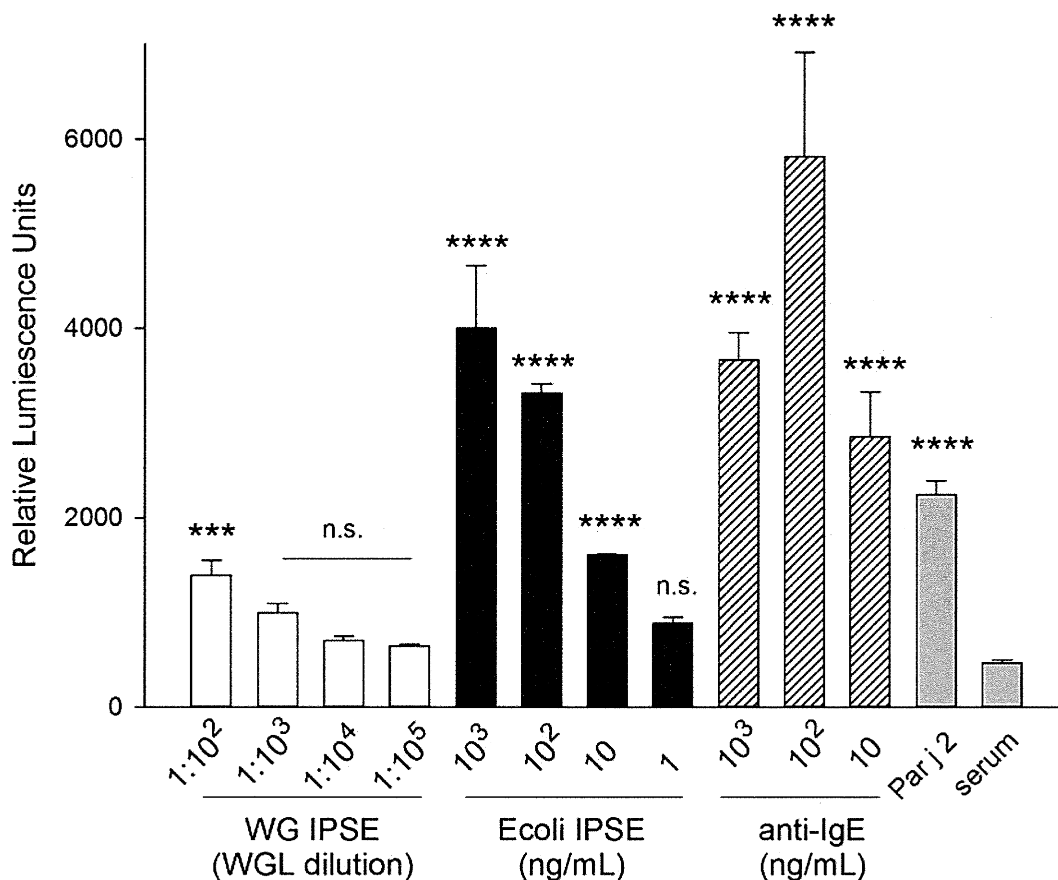



**Figure 7. Activation of RS-ATL8 reporter system by IgE binding factor IPSE/alpha-1 expressed in wheat germ (white bars) and *E. coli* (black bars).** All cells (except IgE only) were sensitized for 16 hours with pooled serum from *P. judaica* allergic individuals diluted 1:50 (v/v). The experiment included positive controls: sensitized with 1 μg/mL IgE and stimulated with the indicated amounts of polyclonal anti-human IgE (hatched bars); stimulated with Par j 2 (100 pg/ml; grey bar), as well as the negative controls (grey bas): serum sensitized/unstimulated cells (serum only). Data are mean ±SD of the readings of three separate wells. Multiplicity adjusted p-values were obtained by ANOVA followed by Dunnett's test for each condition compared with serum only control ****: $p<0.0001$; ***: $p<0.001$; n.s.: not significant.
doi:10.1371/journal.pntd.0003124.g007

dimerization status [34]. However this limitation does not appear to affect this molecule's antigenicity (Fig. 2).

Using a humanised rat basophil cell line also has several advantages [22,23]. It is easier to obtain than e.g. human peripheral blood basophils, which have been notoriously difficult to purify until recently [44], and are still difficult to obtain in sufficient amounts despite these advances due to their rarity. The cells are sensitized only with the desired sera and do not require difficult IgE stripping protocols [45]. Furthermore, known potential issues such as non-responder status [46] due to down regulation of key signalling molecules such as spleen tyrosine kinase (Syk) caused by chronic exposure to low level of allergens [47], which therefore might also be occurring in helminth infection, are avoided. Non-responder status is an issue we came across in a subset of individuals when assessing peripheral blood basophil sensitization status in a cohort experimentally infected with a single dose of ten *Necator americanus* infective stage larvae [21], and could lead to false negative results. Also as human IgG is not thought to bind to rat immunoglobulin receptors, and the rat high affinity IgE receptor does not bind human IgE [48,49], there is no potential for confounding factors which could mask the potential allergenicity of the studied antigens, such as competing IgG4 [50,51], inhibitory co-crosslinking of FcεRIα and FcγRIIB [52], or activation due to IgG-IgE immune complexes [53], as these factors are removed by washing the reporter cell line prior to allergen stimulation. Finally, using the NFAT luciferase reporter for detection of activation, rather than the traditionally used β-hexosaminidase biochemical assay, results in considerably increased sensitivity. The ability of the RS-ATL8 to detect sensitisation with less than 100 pg IgE (Supplementary data S2) compares favourably with previously reported limit of 10 ng/ml upon polyclonal stimulation with an anti-IgE antibody using β-hexosaminidase activity for detection [54]. We have previously assessed alternative methods of measuring activation induced by IgE crosslinking in RBL cells (traditional beta-hexosaminidase assays, Annexin V measurements, CD63 and CD107a levels by FACS, Calcium Influx using Oregon Green or Alexa488 BAPTA-1, as well Lucifer yellow uptake, but none of these methods worked or offered any advantage over luciferase measurements.

Serum samples which had undergone anti-viral treatment with a mixture of detergents had to be diluted at least 100-fold, as higher concentrations of detergents led to complete destruction of cells as assessed by microscopy and the complete lack of luciferase induction. Tween-80 is the major constituent in the viral inactivation detergent used here and has a critical micellar concentration (CMC) of 0.012 mM [55] which lies precisely in

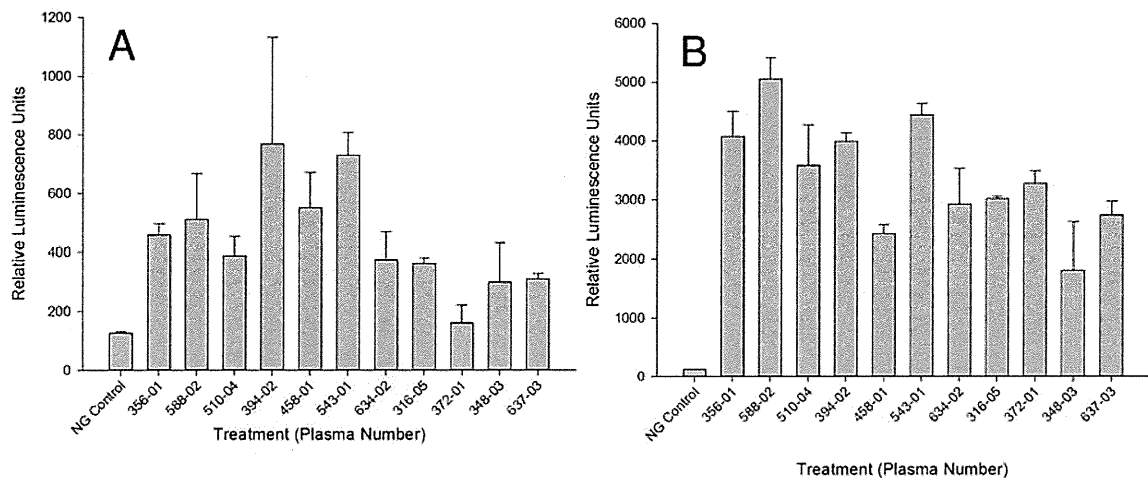



**Figure 8. Screening of SmTAL-1 allergenicity with individual infected individuals' plasma.** A and B) RS-ATL8 cells were sensitised for 16 hours with different unpooled plasma from *S. mansoni* infected individuals with known moderate to high SmTAL1-specific IgE titres determined by ELISA. After a wash, cells were challenged with 1/1000 diluted SmTAL-1 wheat germ lysate translation mixture in (A) or 1 µg/ml anti-IgE (B). Luminescence was measured 4 hours after stimulation. Shown is one experiment as mean of triplicate +/− SD.
doi:10.1371/journal.pntd.0003124.g008

between the concentration of Tween-80 in the 50-fold and 100-fold dilutions of treated sera. Thus a 100-fold dilution of sera will work to reduce serum cytotoxicity for both untreated and virally inactivated sera. As previously shown, serum cytotoxicity in this system is probably in part due to complement activation, as heating the serum at 56°C for 30 min reduced its cytotoxicity [35]. This treatment however irreversibly denatures IgE, abrogating its binding to the high affinity IgE receptor [56]. Our results demonstrate that 2M glucose treatment might represent a suitable way of inactivating complement in sera without leading to loss of

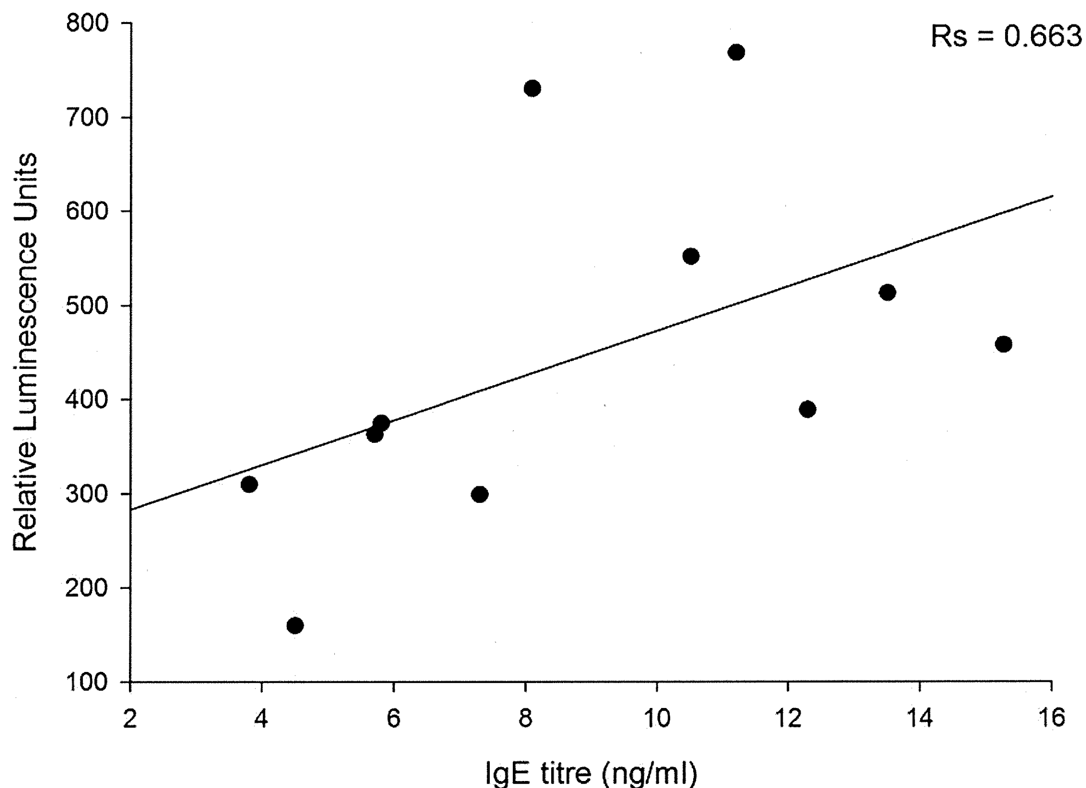



**Figure 9. Correlation between the IgE titre measured in each plasma sample and the intensity of luciferase induction after stimulation with SmTAL-1.** The Spearman rank correlation coefficient was $R_s = 0.663$ ($p = 0.035$, two-sided).
doi:10.1371/journal.pntd.0003124.g009

IgE functionality. However, subsequent experiments with glucose-treated sera clearly showed that these also had to be diluted 20–50-fold to avoid deleterious effects of the high glucose concentration in the cellular assay, or required dialysis-based methods. As this would complicate the workflow unnecessarily, we did not pursue these attempts, and used serum dilutions of 1:50 with virally non-inactivated sera or 1:100 to 1:200 with virally inactivated, detergent-treated sera. The work described in Figures 3–5 and S2, was performed using a well-characterised (ISAC UniCAP, clinical history) serum pool from *P. judaica* allergic individuals, rather than sera from parasite-infected individuals and helminthic allergens. This was mainly due to the unavailability of large amounts of infection serum required for such studies, but also as it allowed us to validate the technology against the current gold standard for specific IgE determination. The results are equally relevant for IgE/allergen combinations studied in a tropical parasite infection context, as the underlying mechanisms of sensitisation, cellular activation and allergenicity are fundamentally the same [57].

The SmTAL proteins are a family of 13 closely related allergen-like molecules. Of these SmTAL1 and 2 have the greatest similarity in amino acid sequence (48% identity). However, in populations from endemic areas, SmTAL1 is reported to be the dominant IgE-inducing antigen whilst an IgE response to SmTAL2 is rare [26,31]. It has been proposed that this is because the response to egg antigen – SmTAL2 – is desensitized by continuous exposure (as eggs die in the host tissues every day); whilst internal adult worm antigen SmTAL1 is only exposed infrequently when adult parasites die [26]. An interesting observation was made when comparing the allergenicity of SmTAL-1 and SmTAL-2 between the bacterial recombinant forms and their wheat germ expressed counterparts. We carried out full titration curves with all four but found that in both cases the WG-expressed form induced significantly higher reporter cell activation than the *E. coli* expressed SmTALs (Supplementary data S4). The reasons for this difference are not clear, but would appear to rule out that LPS contamination of bacterially expressed recombinant allergens could be a source of basophil activation in the used assay.

Taken together, this method offers a robust way for assessing potential allergenicity of *S. mansoni* (or any other parasite) in a format suitable for high-throughput analysis. The novelty of the method presented here lies in the combination of a fast cell-free expression system and an equally fast reporter system which allows expression of candidate allergens in a few hours and detection of activation within three hours, all up-scalable to high-throughput format. This method can be used as an additional safety test when assessing potential vaccine candidates. Perhaps more importantly, when used at the whole genome level, it could be used to unravel the entire allergome of *S. mansoni* and other medically important parasites. Ultimately this could lead to a better understanding of the basis of allergenicity, and in combination with additional cellular studies, to a better understanding of the relationship between parasite-specific IgE and host protection mechanisms at the molecular level [57].

## Supporting Information

**Figure S1** In gel detection of ten different *S. mansoni* antigens (DA18 to DA30, from right to left) expressed *in vitro* using wheat germ lysate. Success of translation was monitored by incorporation of BODIPY-labelled fluorescent Lysine during translation in separate aliquots. Samples were run on 4–20% SDS-PAGE gradient gels under reducing conditions and imaged in a Fujifilm LAS-4000. The left lane (ctrl) includes the wheat germ lysate control without template DNA, indicating fluorescent components produced during *in vitro* translation from endogenous mRNA. The expected molecular weights were: DA30 (14.3.3; Smp_009760): 29.3 kDa; DA29 (IPSE alpha-1; Smp_112110): 13.3 kDa; DA27: (Major Egg antigen; Smp_049300.3): 40.3 kDa; DA26 (Haemoglobinase; Smp_075800): 47.1 kDa; DA25 (Troponin T, Smp_179810): 37.4 kDa; DA23 (triosephosphate isomerase, Smp_003990): 29.0 kDa; DA22 (Sm14 fatty acid-binding protein isoform T20, Smp_095360.3): 11.9 kDa; DA20 (ornithine aminotransferase, Smp_000660): 48.5 kDa; DA-19 (ngng-dimethylarginine dimethylamino-hydrolase, Smp_052560 17.0 kDa); DA-18 (GST class mu; SM26/2 antigen, Smp_102070): 23.5 kDa. Apparent MW may vary from the values described in the literature due to lack of glycosylation, the presence of a $(His)_6$Val tag added at the C-term of each protein, or the removal of an N-terminal signal peptide (see Table S3 in Supplementary data for additional information).
(TIF)

**Figure S2** IgE detection limit of RS-ATL8 reporter cell line. 100,000 RS-ATL8 cells were sensitized overnight with different concentrations of monoclonal IgE (ranging from 5000 to 0.1 ng/mL) in a 100 µl volume. Cells were then stimulated with 1 µg/mL polyclonal anti-IgE. Results are mean +/− s.d. of an experiment performed in quadruplicate determination. T-test results indicate $p<0.001$ significance for all conditions compared with cell sensitized with 1 µg/mL IgE, but not stimulated (IgE only).
(TIF)

**Figure S3** Optimal concentration and minimal concentration of detectable allergen was determined by sensitising RS-ATL-8 cells with the pooled serum (1:50 dilution) from individuals with a monospecific IgE response to the Par j 2 allergen and challenged with recombinant Par j 2, serially diluted from 10 µg/mL to 10 pg/mL in 1:10 dilution increments. Data are mean ±SD of the readings of three separate wells.
(TIF)

**Figure S4** Activation of RS ATL-8 reporter cell line by serial dilutions of SmTAL1 and SmTAL2 expressed in *E. coli* (rTAL1, rTAL2) or using WGL (WG TAL-1, WG-TAL2) as well as negative (medium only, serum only) and positive polyclonal activation (IgE+anti-IgE) control.
(TIF)

**Table S1** Details of S. mansoni infected individual plasma samples used in this study (MUG6 *S.mansoni* infected human sera). 10×30 µl post-treatment with praziquantel (PZQ) plasma samples, which have been virally inactivated as described by Poulsen and Sørensen [32] were pooled in equal amounts. Samples were classified as high vs medium titre groups according to RAST class equivalence. Epg = eggs per gram faeces.
(DOCX)

**Table S2** Characterisation of Par j 2 –specific serum pool by ISAC 103.
(XLS)

**Table S3** Details of genes used in this work. Most proteins have a His-Tag and a terminal Valine added at the C-term. Proteins with a predicted or known classical signal peptide (SS for signal sequence) had the signal removed during cloning.
(XLS)

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## Author Contributions

Conceived and designed the experiments: DW FL DGWA OS EAA NAB XW AM CMF DWD RN MJCA FHF. Performed the experiments: DW FL DGWA OS EAA NAB XW AM CMF FHF. Analyzed the data: DW DGWA OS EAA NAB RN MJCA FHF. Contributed reagents/materials/analysis tools: AM CMF DWD MJD RN GCO. Contributed to the writing of the manuscript: DW FL DGWA MJD AM CMF DWD RN GCO MJCA FHF.

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# Chapter 13

# Use of Humanized Rat Basophil Leukemia (RBL) Reporter Systems for Detection of Allergen-Specific IgE Sensitization in Human Serum

**Daniel Wan, Xiaowei Wang, Ryosuke Nakamura, Marcos J.C. Alcocer, and Franco H. Falcone**

## Abstract

Determination of allergen-specific IgE levels in human blood samples is an important diagnostic technology for assessment of allergic sensitization. The presence of specific IgE in human serum samples can be measured by sensitizing humanized rat basophil leukemia (RBL) cell lines with diluted serum and measuring cellular activation after challenge with the suspected allergens. This has been traditionally performed by measuring the levels of β-hexosaminidase released upon RBL degranulation. Here, we describe the use of two recently developed humanized RBL reporter cell lines which offer higher sensitivity and are amenable to high-throughput-scale experiments.

**Key words** Reporter system, Luciferase, DsRed, RBL, IgE, Allergy, RS-ATL8, NFAT-DsRed

## 1 Introduction

Immunoglobulin E (IgE) is the antibody isotype which is at the center of the pathology of allergic disease. Many of its effects are mediated via the tetrameric high-affinity IgE receptor FcεRI. Because of the key role of IgE, diagnosis of type I allergy has been traditionally focused on measuring the levels of allergen-specific and total IgE in serum or plasma of allergic individuals. This is routinely performed in vitro by measuring IgE binding to solid-phase bound allergens and detection via enzymatically labelled secondary antibodies and fluorescent substrates, using devices available in automated or semiautomated formats from various companies [1]. More recently, new methods have taken advantage of protein array technology (ISAC: ImmunoSolid phase Allergen Chip) [2], allowing simultaneous testing of more than 100 allergens using a small blood sample. Methods based on allergen-specific IgE measurements have high sensitivity, but their clinical relevance is not always

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clear, as they report the ability of IgE to bind allergen but not to induce an allergic reaction. As a result, IgE tests are frequently accompanied by clinically more relevant skin prick tests (SPTs), and a detailed medical history. However SPTs also have a range of disadvantages since the use of certain medications can interfere with the test results resulting in some patients being excluded from this type of test. The residual hazard also requires these tests to be carried out in specialized units. Another more recent type of allergy test is based on the assessment of basophil activation in whole blood (basophil activation tests, BAT) using flow cytometry. These tests work by monitoring the upregulation of CD63 and/or CD203c on the surface of basophils after incubation of heparinized whole blood with the suspected allergen (*see* Chapter 11). There is currently no widely accepted standard protocol for BATs, and these tests need to be performed within a day after blood sampling (whereas serum samples can be stored). As a result, this test is still not widely used clinically, but only performed in specialized, research-active laboratories.

Another technique has been available for about two decades but, due to its perceived limitations, has not found a widespread use. This is the use of humanized rat basophilic leukemia (RBL) cells for monitoring of specific allergen sensitization in patient serum. The RBL cell lines need to be humanized with at least the human α chain of FcεRI [3], as human IgE does not bind to the rodent IgE receptor [4]. A critical issue with this cell line has been the cytotoxicity of certain human sera for the rat cells, requiring high dilutions and resulting in insufficient sensitization and subsequently negative tests. However, a new generation of reporter cell lines is now replacing the previous humanized RBL cell lines taking advantage of sensitive reporter genes such as luciferase or fluorescent proteins. This enables detection of allergen-specific IgE even when using serum dilutions as high as 1:100 [5, 6]. In this chapter, we describe the use of two such humanized RBL reporter systems, RS-ATL8 and NFAT-DsRed, for detection of allergen-specific IgE in human serum samples. Both methods pave the way for high-throughput measurements, and the latter can also be used in conjunction with allergen arrays [7].

## 2 Materials

All reagents and supplies should be sterile. Follow local waste disposal regulations.

### 2.1 Reagents

1. RBL medium: Minimum essential medium (MEM), 10 % v/v heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (if not already present in MEM). Store at 4 °C.

2. RS-ATL8 and NFAT-DsRed reporter cell lines, available from the authors through a Material Transfer Agreement (MTA).
3. G418 sulfate (Geneticin).
4. Blasticidin S (for NFAT-DsRed cells).
5. Hygromycin B (for RS-ATL8 cells).
6. DPBS without $Ca^{2+}$ or $Mg^{2+}$.
7. Trypsin–EDTA or cell scrapers.
8. Patient sera.
9. Allergens.
10. Anti-human IgE.
11. Lysis buffer: 1 % v/v Triton X-100 in DPBS (for fluorescence assay). Store at 4 °C.
12. ONE-Glo Luciferase Assay System (Promega), for luminescence assay. Follow local regulations regarding the handling of suspected carcinogens. Prepare and store as described by the manufacturer. Warm to room temperature before use.

### 2.2 Supplies

1. Graduated pipettes and pipette tips.
2. 25 $cm^2$/75 $cm^2$ cell culture flasks.
3. 50 mL centrifuge tubes.
4. Clear 96-well plates.
5. Black 96-well plates (for fluorescence assay).
6. White 96-well plates (for luminescence assay).

### 2.3 Equipment

1. Pipettors for graduated pipettes/tips.
2. Water bath.
3. Class II microbiological safety cabinet.
4. Cell culture incubator: Humidified incubator set to 37 °C and 5 % $CO_2$.
5. Heat block.
6. Hemocytometer.
7. Centrifuge capable of holding 50 mL tubes.
8. Plate reader for fluorescence or luminescence.

## 3 Methods

### 3.1 General Cell Culture

Work in a class II microbiological safety cabinet using sterile techniques.

1. If starting from frozen stocks, partially defrost a vial of cells by warming in hand, and then add 1 mL pre-warmed RBL medium to fully defrost.

2. Transfer cells to a 25 $cm^2$ flask containing 8 mL RBL medium, and place in a cell culture incubator overnight.
3. When the cells are confluent in the 25 $cm^2$ flask (this may take several days, depending on the viability of frozen cells), transfer cells to a 75 $cm^2$ flask by detaching adherent cells using trypsin–EDTA (*see* **steps 1–9** of Subheading 3.3 below).
4. Maintain cells in 10 mL RBL medium supplemented with 1 mg/mL G418 sulfate and 20 μg/mL blasticidin S (NFAT-DsRed) or 500 μg/mL G418 sulfate and 200 μg/mL hygromycin B (RS-ATL8), in a 75 $cm^2$ flask, in the cell culture incubator.
5. Passage cells when confluent using trypsin–EDTA (*see* **steps 1–10** below). Replacing the flask with 20 % cells will result in confluence after 3 days. Replace with 10 % of cells for confluence (*see* **Note 1**) after 4 days (refresh the medium after 2 or 3 days). Top up the level of liquid in the flask to 10 mL.
6. Freeze stocks of cells in larger batches, i.e., from several flasks. Centrifuge cells at $300 \times g$ for 5 min, and resuspend in RBL medium with 10 % v/v DMSO to obtain a cell concentration of $5 \times 10^6$ cells/mL. Work quickly since DMSO is toxic to cells at room temperature. Aliquot 1 mL cells/cryovial, transferring cryovials to a Mr. Frosty™ (Nalgene) container filled with isopropanol, and place in a −80 °C for several hours or overnight. After freezing, transfer the cryovials to a liquid nitrogen Dewar flask for long-term storage. Defrost a vial to check the viability of the batch.

### 3.2 Treatment of Serum Before Sensitization (Optional)

1. If necessary, heat human serum in an Eppendorf tube at 56 °C for 5 min (*see* **Note 2**) on a heat block and then place it on ice.

### 3.3 Sensitization

Cells are sensitized for 16–20 h by incubating them in RBL medium with the desired dilution of previously heat-inactivated serum (*see* **Note 3**).

1. Pre-warm RBL medium to 37 °C in a water bath.
2. Remove medium from 75 $cm^2$ flask of confluent cells (*see* **Note 4**).
3. To the flask, add 10 mL DPBS without $Ca^{2+}$ or $Mg^{2+}$ (*see* **Note 5**).
4. Tilt the flask to wash the cells with DPBS.
5. Discard the DPBS in the flask.
6. Add 2 mL trypsin–EDTA to the flask.
7. Tilt the flask to ensure that the cells are exposed to the trypsin–EDTA solution.
8. Incubate the flask for 5–15 min at 37 °C in a cell culture incubator, until clumps of cells are visibly detached (*see* **Note 6**).

9. Add 8 mL pre-warmed RBL medium to the flask and resuspend the cell suspension (*see* **Note 7**).
10. Transfer the cells to a 50 mL tube. Some cells can be left behind in the flask for passaging (*see* Subheading 3.1).
11. Count a sample of cells in a hemocytometer and calculate the total cell number.
12. Pellet the cells by centrifuging at $300 \times g$ for 5 min.
13. Resuspend the cells to a concentration of $1 \times 10^6$ cells/mL using RBL medium.
14. Sensitize cells using appropriate concentration of serum (*see* **Note 2**).
15. Pipette 50 μL cells/well of a 96-well plate.
16. Incubate the plate overnight in the cell culture incubator.

### 3.4 Stimulation

1. Check the plate under a light microscope to see if the cells are in healthy state.
2. Prepare appropriate dilutions of allergens or other stimuli in RBL medium. Optimal concentrations may vary for each allergen and will need to be determined experimentally. Use an appropriate amount of anti-human IgE as a positive control (*see* **Note 8**).
3. Remove the medium from the cells in the 96-well plate.
4. Add 50 μL stimuli to appropriate wells of the plate. Do this quickly so that the cells do not dry out when the medium is removed. Smaller steps, e.g., 3–6 wells at a time, can be done instead.
5. Incubate the plate in the cell culture incubator for 24 h (fluorescence assay with NFAT-DsRed) or 3 h (luciferase assay with RS-ATL8).

### 3.5 Measurement of Activation

#### 3.5.1 Fluorescence Assay with NFAT-DsRed

1. Wash the cells once with 50 μL DPBS, remove DPBS, and add 100 μL 1 % Triton X-100 in DPBS to lyse the cells.
2. Tap the plate gently to mix, then transfer the lysate in each well to a black 96-well plate, and measure fluorescence in a plate reader (*see* Fig. 1).

#### 3.5.2 Luciferase Assay (EXiLE Test, See Note 9)

1. Allow the luciferase assay reagent and cells to come to room temperature before mixing. The assay is temperature sensitive and this method allows more reproducible results.
2. Add 50 μL ONE-Glo Luciferase Assay Substrate (Promega) directly to stimulated cells (no need to remove medium containing stimulus).
3. Transfer the 100 μL mixture in each well to a white 96-well plate, and measure luminescence in a plate reader (*see* **Notes 10–12**).

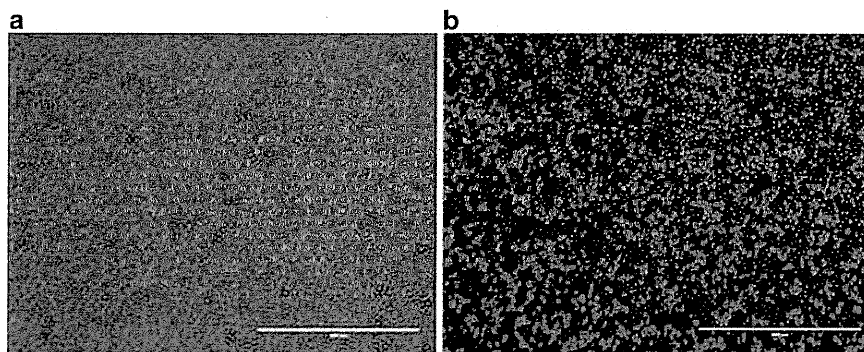


**Fig. 1** Fluorescence microscopy image of NFAT-DsRed reporter cell line sensitized with monoclonal IgE without stimulation (**a**) and 24 h after stimulation with anti-IgE antibody (**b**). Note that not all cells appear to have been activated by this treatment and that the intensity of fluorescence varies between individual cells

## 4 Notes

1. A 1/20 split (5 % cells) is not recommended for routine passaging as this can cause "colonies" to form and unhealthy cells in the middle of the cell clusters.
2. Human sera are frequently cytotoxic for RBL cells (*see* Fig. 2). This is in part due to the presence of activated complement, which is traditionally inactivated by heating at 56 °C for 30 min. This prolonged treatment however also induces irreversible conformational changes in IgE molecules, ablating its ability to bind to FcεRI. A shorter heat inactivation will be sufficient to inactivate complement while preserving the integrity of IgE. We have had good results with this protocol, even with 20 % serum concentration (1:5). Not all sera, however, require heat inactivation. In most cases, a 1:50 or a 1:100 dilution will be sufficient to remove cytotoxicity without any heat treatment. Thus the heat treatment is described as optional.
3. We routinely start the sensitization protocol at 3 pm, and the stimulation at 9 am the following day.
4. Once fully confluent, a 75 $cm^2$ flask contains enough cells to fill two 96-well plates.
5. Cell scrapers can be used to detach the cells instead of trypsin–EDTA. Simply refresh RBL medium and scrape the cells. There is no need for a DPBS wash.
6. You can speed up the detachment process by repeatedly knocking the bottom of the flask with your knuckles, holding it tight with the other hand. Be careful, however, as the use of too much force can split the flask.
7. Make sure that any visible clumps are broken up, by pipetting up and down. We routinely use a 5 mL serological pipette for

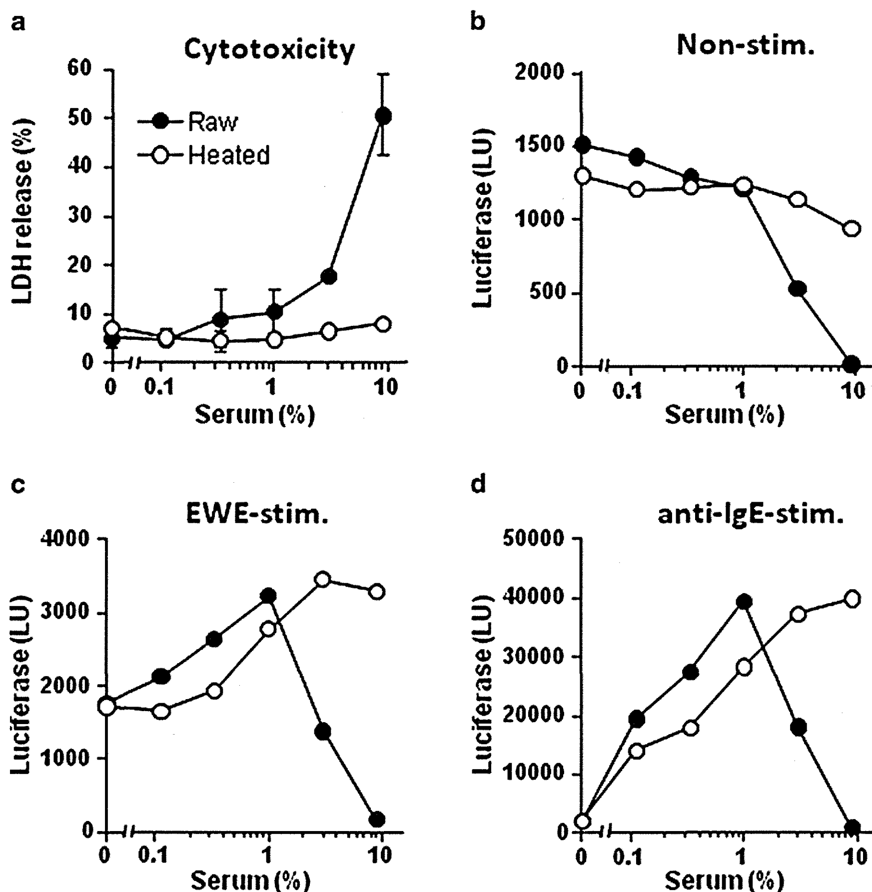


**Fig. 2** Cytotoxicity of human serum on the RS-ATL8 cells. A serum sample of an egg white allergic patient (7 years, male, total IgE 1388 IU/mL egg white sIgE 12.8 $U_A$/mL) was heated at 56 °C for 30 min. The RS-ATL8 cells were sensitized overnight with the serum at various concentrations. (**a**) Cytotoxicity of the sera on the cells as revealed by LDH release assay. EXiLE response after 3-h treatment of (**b**) control medium, (**c**) egg white extract (EWE), and (**d**) anti-human IgE

this purpose, as these have a narrower tapered end. Make sure that you do not create bubbles when pipetting up and down by always leaving some medium in the pipette when expelling the liquid and not taking up any air when aspiring it.

8. The optimal amount of anti-IgE will depend on the type (species, clonality, affinity, etc.) used and needs to be determined empirically. Optimum concentrations can range between 100 ng/mL and 2 μg/mL which therefore need to be determined by testing the anti-IgE antibody over a wide range of concentrations (e.g., at least five orders or magnitude). This should result in a bell-shaped stimulation curve, with a clear suboptimal and supraoptimal stimulation range.
9. This test is now known as EXiLE test, for Ig*E* cross-linking (=*X*)-*i*nduced *L*uciferase *E*xpression [5].

10. In our lab, we have tested different types of multiwell plates. Clear plates are not recommended for final scans due to lower sensitivity than black or white plates. However, if there is no option but to use clear plates, light detection from below (bottom scans) will give higher sensitivity than top scans. Black plates give greatest sensitivity for fluorescence assays, and white plates give greatest sensitivity for luminescence assays. For opaque plates, for obvious reasons, light detection (and fluorescence excitation) must be from above. We found that opaque-walled, clear-bottomed plates do not greatly increase sensitivity. However, different plate readers may give different results. If you have the resources, it may be worthwhile to repeat these tests for the plate readers in your own lab.
11. If cells are sensitized in black/white plates, then there is no need to transfer cell lysates from clear to opaque plates; the entire experiment can be carried out in a single plate. However, we find it essential to examine the cells after an overnight incubation to check for cell death or contamination. A clear-bottomed plate is required for light microscopes.
12. Bubbles can be created by incorrect pipetting technique or sample evaporation, and may interfere with plate readings. Large bubbles can be removed by stabbing with a dry pipette tip (a wet pipette tip cannot burst bubbles). If there are many small bubbles throughout the plate, most can be removed by briefly centrifuging the plate.

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# Laminin γ-1 and collagen α-1 (VI) chain are galactose-α-1, 3-galactose–bound allergens in beef

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**Keywords**
allergens; carbohydrate; food allergy; mammalian meat; α-Gal.

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## Abstract

**Background:** Sensitization to the carbohydrate galactose-α-1,3-galactose (α-Gal) has been reported in patients with beef allergy. However, the proteins responsible for this allergy have not yet been identified. This study aimed to identify beef proteins that predominantly react with serum IgE in Japanese patients with beef allergy.

**Methods:** Sera were collected from 29 patients with beef allergy who had allergic reaction(s) such as urticaria, abdominal pain, vomiting, and anaphylactic shock after ingestion of beef and pork; the sera tested positive for IgE against beef and pork. IgE-binding proteins were detected by immunoblotting sera from the patients and identified using a combination of two-dimensional gel electrophoresis and peptide mass fingerprinting techniques. The involvement of carbohydrate in the binding of IgE to allergens was examined by periodate treatment and an inhibition assay with cetuximab by immunoblotting. Specific IgE binding to cetuximab was measured using the CAP-fluorescent enzyme immunoassay.

**Results:** Two IgE-binding proteins (240 kDa and 140 kDa) were detected in beef extract and identified as laminin γ-1 and the collagen α-1 (VI) chain from *Bos taurus*, respectively. Periodate treatment or the inhibition assay resulted in the loss of IgE binding to these proteins. Immunoblotting with anti-α-Gal antibody revealed the presence of α-Gal on the 240- and 140-kDa beef proteins. The amount of IgE bound to cetuximab was significantly correlated with that to beef in the patients with beef allergy.

**Conclusion:** The carbohydrate moiety (α-Gal) on laminin γ-1 and collagen α-1 (VI) chain are possibly common IgE-reactive proteins in the Japanese patients with beef allergy.

Meat accounts for 3% of the causative foods of immediate-type food allergies in the Japanese population (1). An allergic reaction to mammalian meat, referred to as beef allergy or red meat allergy, develops after the ingestion of mammalian meat such as beef and pork, but not chicken or fish. Investigations of allergens causing a meat allergy have mainly focused on cross-reactive allergens in cow's milk in patients with atopic dermatitis who were sensitized to beef; bovine serum albumin (BSA), bovine immunoglobulin G (IgG), and bovine actin have been identified as the cross-reactive allergens (2–8).

Chung et al. (9) reported that the carbohydrate galactose-α-1,3-galactose (α-Gal), which is present in the heavy chain of cetuximab (a chimeric monoclonal antibody against epidermal growth factor receptor), is a major allergen causing hypersensitivity reaction after the first cetuximab infusion. In addition, they showed that the distribution of IgE antibodies to α-Gal was highly variable depending on the region in the United States (9). Based on this finding, a series of studies have clarified that patients with cetuximab hypersensitivity have a red meat allergy, and α-Gal in red meat is responsible for red meat allergy (10–16). However, red meat proteins bearing α-Gal have not yet been identified.

We screened IgE-binding proteins in red meat (beef and pork) using sera from Japanese patients with beef allergy and identified laminin γ-1 and the collagen α-1 (VI) chain as major allergens possessing an α-Gal moiety.

## Materials and methods

### Patients and sera

Sera were obtained from 29 patients with beef allergy; sera were stored at −20°C until analysis. Beef allergy was diagnosed by the presence of clinical episode(s) of allergic reaction such as urticaria, abdominal pain, vomiting, and anaphylactic shock within 8 h after ingesting mammalian meat, and positive serum-specific IgE tests of beef and pork. Table 1 lists the clinical features of the patients. Serum-specific IgE values were measured by a CAP-fluorescent enzyme immunoassay (CAP-FEIA; ImmunoCAP®; Thermo Fisher Scientific, Uppsala, Sweden). This study was approved by the Ethics Committee of the Shimane University Faculty of Medicine (approval No. 469).

### Preparation of meat proteins

Meat (beef, pork, and chicken) was homogenized in a 10× volume of ice-cold phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The homogenate was centrifuged at 20 000 *g* for 15 min at 4°C, and the supernatant was collected as water-soluble proteins. The pellet was solubilized in 2% sodium dodecyl sulfate (SDS) aqueous solution with constant mixing at 95°C for 5 min and centrifuged at 20 000 *g* for 15 min at 4°C, and the supernatant was collected as water-insoluble proteins. Protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### Immunoblot analysis

For IgE immunoblotting, meat proteins were separated by 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) (25 μg/lane) under reducing conditions and subsequently transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA, USA). The PVDF membrane was incubated with a blocking solution (20 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween-20, and 0.6% polyvinylpyrrolidone) for 1 h and further incubated with patient sera diluted to 10% with blocking solution for 16 h at 28°C. After washing the PVDF membranes with TBST (20 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween-20) three times, bound IgE was visualized on RX-U Fuji medical X-ray film (FUJIFILM Co., Tokyo, Japan) using horseradish peroxidase (HRP)-conjugated goat anti-human IgE antibody (Kirkegaard & Perry Laboratories, Inc., KPL, Gaithersburg, MD, USA) and the ECL prime kit (GE Healthcare UK Ltd, Buckinghamshire, UK). Total protein in the gel was stained with Coomassie Brilliant Blue (CBB).

For α-Gal detection, mouse anti-α-Gal monoclonal antibody (M86 clone; Enzo Life Sciences, Inc., Farmingdale, NY, USA) and HRP-conjugated anti-mouse IgM (μ) antibody (KPL) were used. The chemiluminescent signal was detected as described above.

### Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

To obtain the allergen-rich fraction, water-soluble beef proteins were precipitated with ammonium sulfate at concentrations of 0–20%, 20–40%, 40–60%, and 60–80% at 4°C. Each precipitate was dissolved in PBS, and the suspension was dialyzed twice in PBS at 4°C for 16 h. IgE immunoblot analysis with patient sera revealed that the precipitate obtained with 20–40% ammonium sulfate contained IgE-reactive proteins with relative molecular masses of 240 kDa and 140 kDa.

The allergen-rich fraction was cleaned up with a ReadyPrep 2-D Cleanup kit (Bio-Rad) for isofocusing electrophoresis, and the resultant precipitate was dissolved in a buffer containing 60 mM Tris–HCl (pH 8.8), 6 M urea, 1 M thiourea, 3% CHAPS, and 1% TritonX-100. Residual particles were removed by ultracentrifugation at 200 000 *g* for 20 min at 4°C. Protein concentration was determined using an RC DC protein assay kit (Bio-Rad).

2D-PAGE was performed according to the manufacturer's instructions. First-dimension protein separation (40 μg) was carried out in agarGEL (pH 3–8, ϕ 2.5 × 75 mm; ATTO corp., Tokyo, Japan) at 300 V for 3.5 h. The agarGel was subsequently used for second-dimension SDS-PAGE on a 7.5% acrylamide gel under reducing conditions. For IgE immunoblot analysis, proteins separated by 2D-PAGE were electrophoretically transferred to PVDF membranes and further incubated with 10% patient sera. Bound IgE was detected as described above.

### Identification of beef allergens

Protein spots were manually excised from the CBB-stained gel, subjected to reductive alkylation by iodoacetamide, and digested with trypsin gold (Promega, Madison, WI, USA) using ProteaseMAX™ Surfactant (Promega) following the manufacturer's instructions. Peptides were crystallized with α-cyano-4-hydroxycinnamic acid on a stainless steel plate. The mass spectra of peptides were recorded in the reflectron mode of matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS; AB SCIEX TOF/TOF 5800 system, Framingham, MA, USA). The mass spectrometer was calibrated with angiotensin II, P14R, and adrenocorticotropic hormone fragments 18–39 (Sigma, St. Louis, MO, USA). Data processing was carried out using Data Explorer software ver. 4.10 (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA). For peptide mass fingerprinting (PMF) and the MS/MS ion search, the generated mass lists were searched against the protein data bank of the National Center for Biological Information (NCBI) using the data base search engine MASCOT (Matrix Science, London, UK) after excluding peak lists of known contaminants such as keratin and trypsin. The peptide mass tolerance and fragment mass tolerance were set at ±100 ppm and ±0.3 Da, respectively.

**Table 1** Clinical features of 29 patients with beef allergy

| Case No. | Age (year) | Gender | Symptom(s)* | Causative food(s) | Time to reaction (h)** | CAP-FEIA (kUa/l) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Beef | Pork | Chicken | Cetuximab |
| 1 | 69 | M | U, A | Beef innards and alcohol | 3 | 7.83 | 6.95 | <0.35 | 48.8 |
| 2 | 71 | F | U | Beef meat | 1 | 3.29 | 0.77 | <0.35 | 26.3 |
| 3 | 69 | F | U | Beef meat | 2 | 39.1 | 32.6 | <0.35 | 60.7 |
| | | | U, V, AS | Beef and pork meat | 2 | | | | |
| | | | U, AS | Pork meat | 1.5 | | | | |
| 4 | 58 | F | U | Beef and pork meat | Unknown | 1.83 | 1.28 | n.d.*** | 16.8 |
| | | | U | Beef meat | 1.5 | | | | |
| | | | U | Beef innards | 3 | | | | |
| | | | U | Beef and pork meat | 0.5 | | | | |
| 5 | 47 | F | U, D | Beef meat | Unknown | 3.35 | 3.65 | <0.35 | 8.07 |
| 6 | 70 | M | U | Beef and pork meat | Unknown | 20.7 | 15.3 | <0.35 | 75.5 |
| 7 | 59 | M | U | Beef, pork, and alcohol | 6 | 26.9 | 23.7 | n.d. | 86.4 |
| 8 | 68 | M | U | Pork meat | 5 | 26.1 | 21.3 | <0.35 | 60.1 |
| 9 | 78 | M | U | Boar meat | Unknown | 2.62 | 1.23 | <0.35 | n.d. |
| 10 | 70 | F | U | Beef meat | 1 | 1.93 | 1.72 | <0.35 | 4.03 |
| 11 | 65 | M | U | Beef and pork meat | Unknown | 30 | 20.2 | <0.35 | 122 |
| 12 | 68 | F | A | Beef and pork meat | Unknown | 5.64 | 4.10 | <0.35 | 65.8 |
| | | | U, A, D | Beef innards | Unknown | | | | |
| 13 | 64 | M | U | Beef and pork meat | Unknown | 2.23 | 1.94 | <0.35 | 7.27 |
| 14 | 53 | F | U, AS | Beef and pork meat | 4 | 0.94 | 2.64 | <0.35 | 31.0 |
| | | | U, D | Beef and pork meat | 4 | | | | |
| | | | A, D | Pork meat | 7 | | | | |
| 15 | 57 | M | U | Chinese noodles | 4 | 32.2 | 11.9 | <0.35 | 67.6 |
| | | | U | Pork meat and alcohol | 2 | | | | |
| | | | U, V, A | Beef innards and alcohol | 2.5 | | | | |
| 16 | 70 | F | D | Beef meat | Unknown | 12.4 | 9.12 | <0.35 | 75.3 |
| 17 | 86 | M | U | Beef and pork meat | Unknown | 6.27 | 6.28 | n.d. | 10.0 |
| 18 | 65 | M | U | Raccoon dog meat | 2 | 2.7 | 1.69 | <0.35 | 30.0 |
| | | | U | Beef meat | 2 | | | | |
| | | | U | Boar meat | 4.5 | | | | |
| | | | U | Horse meat | Unknown | | | | |
| | | | U | Boar meat | 1.5 | | | | |
| 19 | 67 | M | U | Beef meat | 1–1.5 | 8.49 | 8.17 | <0.35 | 13.8 |
| | | | U | Beef meat | Unknown | | | | |
| 20 | 60 | F | U | Beef meat | Unknown | 18.7 | 6.07 | <0.35 | 89.7 |
| | | | U | Beef meat | Unknown | | | | |
| 21 | 66 | M | U | Beef innards | Unknown | 1.86 | 1.29 | <0.35 | 2.99 |
| | | | U, D | Beef meat | Unknown | | | | |
| | | | U | Pork meat | 4 | | | | |
| | | | U | Beef and pork meat | Unknown | | | | |
| | | | U | Pork meat | Unknown | | | | |
| 22 | 55 | F | U | Beef meat and alcohol | 5.5 | 31.2 | 14.5 | <0.35 | 257 |
| | | | U | Beef meat and alcohol | 7.5 | | | | |
| 23 | 64 | F | U | Beef and pork | Unknown | 0.94 | 0.69 | <0.35 | 18.1 |
| | | | U | Pork meat | Unknown | | | | |
| 24 | 64 | F | U | Beef meat | 4 | 16.4 | 7.44 | <0.35 | 54.3 |
| 25 | 81 | M | U | Beef meat | Unknown | 6.37 | 5.32 | <0.35 | 10.0 |
| | | | U | Beef and pork meat | Unknown | | | | |
| | | | U | Pork meat | 1 | | | | |
| 26 | 76 | M | U | Beef meat | Unknown | 6.41 | 3.72 | <0.35 | 20.3 |