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# Development of Specific ELISAs for Phase II Drug Metabolizing Glutathione Transferase A Class Isoforms.

Kevin M. Patrie, Toni L. Rader and Denis Callewaert.
Oxford Biomedical Research, Rochester Hills, MI.

## Abstract

Biomarkers that accurately reflect organ toxicity due to exposure to harmful agents are crucial for drug development and for detecting and reducing the incidence of diseases resulting from environmental chemicals. However, most currently used biomarkers fall short of meeting the criteria required to serve as sensitive indicators of toxicity and disease.

Human cytosolic glutathione S-transferases (GSTs) are well documented to be released by cells upon damage due to toxin exposure and their appearance in blood and urine, either as a result of active secretion or release by damaged cells, is a hallmark of organ toxicity. In addition, they exhibit many of the required characteristics of a biomarker upon toxic insults to organs. Indeed, recent human and rodent studies, have indicated that GSTs are more sensitive biomarkers for detecting hepatic injury than the transaminases. Therefore, GSTs have enormous potential to serve as biomarkers for organ damage brought on by exposure to toxic environmental agents, or adverse response to drugs.

In recent years, the difference in tissue distribution of the major GST Classes has been shown to be due to the presence of different isoforms of the major Classes. However, most currently available antibodies directed against a particular GST Class are not capable of distinguishing the different isoforms of that Class. Detecting an increase of an A Class GST in blood samples, for instance, may indicate toxicity to an environmental compound or drug but the target organ(s) can not be determined. Antibodies specific for a particular isoform of a GST Class will permit greater resolution regarding which organs are affected. Towards this end, we have recently developed isoform-specific monoclonal antibodies (mAbs) against GST A1/A2, A3 and A4. Here we present data illustrating the development of sandwich ELISAs utilizing these mAbs and their validation with biological samples, with emphasis on the anti-GST A3 ELISA.

## Methods

**Recombinant GST production:** Bacterial expression constructs containing 6× His-tagged GST A isoform cDNAs were provided by Dr. Bengt Mannervik (Uppsala University, Uppsala, Sweden). Two hundred ml of 2×YT growth media, supplemented with the appropriate antibiotic, was inoculated with a single bacterial colony and the culture allowed to incubate/shake overnight at 37°C. Pelleted bacterial cells were lysed in 50 mM Tris/300 mM NaCl by sonication. Clarified lysis supernatants were passed over a Cobalt-agarose resin to purify the His-tagged GSTs. Purifications were typically ≥ 95%.

**Antibody production:** Peptide antigens corresponding to regions of human GST A1-1, A3-3 and A4-4 that were significantly divergent from each other were conjugated to bovine thyroglobulin and sent to Bethyl Laboratories (Montgomery, TX) for polyclonal antibody production in rabbits. Purified recombinant whole molecule GST A3-3 and A4-4 were used to immunize mice for subsequent monoclonal antibody production (ProMab Biotechnologies, Richmond, CA). Hybridoma supernatants from subclones (one round of limiting dilution subcloning), as well as rabbit antisera, were evaluated for specificity against all GST A isoforms.

**Enzyme Immunoassay testing of Rabbit antisera and hybridoma supernatants:** Purified recombinant GSTs were coated onto 96-well Strip-well plates at 0.5 µg/well. Dilutions of rabbit polyclonal antisera or hybridoma supernatants were incubated in wells then washed 3× followed by the addition of an HRP-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibody. After a final wash, development of the immunoassay was performed using TMB as the HRP substrate and the absorbance from the wells measured at 450 nm.

**ELISA development:** For development of the anti-GST A3-3 ELISA, a purified mouse anti-A3-3 monoclonal antibody was coated onto 96-well Strip-well plates. Wells were blocked and dilutions of purified recombinant human GST A3-3 incubated in the wells in triplicate. After washing, a biotinylated rabbit polyclonal antibody was added. Wells were washed once more followed by the addition of Streptavidin-Poly HRP enzyme conjugate. A final wash was followed with the addition of TMB substrate. Absorbance of the wells was measured at 450 nm after stopping the color development with 3 N sulfuric acid.

**Western Blotting:** Approximately 10 µg of rat serum was separated on a 4-20% Tris-glycine gel and the proteins transferred to a nitrocellulose membrane. The blot was blocked in 3% BSA/ 1% normal donkey serum (in TBS/0.1% Tween-20). A biotinylated GST A3-3 monoclonal antibody was used as the primary antibody. After washing the blot, Streptavidin-Poly HRP was added. The blot was washed once more then incubated briefly in an HRP chemiluminescent substrate. The blot was then exposed to X-ray film.

## Introduction

An ideal biomarker of organ damage exhibits the characteristics of specific tissue localization, high cytosolic concentration and a relatively short half-life. In addition, it should be sensitive enough to detect the early stages of tissue damage. However, most currently used biomarkers for organ toxicity do not meet all these criteria.

Human cytosolic glutathione S-transferases (GSTs) consist of 17 gene products that are divided into seven major Classes (designated as A, M, P, T, Z, O and S), with most Classes comprised of more than one subclass, or **isoform** (e.g. GST A1-1, GST A2-2, etc.), that exhibit different yet overlapping tissue expression patterns. GSTs play prominent roles in Phase II detoxification and biotransformation of xenobiotics and in the metabolism of many endogenous compounds. For these reasons GSTs are of significant interest to pharmacologists and toxicologists.

The appearance of GSTs in biological fluids is reflective of cellular damage due to toxin exposure and is a hallmark of organ toxicity. In addition to their distinct tissue localization and relatively quick release into the bloodstream following organ damage, GSTs have a high cytosolic concentration and a relatively short half-life. These characteristics provide GSTs with the ability to serve as ideal biomarkers for organ damage brought on by exposure to toxic environmental agents or adverse response to drugs. Indeed, in both human and rodent studies, GST A has recently been shown to be a more sensitive biomarker for detecting hepatic injury than the transaminases. The tissue distribution of GSTs in rodents follows a similar pattern to that in humans. Thus, they are capable of fulfilling similar roles as early indicators of organ damage in pre-clinical animal models.

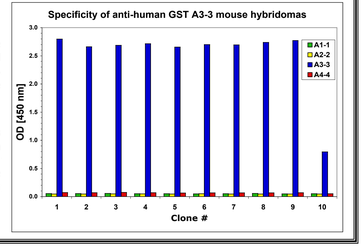
Isoforms of a particular GST Class exhibit differences in tissue expression as well. This relatively recent discovery makes it important that antibodies for GSTs be specific for individual GST isoforms. Currently, most commercially available antibodies for a particular GST Class are not isoform-specific. Therefore, a detectable increase of GST A in blood samples, for instance, could be an indication of toxicity from exposure to an environmental compound or pharmaceutical, yet the organ(s) effected would be unclear. Finer resolution of those organs/tissues targeted by toxins would be achievable with antibodies specific for a particular isoform of a GST Class. It is also important to note that, although more than one GST Class may be expressed in one tissue or organ, a given xenobiotic molecule or drug may have a preferential effect on a particular cell type within the target tissue or organ. Hence, knowledge of the cellular expression of GSTs (via immunohistochemical procedures) will help identify not only which cells are being effected but also may lead to insights of the modality of toxicity brought on by an environmental toxin or drug.

Our goal to develop a panel of highly sensitive GST immunoassays using antibodies specific for not only A Class enzymes but for other GST Classes and isoforms will aid in determining target organs of toxicity with much greater precision and accuracy.

## Evaluation of GST A isoform-specific antibodies

Rabbit polyclonal antisera and mouse hybridoma supernatants were evaluated for their specificity towards human GST A Class isoforms by antigen-down enzyme immunoassays (EIAs).

The graph depicted to the right summarizes a typical EIA that was performed to assess the specificity of ten mouse hybridomas. Splenocytes used for the generation of the hybridomas were derived from a mouse immunized against human GST A3-3 (whole molecule).

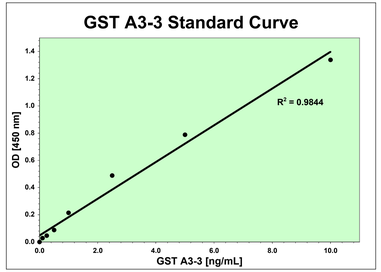



## Antibodies against human GST A Class isoforms

The following table summarizes the antibodies currently in-house at Oxford Biomedical Research. The antibodies highlighted in pink are currently available while most, if not all, of those shaded in green are undergoing further evaluation and will be available in the near future. Immunohistochemistry (IHC) will be among the other applications that the antibodies will be further evaluated for.

| GST antigen | Immunogen | host species | antibody type | Specificity | Application(s) |
|---|---|---|---|---|---|
| human GST A1-1 | Whole molecule | Goat | polyclonal | A1, A3 | WB, ELISA |
| human GST A1-1 | Whole molecule | Rabbit | polyclonal | A1, A2, A3 | WB |
| human GST A1-1 | peptide | Rabbit | polyclonal | A2, A4 | WB |
| human GST A4-4 | peptide | Rabbit | polyclonal | A4 | WB, ELISA |
| human GST A3-3 | Whole molecule | Mouse | monoclonal | A3 | WB, ELISA |
| human GST A3-3 | Whole molecule | Mouse | monoclonal | A3 | WB |
| human GST A4-4 | Whole molecule | Mouse | monoclonal | A1, A2, A3, A4 | WB |
| human GST A4-4 | Whole molecule | Mouse | monoclonal | A4 | WB |
| human GST A4-4 | Whole molecule | Mouse | monoclonal | A4 | WB, ELISA |

## Development of an ELISA for human GST A3-3




An Enzyme-linked Immunosorbant Assay (ELISA) was developed for the detection and quantitation of human GST A3-3. For this indirect sandwich ELISA, a monoclonal antibody specific for GST A3-3 was employed as the Capture antibody and a biotinylated rabbit polyclonal antibody used as the Detection antibody. The enzyme-conjugate was in the form of Streptavidin-Poly HRP. Further optimization is currently ongoing to assess the ELISA's parallelism as well as determine its sensitivity and dynamic range.

## Results & Findings

A range of monoclonal and polyclonal antibodies have been developed (a contractual effort with antibody production facilities) that exhibit specificities for human GST A class isoforms. Among these antibodies are those that are specific for only one A Class isoform.

For each GST A isoform-specific ELISA, a broad range of Blocking Agents (up to 15) were evaluated to determine the one that provided the lowest amount of background interferences (non-specific binding). As a result, the optimal Blocking Agent was empirically determined for each ELISA that employed different antibody pairs. Thus, each ELISA has been developed to maximize signal to noise ratios for optimal sensitivity.

Ability of the antibodies to recognize rat GST A Class enzymes.

Finding that a monoclonal directed against one A Class isoform recognizes all A Class isoforms equally well.

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