Application of Proteomics and Cytomics in Human Neutrophils Functional Studies

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Dedicated with Love to my wife,

<u>Yolanda Virgili</u>

And my two mothers,

<u>Solange Rosa</u>

and

<u>Carmem Villa Sola</u>.

And in Memory of my Father,

<u>João Bezerra Campos Filho</u>

This Dissertation would not have been possible without the timely cooperation from my colleagues, who I would like to take the opportunity to thank individually. They are: Carlos Garcia, Elaine Nascimento, Renan Rodrigues, Pedro Ivo, Nuno Domingos, David Billido, Antonia Odena and my two tutors Wagner Fontes and Eliandre de Oliveira.

Abstract

Biomedical research commonly starts by raising a hypothesis to solve a problem. In this context, scientists select the most appropriate method(s) to answer the question and solve a common dilemma. Over the past decade, we have witnessed a revolution of new technologies in molecular biology – the ***Omics Science***. High-scale technologies such as metabolomics, cytomics, genomics and proteomics are changing the way we study complex biological systems. Current approaches to understanding the functional diversity of an organism preferentially strive for a systems biology approach whereby first the phenotypic classification of a specific cytome is achieved prior to an attempt to perform proteomic analysis. In this context, to better understand the features that involve neutrophil activation and programmed cell death in the pathological and healthy states, this study proposes the integration of cell biology approaches such as flow cytometry with a very robust proteomics platform in an attempt to integrate data at the molecular level with phenotypic data of neutrophils. The application of subcellular fractionation method using digitonin detergent extraction to enrich cytosolic proteins from neutrophils was found reproducible, simple to perform, and inexpensive.

1. Introduction

[1.1] The Neutrophil – an interesting biological puzzle

The immune cells are classified into two distinct lineages with specialized cell types required for proper immune response (Fisher, A. 2002). The myeloid lineage of monocytes, macrophages, dendritic cells and neutrophils carries out the innate immune response. The lymphoid lineage of T cells, B cells and natural killer (NK) cells enables adaptive immunity by distinguishing self from non-self antigens and also providing a memory of foreign proteins seen before (Kindt, T. *et al.,* 2002). Other immune cells, such as eosinophils, basophils and mast cells, also participate in the immune response. The human body contains about five liters of blood. Red blood cells constitute about 45% of this volume and white blood cells (WBC) around 1%, the rest being the liquid blood plasma (Diggs, L. 1985). The white blood cells, or leukocytes, can be sub-divided into: lymphocytes, monocytes and granulocytes (Kindt, T. *et al.,* 2002). The former represents the largest group of leukocytes and can themselves be subdivided in neutrophils, eosinophils and basophils (table 1). Circulating neutrophils are terminally differentiated white blood cells presenting a short-life span and characterized by a multilobed nucleus and a granular cytoplasm (fig. 1 and 2e). The latter is due to the presence of specialized granules containing the host-defense molecules, which is rapidly mobilized following an infectious challenge (Faurschou, M. and Borregaard, N. 2003).

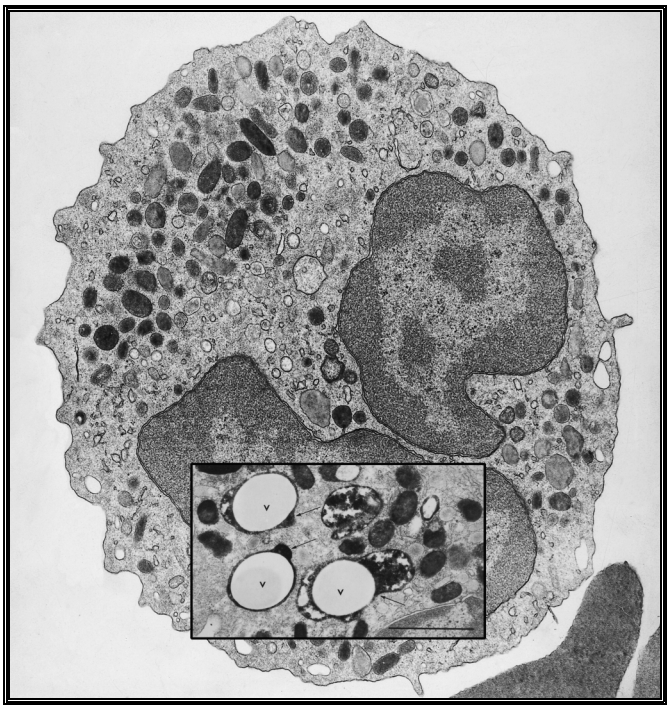


Fig. 1: Transmission Electron Micrograph of human neutrophil. Inset is an image taken from a neutrophil 20 s after the phagocytosis of latex particles opsonized with IgG (V, vacuole). The section was stained for myeloperoxidase (MPO) to reveal the

electron-dense product in the azurophil granules, some of which can be seen degranulating into the phagocytic vacuole (arrows). Bar = 1 μm. (*Image from Segal, AW. et al., 1980*)

Immunologists have long praised the essential role of polymorphonuclear leukocytes, predominantly neutrophils, in the host defense against invading microorganisms. On a per-cell basis, neutrophils make fewer molecules of a given cytokine than do macrophages or lymphocytes, but neutrophils often outnumber mononuclear leukocytes at inflammatory sites by one to two orders of magnitude, and they can therefore be important sources of cytokines such as tumor necrosis factor α (TNFα) at the crucial juncture at which the decision is made to mount an immune response (Nathan, C. 2006). A normal adult produces more than 10 billion neutrophils per day, a figure that can increase as much as tenfold during a period of acute inflammation. The relative abundance of neutrophils, with approximately 50 billion circulating in the bloodstream, and its rapid recruitment to the site of infection make these cells a prominent early player of the innate immune system.

***Table 1*: Cells in Normal Human Adult Peripheral Blood**

Cells	***No. per cm^3***	***Percent of WBC***	***Diameter (μm)***
Platelets	1-3 x 10^8	----	2-3
Erythrocytes (RBC)	4-6 x 10^9	----	6-8
Leukocytes (WBC)	3-6 x 10^6	100%	
• Neutrophils	2-4 x 10^6	50-70%	10-12
• Eosinophils	0.1-5 x 10^5	1-3%	10-12
• Basophils	0-0.1 x 10^6	0-1%	8-10
Lymphocytes	1-4 x 10^6	20-40%	6-12
Monocytes	0.2-1 x 10^6	1-6%	12-15

Source: Diggs LW, *et al.,* (1970). The Morphology of Human Blood Cells, 5th edition. Abbott Laboratories, Abbott Park, IL.

[1.2] Neutrophil Morphology

Cell biologists and immunologists have relied mainly on cell morphology to characterize the five different stages of neutrophil differentiation: ***myeloblast, promyelocyte, myelocyte, banded cells, and segmented cells*** (fig. 2a-e) (Bainton, D. 1971). Differentiation of myeloblasts into mature neutrophils does not result from a simple state transition; instead, neutrophil differentiation appears to be a multi-step process characterized by the sequential formation of ***azurophil***, ***specific*** and ***gelatinase granules*** (granulopoiesis) and their constituent granule proteins (Faurschou, M. and Borregaard, N. 2003). The various subsets of granules contained within the neutrophil are key effectors of the neutrophil immune response.

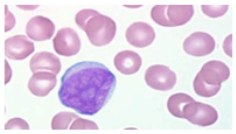
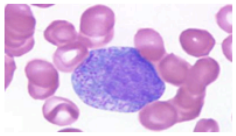
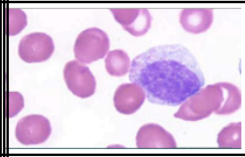
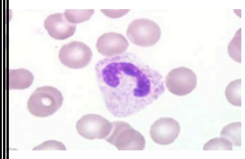
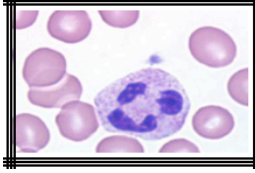
	**(A)** Myeloblasts lack cytoplamic granulation. The nucleus is composed of very fine nonaggregated chromatin that stains light blue to reddish-purple with Wright's stain. Two to five distinct nucleoli are usually present.
	**(B)** Promyelocytes are characterized by large peroxidase-positive, reddish-purple granules (eosinophils and basophils) that reacts with Sudan black stain. The cell size, 12 to 20 µm, is the same as that of a myeloblast, but the nucleus to cytoplasm ratio is less, usually from 3:1 to 2:1. The chromatin is still fine, but some aggregation is evident. One to three nucleoli are also visible, although these can be obscured by heavy granulation.
	**(C)** Myelocytes (diameter = 10 to 18 µm) present a nucleus to cytoplasm ratio of 1:1. This nucleus is oval or round, and often eccentrically located. Chromatin is finely granulated in early myelocytes and more aggregated in later cells.
	**(D)** Band neutrophils (diameter = 9 to 15 µm) comprise approximately 1 to 3% of the peripheral leukocytes. The nucleus forms a "U" or curled rod prior to segmentation. The chromatin pattern is coarse and clumped. The cytoplasm is moderate to abundant with a few nonspecific granules and many specific granules.
	**(E)** Segmented neutrophils are the mature phagocytes and comprise 40-75 % of the peripheral leukocytes. They are usually 9 to 16 µm in diameter. The nuclear lobes, normally numbering from 2 to 5, may be spread out so that the connecting filaments are clearly visible, or the lobes may overlap or twist. The chromatin pattern is coarse and clumped. The cytoplasm is abundant with a few nonspecific granules and a full complement of rose-violet specific granules.

Fig. 2: Blood films on glass slides of the different stages of neutrophil differentiation stained with a Romanowsky stain. Source: (1) Clinical Chemistry and Hematology Laboratory at the New York State Department of Health - Wadsworth Center; and (2) S.S. Spicer and J.H. Hardin, 1969.

Granulopoiesis is initiated in early promyelocytes, when immature transport vesicles bud off from the Golgi and fuse. The resultant early-appearing granule is rich in antibacterial proteins such as elastases, defensins, and myeloperoxidases (MPO), and referred to as "azurophil granules" (or "primary granules"). At the promyelocyte/myelocyte transition, when the production of MPO ceases, peroxidase-negative granules begin its sequential formation. Appearance of "specific granules" (or "secondary granules") takes place during myelocyte and metamyelocyte stages, and has a high content of lactoferrin; in addition, synthesis of the fMLP receptor and CD11b is initiated at this stage. Gelatinase granules are formed in band and segmented neutrophils, and present a high content of gelatinase (Faurschou, M. and Borregaard, N. 2003). Finally, secretory vesicles containing mainly plasma proteins are formed in segmented neutrophils (Cowland, J. and Borregaard, N. 1999). Secretory vesicles are generated by endocytosis, resulting in membranes rich in receptors, signaling proteins, and adhesion molecules, whereas their lumen contains plasma (Faurschou, M and Borregaard, N. 2003; Lominadze, G. *et al.,* 2005). In conclusion, the proteome of each subpopulation of granules is formed as a consequence of an orchestrated gene expression program, a mechanism known as "targeting by timing" (Borregaard, N. and Cowland, JB. 1997). The overlap between the timing of synthesis of different granular proteins gives rise to some overlap of contents among different granule subsets (Le Cabec, V. *et al.,* 1996). However, although all granule subsets share common features, there are substantial differences

with respect to protein content and the extent of protein release to phagosomes and extracellular environment.

Azurophil granules

Despite its early recognition as specialised lysosomes, azurophil granules are nowadays probably best regarded as regulated secretory granules (Cieutat, A. *et al.,* 1998). Like lysosomes, azurophil granules contain granulophysin (CD63) in their membrane. However, in contrast to lysosomes, they do not express the lysosome-associated membrane proteins LAMP-1 and LAMP-2 (Cieutat, A. *et al.,* 1998), and the sorting of proteins to azurophil granules does not involve the mannose-6-phosphate receptor system essential for lysosomal enzyme targeting (Dahms, N. *et al.,* 1989; Nauseef, W. *et al.,* 1992). Azurophilic granules are the largest of the human neutrophilic granules, measuring 0.3 μm in diameter (Nüsse, O. and Lindau, M. *et al.,* 1988; Lollike, K. *et al.,* 2002). They are also referred to as primary or peroxidase-positive granules. Each human neutrophil contains 1300 azurophilic granules based on morphological data (Nüsse, O. and Lindau, M. *et al.,* 1988). Based on their time of appearance during myelopoiesis, azurophil granules are often divided into two major subsets, namely early- and late-appearing azurophil granules, which are formed from the beginning of the promyelocytic stage and near the promyeocyte/myelocyte transition, respectively (Sengelov, H. *et al.,* 1993; Arnljots, K. *et al.,* 1998).

Azurophil granules contain a highly packed matrix of acid mucopolysaccharide and highly cationic myeloperoxidase. These granules contain the largest number of luminal bactericidal proteins such as the α-defensins and myeloperoxidase (MPO, the iron-containing enzyme that colours pus green.), while there is a paucity of membrane, cytoskeletal, and GTP-binding proteins. Many proteins of azurophil granules are synthesized as proforms, which go through proteolytic trimming on arrival at the granule compartment. This seems to be important to prevent misfolding, autotoxicity or retention of the mature proteins during their passage through the biosynthetic machinery of the cell (Garwicz, D. *et al.,* 1998; Liu, L. and Ganz, T. 1995). After removal of amino-terminal propeptides, the proteins are stored in their active conformations (Lindmark, A. *et al.,* 1998; Garwicz, D. *et al.,* 1998).

Peroxidase-negative granules

Historically, specific and gelatinase granules have been also designated peroxidase-negative granules (Kjeldsen, L. *et al.,* 1992). Although widely employed, this nomenclature may be misleading since myeloperoxidase has been found in these granule subsets as well (Lominadze, G. *et al.,* 2005). Recently, two independent approaches were used to determine MPO presence in the neutrophil granule subsets. Theilgaard-Monch *et al.,* (2005) found that MPO expression is downregulated during transition from promyelocytes (formation of azurophil granules) to myelocytes/metamyelocytes (formation of specific granules). By peering the microarray data, one can note that MPO expression is not completely abrogated during this transition, whereas transition from myelocytes/metamyelocytes to bone marrow PMNs seems to correlate with complete abrogation of this gene. MPO protein expression in peripheral blood neutrophils has also been assessed by ELISA method (Lominadze, G. *et al.,* 2005). This study showed that 73% of total MPO was present

in azurophil granules, 20% was in specific granules, and 7% was in gelatinase granules. Similar distribution has been also reported by Kjeldsen *et al.,* (1994; 1999). However, these studies are not conclusive and absolute quantification has not been carried out to date. Furthermore, this supposedly MPO detection in specific and gelatinase granules may be a result of contamination during granule separation process. Gelatinase granule membranes contain a large number of membrane receptors and adhesion molecules. Therefore, the large amount of actin cytoskeleton and cytoskeletal regulatory proteins in these granules supports its role in enhancing the plasma membrane expression of molecules necessary for neutrophil adherence to and migration through inflamed vascular endothelium.

Specific granules contain large amounts of moderately cationic luminal proteins. The greater complexity of the specific granule lumen and the presence of a significant number of transmembrane and membrane-associated proteins suggest that these granules represent a transitional phase that can contribute to neutrophil activation through exocytosis or provide bactericidal proteins to phagosomes. Consistent with this notion, gelatinase and specific granules contain overlapping sets of proteins, which include lactoferrin, lipocalin, lysozyme, LL37, three metalloproteases (MMP8, MMP9 and MMP25), and several other antimicrobial proteins, matrix-degrading enzymes and membrane receptors (Lominadze, G. *et al.,* 2005). From a total of 247 proteins identified from the three granule subsets in a recent study, Lominadze *et al.,* (2005) reported an overlap of 79 proteins between gelatinase and specific granules, but only five proteins overlap between specific and azurophil granules. Moreover, a total of 86 proteins were identified only from gelatinase granules, 28 proteins only from specific granules, and 26 proteins only from azurophil granules. In contrast to azurophil granules, specific granules store proteins as intact proforms.

[1.3] Neutrophils in Action

The remarkable capacity of detecting and eradicating invading pathogenic microorganism without destroying self-tissues requires a well-orchestrated innate and adaptive immune response. Activation of immune response with subsequent eradication of microbial pathogens is a complex, multi-step process (fig. 3). As a first step, neutrophils are rapidly recruited from circulation and bone marrow reserves to sites of infection by host and pathogen-derived stimuli (chemotatic factors) such as leukotriene LTB_4 (Freeland, H. *et al.,* 1988) platelet-activating factor (PAF) (Mencia-Huerta, J. *et al.,* 1983); tumor-necrosis factor α (TNFα) (Williams, C. and Coleman, J. 1995), interleukin-8 (IL-8) (Gordon, J. *et al.,* 1990), *N*-formylmethionyl-leucyl-phenylalanine (fMLP) (Le, Y. *et al.,* 2002) and lipopolysaccharide (LPS) (Fessler, M *et al.,* 2002). In addition to recruiting neutrophils, many chemotactic factors, including lipopolysaccharide (LPS), tumor necrosis factor-alpha (TNFα), and platelet-activating factor (PAF), purportedly modulate neutrophil responses to subsequent stimuli, a process known as *neutrophil priming*. (Brazil, T. *et al.,* 1998) During recruitment and migration to infection focus, neutrophils produce a number of factors to orientate their migration, ensure their survival in the hostile inflammatory milieu, recruit additional neutrophils and 'inactivate' their own toxic products (Botha, A. *et al.,* 1995).

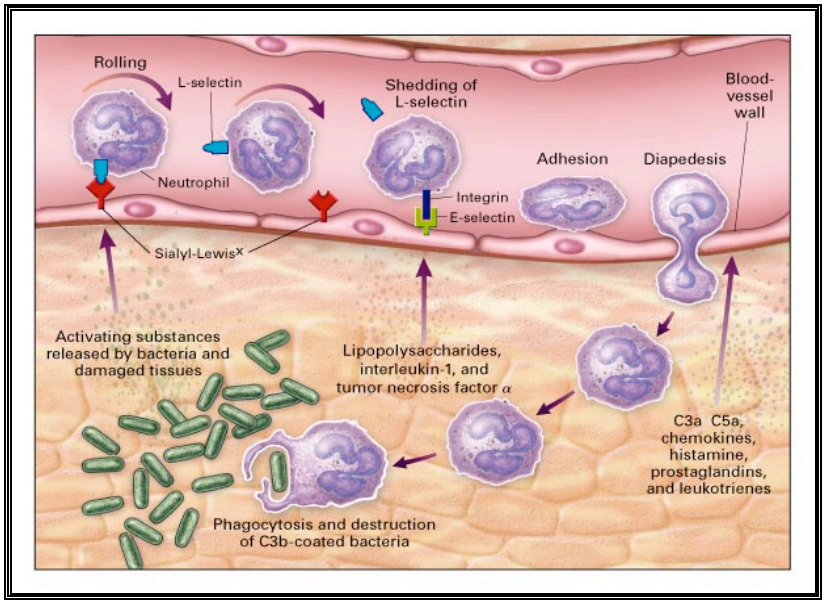


Fig. 3: Multi-step activation process of neutrophils

The migration of neutrophils from the blood to target tissues ***(extravasation)*** involves tethering and rolling of neutrophils on the blood-vessel wall, firm adhesion and crossing through the endothelial barrier (***diapedesis)*** (Cinamon, G *et al.,* 2001). Adherence of circulating leukocytes to the vascular endothelium is facilitated by the expression of several membrane proteins such as CEA-related cell-adhesion molecules (CEACAMs), selectins, immunoglobulin superfamily members, and integrins. In addition to these classical interactions, other cell-surface-expressed enzymes with catalytic domains outside the plasma membrane - ectoenzymes such as CD26, CD38, and vascular adhesion protein 1 - were recently reported to take part in the different steps of the extravasation cascade (Goding, J. 2000). The adhesive interactions of neutrophils are usually dynamic and transient. In fact, a high degree of flexibility in cell adhesion and mobility should be attained to allow neutrophils to circulate in the blood without pathologically adhering to the vascular wall.

Several studies have underscored the importance of a small-scale respiratory burst and granule exocytosis to release certain neutrophil proteases and adhesion molecules as a precondition to neutrophil recruitment to inflammatory loci (Nathan, C *et al.,* 1993; Raptis, SZ *et al.,* 2005). The neutrophil–endothelial interactions have been shown to trigger mobilization of secretory vesicles from neutrophils (Borregaard, N *et al.,* 1994). Secretory vesicles are mobilized at the earliest phases of the neutrophil-mediated inflammatory response in response to a wide variety of inflammatory stimuli (Faurschou, M and Borregaard, N. 2003; Lominadze, G *et al.,* 2005). These vesicles constitute a reservoir of membrane-associated receptors including the β2-integrin CD11b/CD18 (Mac-1, CR3), the complement receptor 1 (CR1), receptors for formylated bacterial peptides (formylmethionyl-leucyl-phenylalanine (fMLP)-receptors), the LPS/lipoteichoic acid-receptor CD14, the FcγIII receptor CD16, and the metalloprotease leukolysin, all of which are incorporated in the plasma membrane after exocytosis.

The disassembly of extracellular matrix is believed to be crucial for neutrophil extravasation and migration, and to facilitate the neutrophil-bacterial contact (Borregaard, N. and Cowland, J. 1997; Owen, C. and Campbell, E. 1999; Kang, T. *et*

al., 2001). The release of reactive oxygen species such as hydrogen peroxide, hypohalites and chloramines activates matrix metalloproteinases (MMPs), and inactivates protease inhibitors (Henson, P. and Johnston, R. 1987; Weiss, S. 1989). MMPs play fundamental roles in a wide range of mammalian physiological and pathological processes by degrading major structural components of the extracellular matrix including collagens, fibronectin, proteoglycans, laminin and gelatin.

Neutrophil cytotoxic arsenal

Neutrophils have been long praised as a crucial line of defense against bacterial infections because of their rapid release of a potent arsenal of cytotoxic agents that can be mechanistically divided into ***oxygen-dependent*** or ***oxygen-independent***. The latter encompasses the contents of the three neutrophil granule subsets and the secretory vesicles, whereas the former stems from the 'oxidative burst', which takes place after NADPH oxidase complex assembles at the phagosomal or cellular membranes. Production of ***reactive oxygen species*** (ROS) as well as proteases and its subsequent release by neutrophils are stringently controlled to protect surrounding cells and tissues. However, overwhelming and protracted neutrophil activation at sites of infection can ensue in damage to healthy tissue as well (Brown, K. *et al.,* 2006; Nathan, C. 2006; Parsey, M. 1998; Weiss, S. 1989; Henson, P. and Johnston, R. 1987; Holman, J. and Seba, T. 1988). To optimize defense capabilities while minimizing damage to host tissues, neutrophil responses must be spatially and temporally regulated. Thus, later in the inflammatory process, neutrophils undergo to a drastic functional switch to generate signals to retard their own accumulation; to suppress their own activation; to attract and program other immune cells such as macrophages and lymphocytes; and to promote their own death.

Human neutrophils contain three types of granules and the secretory vesicles that are mobilized (exocytosis) hierarchically during neutrophil extravasation from the bloodstream to the site of inflammation (Sengeløv, H. *et al.,* 1995; Borregaard, N. and Cowland, J. 1997). The exocytosis of neutrophil granules is a meticulously orchestrated sequence of events triggered by bacterial and host molecules such as cytokines. Therefore, exocytosis gradually changes the functional state of the neutrophil, and is crucial for effective killing of invading microorganisms. The different neutrophil granules containing ***antimicrobial proteins*** can discharge their contents either into the phagosome or into the extracellular media. The secretory vesicles, the gelatinase granules (also known as tertiary granules), and the specific granules (also known as secondary granules) function as easily accessible reservoirs of various receptors, whereas azurophilic granules (also known as primary granules) are thought to primarily fuse with the phagosome (fig. 4).

In neutrophils, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase acts as a multicomponent enzyme complex that generates relatively large amounts of superoxide anion (~2 nmol/min per 10^6 neutrophils) (Babior, B. 1999; Lambeth, J. *et al.,* 2000; Babior, B *et al.,* 2002) and other secondarily derived ROS. Reactive oxygen species (ROS) and their derivatives products generated by neutrophils during respiratory burst include superoxide, singlet oxygen, ozone, hydrogen peroxide, hypohalous acids, chloramines and hydroxyl radicals (Klebanoff, S. 2005) (fig. 4). The role of ROS in immunity is believed to be mainly microbicidal, although

controversial reports have come into light in the last years (Reeves, E. *et al.,* 2002). In contrast to many signal transduction systems in which agonist-induced transcription of genes initiates a vigorous biochemical cellular response, the NADPH oxidase is spatially regulated, whereby the dormant oxidase is maintained in an inactive state by partitioning the components into a cytoplasmic fraction, and a membrane-bound flavocytochrome b_{558}. The latter is a stable heterodimer composed of the $p22^{phox}$ and $gp91^{phox}$ subunits, which is localized both in the plasma membrane (around 5%) and in the membranes of mobilizable intracellular granules (around 95%) in the rest neutrophils (Borregaard, N. *et al.,* 1983; Sengeløv, H *et al.,* 1992). The cytoplasmic phox components exist as a complex (M_r 250kDa) stabilized through multiple SH3-domain-mediated interactions of its factors, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, the small regulatory GTPases Rac2 and Cdc42, and the newly identified p29 peroxiredoxin. Upon neutrophil stimulation, several serine residues within the polybasic region of $p47^{phox}$ become phosphorylated resulting in a conformational change in $p47^{phox}$, unfolding the protein and exposing the now interactive SH3 domains of $p47^{phox}$ to eventually interact stably with proline-rich regions (PRRs) on the cytoplasmic tail of $p22^{phox}$ (Nagasawa, T. *et al.,* 2003), thereby resulting in translocation to the membrane and activity of a functional oxidase (Shiose, A. and Sumimoto, H. 2000).

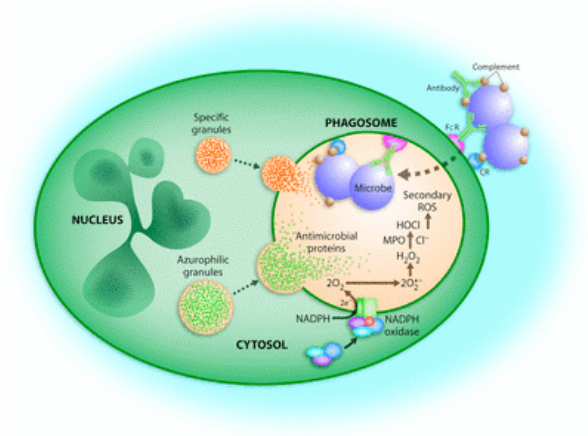


Fig. 4: *Oxygen-dependent* and *oxygen-independent* mechanisms of neutrophil defense.

The reactivity between ROS and proteins has been investigated in the past years leading to the postulation that ROS partake in several cell signaling events (Nathan, C. 2003). Hydrogen peroxide (H_2O_2) was shown to convert free L-tyrosine in the human blood plasma to tyrosyl radical, which may react with tyrosine residues of target proteins to produce o,o'-dityrosine bonds (Heinecke, J. *et al.,* 1993; Marquez, L. and Dunford, H. 1995; Jacob, J. *et al.,* 1996). Peroxynitrite and HOCl can also give rise to the formation of chlorotyrosine and dityrosine, respectively (Hazen, S. *et al.,* 1997; Kettle, A. 1996; Pfeiffer, S. *et al.,* 2000). These protein modifications have been associated with organ dysfunction in several diseases (Heinecke, J. 2002). A recent study showed that human neutrophils can moduate their proteome via a tyrosine oxidation pathway induced by pro-inflammatory mediators. In addition, upon neutrophil stimulation with TNFα, IFNγ or PMA, a limited number of membrane-associated proteins became tyrosylated (Avram, D. *et al.,* 2004). ROS

has been also shown to mediate temporary inactivation of tyrosine phosphatases by reversible oxidation of their active-site cysteine sulphydryls (Tonks, N. 2005).

The ability of neutrophils to ingest and kill microorganisms is crucial to innate immunity and host defense. Phagocytosis is an inherently complex process that requires phagocyte-microbe contact and is accompanied by activation of signaling pathways that orchestrate rearrangement of the actin cytoskeleton, extension of the plasma membrane, and engulfment. Neutrophil-microbe contact is initiated via a plethora of membrane receptors 'provided' by the aforementioned fusion of secretory vesicles, gelatinase and specific granules with the plasma membrane during the exocytosis process. Azurophilic and specific granules fuse with the phagocytic vacuole after microbe uptake (Segal, A. *et al.,* 1980). It has been estimated that the granule protein makes up about 40% of the vacuolar volume (Hampton, M. *et al.,* 1998), which gives a concentration of about 0.5 g ml^{-1} (Reeves, E. *et al.,* 2002). The phagosome volume depends on the size of the engulfed microbe, but will also increase dramatically over time. In vitro, the phagosome volume increases about 3-fold over 15 min in neutrophils (Reeves, E. *et al.,* 2002), and this increase is mostly attributable to the addition of specific granule contents, which are already present in 50% of phagosomes within 30 seconds after ingestion of bacteria (Bainton, D. 1973), and later of azurophil granule contents. Fusion of specific granules adds 0.26 fl and azurophilic granules add another 0.92 fl to the phagosome, which together would triple its volume if it started out at 0.6 fl. These estimates are based on the dimensions and numbers of granules in neutrophils, 4600 specific granules 0.13 μm in diameter and 1300 azurophilic granules 0.3 μm in diameter (Nüsse, O. and Lindau, M. 1988), and the assumption that all granules fuse with the first 20 phagosomes, which seems a reasonable synthesis of several observations (Hampton, M. *et al.,* 1998; Bainton, D. 1973). It is also important to note that multiple phagosomes may coalesce, forming much larger structures (Bainton, D. 1973).

[1.4] The Neutrophil Implication in Pathological Processes

Overwhelming neutrophil activation has been suggested to play a major role in several inflammatory-related disease such as the pathogenesis of organ dysfunction in sepsis (Brown, K. *et al.,* 2006). Such premise has been the subject of intense scrutiny in the last years, as researchers have sought to develop efficient therapies to control neutrophil-mediated healthy tissue damage in septic patients. Although limiting the inflammatory response attributable to neutrophils appears to represent a promising avenue for therapeutic intervention in sepsis (Abraham, E. 2003), neutrophils are essential components in the activation of innate immune response for detection and eradication of invading pathogenic microorganisms.

Evidence to support a pivotal role of neutrophils in sepsis-induced organ dysfunction derives from both experimental models and clinical studies. Postmortem pathological examination of specimens from septic patients with multiple organ failure reveals neutrophil infiltrations in compromised organs (Nuytinck, H. *et al.,* 1998; Thijs, A. and Thijs, L. 1998; Brealey, D. and Singer, M. 2000). The extent of neutrophil infiltrates correlates with impaired lung function and with high concentrations of neutrophil-derived proteolytic enzymes in the bronchoalveolar lavage from patients with acute respiratory distress syndrome (ARDS), a severe form of acute lung injury that may be associated with sepsis (Windsor, A. 1993; Ware, L. and Matthay, M. 2000; Steinberg, K. *et al.,* 1994; Adams, J. *et al.,* 2001).

Experimental interventions that deplete or inhibit the activity of neutrophils attenuated organ dysfunction and improved survival in animal models (Nathens, A. *et al.,* 1997; Abraham, E. 2003). These findings concur with clinical observations in patients with systemic inflammatory response where removal of blood neutrophils (by leucodepletion filters) enhances respiratory and renal function (Treacher, D. *et al.,* 2001). Moreover, neutrophils retrieved from both lungs and blood of patients with sepsis exhibit increased production of ROS (Baldwin, S. *et al.,* 1986); delayed apoptosis (Matute-Bello, G. and Liles, W. 1997; Feterowski, C. *et al.,* 2001); and increased predisposition to endothelial binding as evidenced by increased expression of β2 integrins (Brown, K. *et al.,* 2001; Chollet-Martin, S. *et al.,* 1996).

Neutrophil turnover is a healthy and critical mechanism to dampen local tissue destruction, diminish further inflammation and recruitment of immune cells, and facilitate termination of the innate immune response by limiting pro-inflammatory capacity (Matute-Bello, G. and Liles, W. 1997; Feterowski, C. *et al.,* 2001).

[1.5] Proteomics Technologies – Advances and Caveats

Proteomics defined in its most broad terms is the understanding of the protein expressed from biological compartments such as cell, tissue, or organisms in term of identification, quantify, post-translational modification, protein–protein interaction, sub-cellular localization activities and their biological functions. The introduction of novel protein separation techniques (Gorg, A. *et al.,* 2004; Washburn, M. *et al.,* 2001), the advent of mass spectrometers with higher mass accuracy, dynamic range and resolution (Shevchenko, A. *et al.,* 2000; Medzihradszky, K. 2000; Wilm, M. and Mann, M. 1996; Wilm, M. *et al.,* 1996; Syka, J. *et al.,* 2004), the improvement of computational data analysis tools (Clauser, K. *et al.,* 1999; Creasy, D. and Cottrell, J. 2002; Mann, M. and Wilm, M. 1994) and the proliferation of genomic and proteomic databases (Henzel, W. *et al.,* 1989; Mann, M. *et al.,* 1994; Pappin, D. *et al.,* 1993; Yates, J. *et al.,* 1993; Lander, E. *et al.,* 2001) are likely the main reasons for the increase in number and size of proteomic datasets. However, it has become evident that proteins are much more complex, diverse and dynamic than was originally anticipated (Aerbersold, R. and Mann, M. 2003); and despite the technological advances, there are important biological and technological caveats in proteomic analysis of complex material such as biological samples.

Several different protein prefractionation techniques can be combined with various analytical techniques. Gel-based methods such as one-dimensional and two-dimensional polyacrylamide gel electrophoresis (1D-PAGE and 2D-PAGE or 2-DE, respectively) are arguably the most popular and versatile methods of protein separation. Gel-independent approaches focus mainly on chromatographic separation techniques in combination with fast and sensitive MS.

Two-dimensional polyacrylamide gel electrophoresis (2-DE) combines isoelectric focusing (IEF), which separates proteins according to their isoelectric point (pI), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates them further according to their molecular mass. In an electric field, a protein will migrate toward the pH region on the IPG strip in which the pH equals its isoelectric point. After the first dimension separation, the IPG strip is laid atop the second dimension polyacrylamide sodium dodecyl sulfate gel (SDS-PAGE), and the proteins are run in an orthogonal direction, to achieve separation by size of fully

unfolded, SDS coated protein molecules. The major steps of the 2-DE-based proteomics workflow include: (i) sample preparation and protein solubilization; (ii) protein separation by 2-DE; (iii) protein detection and quantitation; (iv) computer-assisted analysis of 2-DE patterns; and (v) protein identification usually by mass spectrometry (Gork, A. *et al.,* 2004).

Sample preparation for 2D gel electrophoresis can be divided into three basic steps, namely cell disruption, inactivation or removal of interfering substances, and solubilization of the proteins. Protein extraction is a crucial step in any proteomics strategy because of its influence in yield, biological activity, and the structural integrity of the specific target protein. Several techniques for cell disruption are available such as osmotic lysis, freeze- thaw cycling, detergent lysis, enzymatic lysis of the cell wall, sonication, grinding with (or without) liquid nitrogen, high pressure (*e.g.* French press), homogenization with glass beads and a bead beater, nitrogen cavitation, or a rotating blade homogenizer. Neutrophil cell membranes are weak and thus easily disrupted by gentle treatment with mild detergents. This is best accomplished by using lower concentrations of a nonionic detergent such as Triton X-100. Detergents also prevent hydrophobic interactions between the hydrophobic protein domains; hence, avoiding loss of proteins due to aggregation and precipitation.

The most common interfering compounds in sample preparation are proteolytic enzymes, salts, lipids, nucleic acids, polysaccharides, and/ or highly abundant proteins. Salt ions may interfere with electrophoretic separation and should be removed if their concentration is too high (>100 mM). Salt increases the conductivity of the IEF gel, thereby prolonging the time required to reach the steady-state. High amounts of lipids may interact with detergent and thus reduce its effectiveness. Lipids can also form complexes with proteins, reducing its solubility. The presence of polysaccharides and nucleic acids can result in poor focusing in the acidic region of the IEF gel, and obstruction of the pores of the polyacrylamide gels due to an increase in the viscosity of the solutions.

To assure protein solubilization, 2D electrophoresis sample buffer usually contain reducing agents and high concentrations of chaotropes. The chaotropes urea and thiourea are often used at concentrations of 7 M and 2M, respectively. The former is quite efficient in disrupting hydrogen bonds, leading to protein unfolding and denaturation. Nevertheless, it has been shown that hydrophobic interactions are more efficiently broken by thiourea (Rabilloud, T. *et al.,* 1997). Due to its poor solubility in water, thiourea is used in concentrated urea solutions to increase its solubility. Reducing agents are necessary for cleavage of intra- and intermolecular disulfide bonds to achieve complete protein unfolding. The most commonly used reductants are DTT or dithioerythritol (DTE) are commonly used at concentrations ranging from 20 mM to 100 mM. A disadvantage of these agents is that in a pH between 8.5 and 9, they are ionized and behave as a weak acid; and therefore, run short in the alkaline gel area due to migration to the anode during IEF.

Last but not the least, many cell types, particularly the neutrophils, have a powerful arsenal of proteases stored in their organelles/granules. Hence, these proteases must be inactivated to prevent protein degradation that otherwise may result in artifactual spots and loss of high *M*r proteins. To prevent proteolysis of sample proteins, protease inhibitors are usually added to lysis buffers.

Two-dimensional PAGE delivers a map of intact proteins, which reflects

changes in protein expression level, isoforms or posttranslational modifications (PTMs). Depending on the gel size and pH gradient used, 2-DE can ***theoretically*** resolve more than 5,000 proteins simultaneously (~1,000 proteins routinely). Nonetheless, there is well-justified concern about the overall reproducibility of 2-DE when it is used as the sole method to identify qualitative and quantitative differences in protein expression in samples of interest (Horgan, G. 2007; Campostrini, N. *et al.,* 2005). In addition, 2DE-based approaches are biased against membrane proteins, low abundance proteins, and proteins at the extremes of isoelectric point and molecular mass (Pedersen, S. 2002; Rabilloud, T. 2003; Helling, S. *et al.,* 2006). For example, Lominadze *et al.,* found that the presence of large amounts of basic proteins in neutrophil granules produced poor isoelectric focalization. To overcome these limitations, several strategies have been employed in the past years to increase the number and certainty of the protein identifications. One example of such proteomics platform is the multidimensional protein identification technology [MUDPIT] (Washburn, M. *et al.,* 2001), which combines peptide separation by strong cation exchange and reverse phase two-dimensional chromatography, followed by identification by mass spectrometry.

Protein Visualization on 2D gels – Staining Procedures

Several detection methods are widely used for protein visualization in polyacrilamide gels that use labels that are fixed to the protein molecules. In general, visualization dyes and procedures can be divided into three major categories: (1) detection by light absorption of ***organinc dyes*** such as Coomassie Brilliant Blue; (2) detection by fluorescence of ***fluorescent probes*** such as Sypro dyes or CyDyes; and (3) ***metal ions*** permit protein visualization either by differential salt binding (e.g. reverse staining with zinc-imidazole) or by metal ion reduction such as silver stains (Simpson, R. 2003). The latter has gained wide popularity because of its excellent sensitivity (in the low nanogram range), flexibility (can be completed in a time range from 2 h to 1 d), and mass spectrometry compatibility. Silver staining was first introduced in 1979 by Switzer *et al.,*; however, issues related to background, dynamic range and reproducibility resulted in many improvements of the original protocol. By 1994, Rabilloud *et al.,* (Rabilloud, T. *et al.,* 1994) found more than 100 different silver staining protocols in the literature.

The principle of silver staining is straightforward. Silver ions bind to proteins and, under appropriate conditions, are reduced to elementary Ag developing a visible image typically in brownish-grey-black color (Rabilloud, T. *et al.,* 1994). Despite its wide use, silver staining is somewhat tricky. Unlike staining with organic dyes, silver staining goes against general thermodynamics. Proteins bind silver ions, and this binding decreases the reactivity of the ions, which leads to the formation of the commonly observed 'hollow' or 'doughnut' spots (Chevallet, M. 2006). Moreover, silver ions bind preferentially to basic amino acids of proteins; hence, it may cause a higher affinity of basic proteins for silver ions decreasing the staining linearity. Silver staining methods have been reported to be linear over a 40-fold range in protein concentration depending on certain physical-chemical properties of a given protein (Merril, C. *et al.,* 1984).

In general, depending on the silver impregnation, the silver staining methods can be divided into simple silver nitrate or silver-ammonia. The former is relatively simple and compatible with mass spectrometry; in addition, it presents good

consistency. Basic proteins are less efficiently stained than acidic ones with this type of stain (Rabilloud, T. 1999). Silver-ammonia stains are more sensitive than silver nitrate stains, in the range of 2-4 ng (Simpson, R. 2003). In contrast to simple silver stains, ammonical silver stains basic proteins more efficiently than acidic ones (Rabilloud, T. 1999).

Differential Gel Electrophoresis (DIGE)

Many of the disadvantages and shortcomings of 2D electrophoresis (2DE) have been overcome by 2D difference gel electrophoresis (***DIGE***) (Unlu, M. *et al.,* 1997). This technology uses three spectrally distinct fluorescent cyanine dyes (CyDyes - Cy2, Cy3, and Cy5) that match in charge and mass (Viswanathan, S. *et al.,* 2006). Hence, up to three samples can be differentially labeled and then run together on a single 2D polyacrylamide gel. The generated protein map of each sample labeled with different CyDye DIGE fluors can then be visualized separately by imaging (scanning) the gel at CyDye-specific excitation wavelengths. A CyDye fluor generates a digital image of the proteomic pattern in an individual sample that is then compared to the other samples (CyDye images) in the same gel by DeCyder differential analysis software (from Amersham).

Each CyDye DIGE fluor minimal dye has an NHS-ester reactive group, which covalently attaches to protein amino-termini and the epsilon amino group of lysine residues in proteins via an amide linkage (Marouga, R. *et al.,* 2005). The relatively high lysine content of most proteins makes this amino acid suitable for this labeling strategy, in which very small amounts of dye are used. In addition, it has been reported that only 3–5% of the total protein present in the sample is labeled. When bound to a lysine residue, the single positive charge on the CyDye DIGE fluor minimal dyes replaces the single positive charge present in lysine at neutral or acidic pH, thus ensuring that the pI of the protein is not significantly altered. In addition, the dyes are size-matched, adding approximately 500 Da to the labeled protein. Consequently, the same protein labeled with any of these dyes will migrate to the same position on a 2D gel. The CyDye fluor minimal dyes are highly sensitive – Cy2 = 0.075 ng, Cy3 = 0.025 ng, and Cy5 = 0.025 ng – than silver staining (~1 ng). In contrast to silver staining, CyDyes have a high dynamic range resulting in more reliable quantification of protein spots than that obtained using standard colorimetric staining methods.

Overall, the use of CyDye fluor dyes has the ability to substantially reduce the effects of gel-to-gel variation on the quantification of a protein spot on a gel, increasing therefore, the confidence that a difference in fluorescence intensity between two samples is due to biological rather than experimental variation (Marouga, R. *et al.,* 2005; Gharbi, S. *et al.,* 2002). This greater quantitative accuracy is enabled by the ability to multiplexing, and the possibility to use an internal standard. The latter consists in pooling equal amounts of each biological sample in the experiment, and labeling it with one of the CyDye DIGE fluor dyes, usually the Cy2 for minimal labeling. The internal standard is then run on every single gel along with each individual sample labeled. The advantages of using an internal pooled standard for 2D gel electrophoresis have been described in the literature (Alban, A. *et al.,* 2003; Friedman, D. *et al.,* 2004; Knowles, M. *et al.,* 2003). Most notably, every protein from all samples will be represented in the internal standard. Hence, the abundance of each protein spot on a gel can be measured relatively to its corresponding spot in the internal standard present on the same gel. In addition to accurate quantitation, this

strategy enables partition of experimental variation from inherent biological variation.

Gleaning Biological Relevancy through Differential Statistical Analysis

The aim of the differential analysis is to detect the proteins whose abundance differs according to the condition. The detection of differences in protein abundance in most cases relies on a statistical procedure that compares the differences of observed spot volumes. In statistical terms, this means to test simultaneously a large number of hypotheses – for each spot, we have to test the hypothesis that the spot volume does not differ according to the condition. In this context, the experimental design must properly estimate whether the variability in spot volume come from the biological and technical variation.

Statistical analysis to detect differential expression among 2D gels usually begin with filtering out interesting protein spots whose volume difference between two conditions is significant with a type I error cutoff predefined at $\alpha = 5\%$. The statistical testing procedure consists of choosing a *test statistic* and deciding if the hypothesis is rejected or not. In general, two approaches may be employed – ***Spot-by-spot*** and ***Global*** approaches. The former approach (also referred to as ***Univariate analysis***) is based on the data observed for the spot *'s'* only, whereas the latter approach (also known as ***Multivariate analysis***) is based on the results of an analysis of variance considering all the observations together. The spot by spot analysis is commonly available in several software packages used for 2D gel image analysis. Because univariate statistical analyses, such as the Student's t-test and the analysis of variance test (ANOVA), treat each individual variable independently, they cannot easily capture information about correlated trends. In addition, a large number of replications is necessary since this type of analysis uses information coming from only one spot. In the spot by spot analysis, we assume that the volumes of the spots within an experiment are distributed as Gaussian independent variables with mean and variance. To test the variance of given spot, the mean of such spot within a given condition should be compared to the mean in other conditions.

Non-parametric tests such as the Mann–Whitney (or Kolmogorov) test can be quite handy in gel analysis since it does not need to assume Gaussian distribution. The Mann–Whitney test consists in testing the degree of 'similarity' of the distributions function among the observations of spot *'s'* under a given condition. This test is based on the ranks of the observations rather than on the observations. As with the aforementioned Gaussian-dependent univariate tests, the Mann–Whitney test lacks power when the number of observations is small; thus, a minimum of seven replications by condition has been recommended (Meunier, B. *et al.,* 2005).

Alternatively to the use of univariate analyses for 2DE analysis, the use of multivariate statistics and pattern recognitions techniques consider a group of variables together rather than focusing on only one variable at a time; in other words, it uses information from all the data for testing each hypothesis. The Multivariate approaches include dimension reduction methods, such as principal components analysis (PCA), partial least squares (PLS), hierarchical cluster analysis, linear discriminate analysis, multivariate analysis of variance (MANOVA), and machine learning methods, such as self-organizing maps including neural nets, genetic programming, and support vector machines. Multivariate techniques in general cope better with " long and lean" data sets, where the number of experiments is very much

smaller than the number of variables (e.g., proteins). Such multivariate approaches are also robust to random variation and experimental error in the system, multi co-linearity (where variables may appear to be collinear as a result of the size of the matrix) and a degree of missing data. It is important to keep in mind that multivariate and machine learning methods can still have problems with long lean datasets, and thus any model produced must be tested by robust cross validation using a 'train and test' procedure.

The past decade has brought stunning achievements in genome technology, and consequently data analysis; most notably, the partnership among bioinformaticians, statistician biologists to develop tools for gene expression analysis by microarrays. Although the microarray scientific community has achieved great advances in the field of differential analysis of gene regulation, proteomics researchers still struggle to analyze 2D gels. Albeit feasible, the use of statistical tools made available by the microarray community is not straightforward for 2DE analysis. One reason is that the 2DE data present a greater variability due to the complexity of the image analysis (Dowsey, A. *et al.,* 2003). Moreover, the number of replication is generally small, while the number of missing data may be way too large, up to 50–60% (Roy, A. *et al.,* 2003).

Successful application of microarray data analysis methods in 2DE data comes from the use of false discovery rate (FDR) methods such as ***significance analysis of microarray*** (SAM) to correct the observed significance levels (Meunier, B. *et al.,* 2005). In another application, Kim Kultima and collaborators (Kultima, K. *et al.,* 2006) have adapted a microarray-based permutation test (Subramanian, A. *et al.,* 2005; Tian, L. *et al.,* 2005) to produce cut-off levels for finding differentially expressed proteins, namely DEPPS (Differential Expression in Predefined Protein *Sets)*.

Mass Spectrometry in Proteomics

During the past two decades, mass spectrometry (MS) has increasingly become the method of choice for analysis of complex protein samples, particularly for protein identification, peptide sequencing, identification and location of post-translational modifications of proteins (Aerbersold, R. and Mann, M. 2003). Mass spectrometry can provide information about specific protein structural modifications without *a priori* knowledge of the modification. The term MS encompasses a wide range of very different methods of analysis, each with its own unique characteristics. However, all mass spectrometers have certain features (components) in common: an ***ionization source***, a ***mass analyzer*** and a ***detector*** (Aerbersold, R. and Mann, M. 2003). The ionization source is the region of the instrument in which the sample of interest is ionized, with a positive or negative charge, and then desorbed into the gas phase. The mass analyzer is where the gas phase ions created in the source region are guided through the instrument to the detector, where their mass-to-charge (m/z) ratio is measured (Mann, M. *et al.,* 2001).

The primary function of a mass spectrometer is to separate charge-bearing molecules according to molecular mass and to measure their mass numbers. These measurements are carried out in the gas phase on ionized analytes rather than molecules because mass separation relies on the properties of charged particles moving under the influence of electric and magnetic fields, usually in high vacuum

where the mean free path is sufficient to ensure that they mostly travel without collisions. Ions may be positively or negatively charged, although most mass spectrometric experiments are performed on positive ions. Positive ions can be formed either by removal of one or more electrons from each molecule or by the addition of one or more cations (Gross, J. 2004). A charged species that is formed by the removal or attachment of an electron is referred to as a ***molecular ion***, whereas ionization that is achieved by addition or removal of a charged atom or molecule such as H^+ or NH^{4+} gives a species that is termed a ***quasi-molecular ion***. (Todd, J. 1991). Most often, proteins are ionized by addition of a H^+ and then analyzed by mass spectrometry.

As ions are actually analyzed in the vacuum of the mass spectrometer, arguably the most important reaction in MS is the one that converts analytes of interest into gas-phase ions. A fundamental problem in biological mass spectrometry was how to transfer highly polar, completely non-volatile molecules with a mass of tens of kDa into the gas phase without destroying them. This problem was solved by the advent of 'soft' ionization techniques such as ***matrix-assisted laser desorption/ionization*** (***MALDI***) (Karas, M. *et al.,* 1988) and ***electrospray ionization*** (***ESI***) (reviewed in Fenn, J. 2003). These techniques have indeed revolutionized biological mass spectrometry and now account for virtually all applications of MS for the analysis of peptides and proteins (Yates, J. 1998), DNA and RNA (Gross, J. *et al.,* 2000). Soft ionization methods transfer less vibrational energy and form ions having lower internal energies as compared to electron ionization.

MALDI is a solid-state sputtering/desorption method that produces ions by laser bombardment of crystals containing a small amount of analyte mixed with a large excess of ***ultraviolet-absorbing matrix***, which is normally a low-molecular-weight aromatic acid (Gross, J. 2004). On irradiation with a pulsed laser beam of the appropriate wavelength, the excess matrix molecules sublime and transfer the embedded non-volatile analyte molecules into the gas phase. Most commercially available MALDI instruments employ a nitrogen laser that gives 3-ns length pulses at a frequency of 337.1 nm (Mann, M. *et al.,* 2001). Whether the analyte is preionized in the solid state or becomes ionized in the plume, perhaps by excited state proton transfer, has not been yet established (Zenobi, R. and Knochenmuss, R. 1998).

Ions formed by MALDI have higher internal energies than those from ESI, and the desorption process helps free the analyte ions from undesirable impurities, just as it frees them from the matrix. When carried out in high vacuum and at high accelerating voltage, the pulsed nature of laser radiation produces ions in pulses that are well suited to ***Time-of-Flight (TOF) analysis*** (Baldwin, M. 2005). Compared with ESI, MALDI ionization appears to be relatively inefficient since ESI adds protons to all the basic sites in the analyte molecules, whereas the ions observed from MALDI are typically only singly charged. It is reasonable to conclude that either most analyte molecules remain uncharged or the ions become neutralized in MALDI and, therefore, do not contribute to the mass spectrum. However, in most applications, MALDI ions are formed in a high vacuum and with high kinetic energies, so they are transmitted through the mass spectrometer with high efficiency, whereas ESI ions are formed at atmospheric pressure and many are lost in the transfer to the vacuum system of the mass analyzer (Gross, J. 2004). In addition, MALDI is almost always used with TOF analysis, which maximizes the sensitivity of detection (Mann, M. *et al.,* 2001).

A major ***advantage*** of MALDI is its high level of sensitivity, often providing data from sub-femtomole (< 1 x 10^{-15} moles) amounts of sampling loading, can be observed in the MALDI-TOF setting (Baldwin, M. 2005). Another significant advantage of using MALDI for limited quantities of sample is the ability to study the sample for as long as a sufficient portion remains on a target. Another favorable practical attribute of MALDI lies in its relatively high tolerance to salts and buffers. Unlike ESI, ions can be formed by MALDI from samples that contain physiological levels of salts (Baldwin, M. 2005). Although MALDI has significant advantages, it also has some ***drawbacks***. The first caveat comes from the sample preparation strategies, which are quite heterogeneous. Thus, it is often necessary to identify a ‘‘sweet spot’’ among the crystals to obtain the best spectra. In addition, the presence of a matrix causes a large degree of chemical noise, which hurdles the analysis of samples with low molecular weights by MALDI (Karas, M. *et al.,* 1988).

MALDI-TOF Mass Spectrometers

Conceptually, the principle of linear time-of-flight (TOF) mass spectrometer is straightforward and probably the simplest compared to other mass analyzers. In TOF analysis, ions being pulsed into a TOF drift tube by an accelerating potential (for example, 1–20 kV) results in ion velocities that are inversely proportional to the square root of m/z (Gross, J. 2004). For a nitrogen laser, the pulses have a half-width of 3 ns, so all ions are formed within this short period. As all the ions with same charge obtain the same kinetic energy after acceleration, the lower *m/z* ions achieve higher velocities than the higher *m/z* ions. After the ions are accelerated, they travel through a fixed distance (typically 0.5–2.0 meters) before striking the detector. Typical flight times are tens of microseconds, so the length of the initial pulse is relatively insignificant. Thus, by measuring the time it takes to reach the detector after the ion is formed, the *m/z* of the ion can be determined. In practice, TOF offers mass resolution in the thousands and mass accuracies in the tens of parts per million (ppm) (Strupat, K. 2005).

A common issue frequently seen in the early MALDI-TOF instruments was the scattering and dissociation of many ions during acceleration, with consequent broadening of the mass spectral peaks, as these ions were accelerated as soon as they were desorbed by the laser pulse in the ion source. To overcome this problem, delayed ion extraction (DE) was introduced in the 1990s in order to compensate for the initial velocity distribution (Vestal, M. *et al.,* 2000) of MALDI-produced ions (Juhasz *et al.,*, 1997). In delayed extraction, a grid is placed in front of the MALDI target, and by applying a high potential to both the target and the grid, an initial desorption occurs in a field-free region. In general, the ions of interest are heavy and slow-moving, whereas most of the neutrals are relatively light molecules formed by thermolysis of the matrix and can diffuse away rapidly. After a delay period of perhaps 100 ns, the grid voltage is instantaneously reduced, causing the ions to be accelerated into the drift tube of the mass spectrometer, experiencing fewer collisions (Strupat, K. 2005).

In a linear TOF, ions of a single mass accelerated through the same potential should all have the same flight time and should arrive at the detector simultaneously. In practice, the kinetic energy imparted by the accelerating voltage is superimposed upon a range of energies arising from the laser desorption process (Strupat, K. 2005). To improve performance of TOF instruments, a series of electrostatic mirrors, namely

reflectron placed at the far end of the drift tube is commonly used (Mann, M. *et al.,* 2001). The reflectron provides a linear potential gradient from ground to just above the accelerating voltage, which slows the ions and then reverses their direction of flight, accelerating them back toward a detector situated at a slightly offset angle. The fastest ions penetrate further into the reflectron and travel a longer distance before reaching the detector than the slowest ions. Through a suitable choice of geometry, it is possible to have all ions of each unique mass arriving at the detector simultaneously, even though they have slightly different velocities. Therefore, the purpose of the reflectron is to compensate for small differences in the velocities of ions with the same *m/z*. These differences in velocity are caused by a number of different factors, but are inherent in the experiment. Therefore, the reflectron increases the resolution of TOF spectrometry. The reflectron is predominantly used to analyze smaller species such as peptides, for which increased resolution and more accurate mass measurements represent a substantial advantage (Clauser, K. *et al.,* 1999).

The state-of-the-art MALDI–TOF instruments are normally equipped with both a linear and a reflector port, and with the possibility for delayed ion extraction in the MALDI source. In particular, with the combination of the DE source, calibration and the reflectron, modern high-performance MALDI-TOF instruments easily attain an resolution power of 10,000 or more for peptides in the mass range up to 5000 Da (Vestal, M. *et al.,* 2005), and allows mass measurements to be made with an accuracy of 10 to 50 ppm (0.01 Da to 0.05 Da in 1000 Da) or better. Such a high mass accuracy dramatically increases the specificity of database interrogation, and identification of proteins can be achieved unambiguously if at least five peptide masses are determined with better than 50 ppm accuracy (Clauser, K. *et al.,* 1999, Jensen, O. *et al.,* 1996; Shevchenko, A. *et al.,* 1996). In general, MALDI-TOF is used for the analysis of simple peptide mixtures, such as the peptide obtained from an interest of single spot that separated on 2DE.

Mass Spectrometry Proteomics Strategies

There are two main approaches to characterizing proteins by mass spectrometry: ***bottom up*** and ***top down*** **(**Sze, S. *et al.,* 2002). In the former approach, proteins present in biological samples are enzymatically digested, peptide fragments are detected by MS, and information about the protein identity and modifications is assembled from the peptide data. Although peptides can be easily analyzed by MS, digested samples are quite complex making bottom-up approaches limited by this complexity. In practice, few peptides are detected per protein identification in bottom-up approaches; hence, a large portion of the protein sequence remains undetected. On the other hand, the top-down approaches to protein analysis by MS assay native proteins. Although the collision-induced dissociation (CID) techniques that readily give sequence information for peptide ions are generally not applicable to species as large as intact protein ions, the fragmentation mechanisms of electron-capture dissociation (ECD) and electron-transfer dissociation (ETD) make direct protein sequencing a realistic possibility (Horn, D. *et al.,* 2000). Here, the attachment of a low thermal energy electron to one of the positive charges in a multiply charged ion with a recombination energy of about 6 eV gives a radical ion that will rapidly fragment before energy equilibration occurs. Such species are classified as distonic ions because the charge and radical sites are distinct from each other. As in the

well-known radical cation reactions of species formed by electron impact, alpha-cleavage occurs not at the site of the charge but one bond removed, giving predominant c- and z-ions for cleavage between the amide nitrogen and the α-carbon. This differs from CID techniques normally used in bottom-down approaches that predominantly produce b- and y-type ions.

Data Mining in Proteomics

Bioinformatics tools play a fundamental role in proteomics studies enabling the analyses of complex sample and organization of the data in a useful biological context. In the past decade or so, several websites and software have been created to help the proteomics community to better interpret, automate and organize proteomics data. Two dimensional gel databases allow researchers to examine into any region of 2D gel and seek a desired pattern with the corresponding MW and p*I* scale. Some examples of 2D gel databases include the SWISS-2DPAGE, WORLD-2DPAGE (http://www.expasy.ch/ch2d/2d-index.html) and 2DWG Image Meta-database (http://www-lecb.ncifcrf.gov/2dwgDB).

Protein identification by mass spectrometry relies very much on bioinformatics. Firstly, experimentally collected MS data must be interrogated against proteins and/or genes databases in order to generate the most likely protein identity. There are three different approaches to identify proteins using mass spectrometry data. In the first method, ***peptide mass fingerprint (PMF)***, the molecular weights of the peptides resulting from digestion of a protein by a specific enzyme are used to interrogate the database for possible matches (Wilkins, M. *et al.,* 1998). In the second method, a ***sequence query*** is performed using MS data comprising of mass values combined with amino acid sequence. Last but not the least, MS/MS ion from one or more peptide is used to search the database and find matches (Clauser, K. *et al.,* 1999). Some of the protein databases and existing software for protein identification by PMF, sequence query and MS/MS ions are liste on table 2.

Neutrophil Proteomics – Lessons from a technical perspective

As proteomics remains a rapidly developing field with many different experimental approaches and different ways of searching and interpreting the data, a large variability in protein identifications across studies is not a surprising feature given the lack of experimental reproducibility and standards in the field. Additionally, the proteomic databases mainly contain information on the most abundant, technique-biased separable proteins, whereas low abundance, difficult-to-separate proteins such as regulatory signaling proteins, hydrophobic transmembrane receptors and basic nuclear proteins are usually under-represented in most proteomic studies. These limitations pose one of the main hurdles in proteomics, namely impractical inter-laboratory data comparison. Given the enormous proteomic complexity, it is likely that no individual technology would have the capability to tackle the complexity of the human proteome single handedly. Different analytical platforms may target different protein and peptide subsets and thus integration of results from a diversity of platforms is more likely to give a full picture (Hanash, S. and Celis, J. 2002; Omenn, G. *et al.,* 2005).

Table 2: Most commonly used software for protein identification by peptide mass fingerprint (PMF), sequence query and MS/MS ions

MS data	**Websites**
PMF	
Mascot	http://www.matrixscience.com/search_form_select.html
MS-FIT *prospector*	http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm
PeptideSearch	http://www.mann.embl-heidelberg.de
ProFound	http://bioinformatics.genomicsolutions.com/service/prowl/profound.html
PeptIdent	http://us.expasy.org/tools/peptident.html
ProteinLynx	http://www.waters.com
IonIQ	http://www.proteomesystems.com
Sequence query	
Mascot	http://www.matrixscience.com/search_form_select.html
MS-Seq *prospector*	http://prospector.ucsf.edu/ucsfhtml4.0/msseq.htm
Multident (TagIdent)	http://www.expasy.or/tools/multilident/
PeptideSearch	http://www.mann.embl-heidelberg.de
PepSea	http://www.unb.br/cbsp/paginiciais/pepseaseqtag.htm
MS/MS ions	
Mascot	http://www.matrixscience.com/search_form_select.html
MS-Tag *prospector*	http://prospector.ucsf.edu/ucsfhtml4.0/mstagfd.htm
Omssa	http://pubchem.ncbi.nlm.nih.gov/omssa/index.htm
Phenyx	http://www.phenyx-ms.com
X!Tandem	http://thegpm.org/TANDEM/index.html
Sequest	http://www.thermo.com
PepFrag (Prowl)	http://prowl.rockefeller.edu/PROWL/pepfragch.html
GutenTag	http://fields.scripps.edu/GutenTag/index.html

[1.6] Proteomics Meets Cell Biology

Proteomics can be applied in a hypothesis-free fashion to generate data used to elucidate protein function and regulation in the cellular context. The translation from genome to proteome is highly desirable to provide insights into the functional level of the ***cytome*** (Human Cytome Project, www.cytomics.info); that is, the study of molecular single cell phenotypes that results from the genotype and the environmental condition. The driving force for integrating cytomics into proteomics is the need to generate pure populations of cells from highly heterogeneous biological mixtures. In addition, acquiring additional layers of information from the cells being analyzed by proteomics can provide a better understanding of protein function pos-data analysis.

Neutrophil Proteomics – Lessons from a biological perspective

Reports on immune cell proteomics are dated as early as 1982 when Gemmell and Anderson (Gemmell, M. and Anderson, NL. 1982) separated the three major components of the leukocyte population (lymphocytes, monocytes, and granulocytes) and subjected them to 2-D gel electrophoresis. At the same time Willard *et al.,* (Willard, K. *et al.,* 1982) undertook one of the first attempts to find disease biomarkers in patients with rheumatoid arthritis, and carried out a 2-D PAGE analysis of leukocyte proteins. Proteomic analysis of neutrophils has been reported in human, bovine and rat. Kasper *et al.,* used 2-D PAGE to compare the proteomic profile of neutrophils from healthy donors and patients with severe chronic neutropenia. Using analysis tools available back then, the authors were able to identify by internal sequencing one protein that was overexpressed in disease, tropomyosin (Kasper, B. *et al.,* 1997).

By combining two-dimensional gel electrophoresis (2-DE) and mass spectrometry, Boussac and Garin identified a limited number of proteins secreted by neutrophils in a calcium-dependent manner (Boussac, M. and Garin, J. 2000). Piubelli *et al.,* took a similar 2-DE-MS approach to identify 52 major protein spots in rat neutrophils including multiple post-translational modifications of the same protein (Piubelli, C. *et al.,* 2002). The proteome of neutrophils was also analyzed following LPS challenge and compared with unstimulated neutrophils by 2-DE and proteins identified by MS (Fessler, M. *et al.,* 2002). This study yielded a list of up-regulated modulators of inflammation, signaling molecules, and cytoskeletal proteins. The same approach was later on used to profile proteomic changes in neutrophils of rats chronically treated with nicotine (Piubelli, C. *et al.,* 2005). A technically different approach, one-dimensional gel electrophoresis followed by LC-MS/MS, was employed by Lippolis and Reinhardt (2005) to identify over 250 proteins from bovine neutrophils.

Recently, two groups have applied a subcellular fractionation approach to identify novel granule proteins in neutrophils (Lominadze, G. *et al.,* 2005) and to better understand the dynamics of phagosome maturation in these cells (Burlak, C. *et al.,* 2006). The former analyzed subcellular fractions of neutrophils enriched for azurophil, specific and gelatinase granules, separated in a Percoll discontinuous gradient. Using two independent proteomic platforms (2D PAGE-MS and 2D HLPC ESI-MS/MS), these authors identified 286 proteins on the three granule subsets assigned to various functional gene categories, including kinases and phosphatases, transporters, structural proteins, signal transducers, host defense and enzymes.

Burlak and coworkers purified neutrophil phagosomes containing latex beads and identified more than 198 unique proteins (excluding protein isoforms and subunits translated from the same gene) associated with the phagosome by two-dimensional electrophoresis and mass spectrometry (Burlak, C. *et al.,* 2006). This study reported the identification of many proteins not previously known to be associated with phagosomes, as well as many novel proteins. In a similar approach, macrophage phagosomes containing latex beads were purified and more than 140 proteins associated with the phagosome were identified by similar proteomic techniques (Desjardins *et al.,* M. 2003).

Tackling the Proteome Complexity

One of the major hurdles associated with proteomic analysis of complex samples is the high ***dynamic range*** of proteins, which may be on the order of 10^6-10^{11} depending on the biological source (e.g., cells [Corthals, G. *et al.,* 2000], plasma [Anderson, N.L. and Anderson, N.G. 2002], bronchoalveolar lavage fluid [Wattiez, R. and Falmagne, P. 2005] and cerebrospinal fluid [Hu, Y. *et al.,* 2005]). These figures are at least three orders of magnitude beyond any proteomic analytical method (e.g., 2D gel, mass spectrometry, liquid chromatrography). The complexity becomes such that identification of low-abundance proteins are inevitably masked by those expressed in high abundance (Patterson, S.D. and Aebersold, R.H. 2003). Since most of the regulatory proteins such as kinases, GTPases and certain membrane receptors are present in low copy numbers, important layers of information are missing from studies of whole cell proteomics. Another important shortcoming in proteomic analysis is regarded to the size of the proteome. Despite a relatively modest number (~25,000) of putative protein-coding genes (Lander, E. *et al.,* 2001) and the total number of gene products present in any given cell to be estimated around 10,000, the effective number is likely to be several fold higher owing to splice variants and the wide variety of possible post-translational modifications (Huber, L. 2003).

The combination of large-scale proteomics studies with traditional cell-biology techniques, a strategy known as ***Subcellular Fractionation*** (or ***Organelle Proteomics, or Subcellular Proteomics***), has provided an interesting means to reduce complexity of cellular proteome and partially overcome the abovementioned resolution limitation of proteomic separation technologies (Wu, C. *et al.,* 2004; Blonder, J. *et al.,* 2002; Brunet, S. *et al.,* 2003; Yates, J. *et al.,* 2005). The underlying purpose of subcellular prefractionation is to isolate cellular compartments into distinguishable fractions to further analysis. After plasma membrane disruption, subcellular compartments can be fractionated using a variety of approaches including differential or gradient centrifugation, immunodepletion and free-flow electrophoresis methods.

The total protein content of cells or biofluids can also be simplified by fractionation methods based on affinity procedures to isolate groups of proteins displaying similar features (thiol-based isolation of cysteinyl proteins, hydrazide-based isolation of glycoproteins, metal chelating solid phases or specific antibodies to isolate phosphoproteins, etc.). This strategy is commonly known as ***Subproteomic Enrichment*** (or ***Subproteomic Fractionation***). Although information on protein localization is lost with this strategy, complexity can be greatly reduced at the protein or peptide level and number of identifications increased substantially including low-abundance proteins (Liu, T *et al.,* 2006). In addition to reducing sample complexity,

subproteomic enrichment at the posttranslational modified proteins can provide insight into signaling transduction in cells. In general, subcellular and subproteomic analyses are helping to unravel novel concepts in cell biology, leading to a better understanding of complex molecular processes in health and disease.

Subcellular Proteomics

Combining large-scale proteomics approaches with traditional cell-biology techniques is providing a strategy for mapping proteins in organelles (de Hoog, C. and Mann, M. 2004) and in biofluids (Liu, T. *et al.,* 2006, Andersen, N.L. *et al.,* 2002, Krapfenbauer, K. *et al.,*, 2003, Mootha, V *et al.,*, 2003, Nielsen, P. *et al.,*, 2005, Schirmer, E. *et al.,*, 2003 and Wu, C. *et al.,*, 2004b). Protein catalogs are now available for virtually all subcellular compartments (phagosome, nucleus, mitochondria, plasma membrane, etc) from a variety of cells (Wu, C. *et al.,* 2004b; Kikuchi, M. 2004; Desjardins, M. 2003; Garin, J. 2001). This reductionist approach has also provided a valuable insight into signaling transduction and protein trafficking during many cellular functioning processes.

Subcellular Fractionation Methods

Most subcellular fractionation procedures begin by disrupting cellular plasma membrane. In this case, one must achieve, reproducibly, the highest degree of cell "breakage" using the minimum of disruptive forces, but at the same time preserve the integrity of the other organelles. The most common strategy is to homogenize cells in the absence of detergents using a soft plasma membrane disruptive method. Currently, there is no gold standard methodology for such process. In addition, the cell membrane disruptive conditions for a given cell type should be pre-determined on a 'case-by-case' and 'trial-and-error' basis making reproducibility a common issue.

The use of osmotic pressure or detergent interaction to disrupt cell membranes has been efficiently employed for decades (Trautmann, M. *et al.,* 1992; Carboni, L. *et al.,* 2002; Henningsen, R. 2002; Rodgers, W. 2002; Han, D. *et al.,* 2001). Following cell disruption, subcellular compartments can be enriched by a variety of methods including differential centrifugation, centrifugation in sucrose or percoll gradient, lectin affinity chromatography, silica beads or biotinylation and interaction with immobilized streptavidin (Zhang, W. *et al.,* 2003). Centrifugation is arguably the most common method used in subcellular fractionation. It separates components on the basis of their size and density by using different time periods and velocities of centrifugation. The rate at which a given particle sediments in a centrifugal field will depend mainly on the particle's size (d). In addition, this rate is also determined by the difference in density between that of the particle and that of the liquid (p_p-p_l) (equation 1).

Equation 1

$\mathbf{v=d^2(p_p-p_l)g / 18\mu}$
where v=velocity of sedimentation; d=diameter of particle; p_p=density of particle; p_l =density of liquid; μ=viscosity of liquid; and g=gravitational force.

In ***differential centrifugation*** strategies, sequential (increasing gravitational force) steps of centrifugation are applied to cell lysate (after membrane disruption). In the past years, differential centrifugation technique has been largely surpassed by centrifugal fractionation in gradient media such as sucrose and ***Percoll***. The former, although widely used, may impart an important technical issue for subcellular fractionation. Due to its hypertonicity, sucrose causes subcellular organelles to shrink leading to alteration of their density and in lack of granule membrane integrity (Borregaard, N. *et al.,* 1983). In contrast, Percoll gradient can be made iso-osmotic by addition of buffer, thus avoiding shrinkage of organelles during fractionation (Borregaard, N. *et al.,* 1983). Another advantage of Percoll over sucrose gradients is that Percoll can be made as a self-generating density medium, resulting in a continuously increasing density towards the bottom of the tube. Alternatively, one can prepare discontinuous gradients to increase resolution in specific range(s) of density. A number of efficient Percoll density gradient protocols for neutrophil fractionation have been developed in the past two decades (Kjeldsen, L. *et al.,* 1994, Lominadze, G. *et al.,* 2005; Burlak, C. *et al.,* 2006)

Using Detergents to Fractionate Cellular Compartments

Cell lysis with detergent is arguably the most common method for membrane disruption. Like the components of biological membranes, detergents have hydrophobic-associating properties as a result of their nonpolar tail groups; nevertheless, detergents are themselves water-soluble. Given these traits, detergents are thought to work by solubilizing proteins from their lipid-surrounding environment. The success of the purification by detergent relies greatly on the choice of detergents and their concentrations. The detergent(s) choice for a particular experiment (cell type, downstream applications, overall goal, etc) is very much a 'trial-and-error' procedure. A given protein that is solubilized by a certain detergent may not be solubilized by a different detergent. This premise is the basis of a well-established detergent-based method for sequential protein extraction – ***Differential Detergent Fractionation*** (DDF). DDF has been used as a rapid, practical method of partitioning subcellular compartments (Lenk, R. *et al.,* 1977; Lenstra, J. and Bloemendal, H. 1983; Fey, E. *et al.,* 1984; Ramsby, M. and Makowski, G. 2003). DDF is based on the use of different detergents to sequentially extract cellular proteins while preserving the cell architecture intact. Often, differential protein extraction is carried out in PIPES buffers containing first digitonin, then Triton, and finally Tween/deoxycholate. This procedure yields four biochemically and electrophoretically distinct fractions (fig. 5): (1) cytosolic proteins and extractable cytoskeletal elements; (2) membrane and organelle proteins; (3) nuclear membrane proteins and extractable nuclear proteins; and (4) detergent-resistent cytoskeletal filaments and nuclear matrix proteins.

In the past years, DDF, and variations therof, has been used in a number of gel- and LC-based proteomic studies (Ramsby, M. *et al.,* 1994; Abdolzade-Bavil, A. *et al.,* 2004; Ramsby M. and Makowski G. 2003, 112, 53-66; Blonder, J. *et al.,* 2002; Lehner, I. *et al.,* 2003; McCarthy, F. *et al.,* 2005). Overall, DDF compares favorably with some of the methods for isolating membrane proteins (Blonder, J. *et al.,* 2002; Lehner, I. *et al.,* 2003; McCarthy, F. *et al.,* 2005). Although membrane proteins are expected to be found almost exclusively in the Triton X-100 fraction, a recent LC-based proteomic study (LC-ESI-MS/MS) showed that a number of membrane

proteins are also present in other DDF fractions; yet there is a higher proportion of membrane proteins in the Triton X-100 fraction (e.g., at least a 4-fold increase in membrane proteins of Triton X-100 fractions compared with the digitonin fractions).

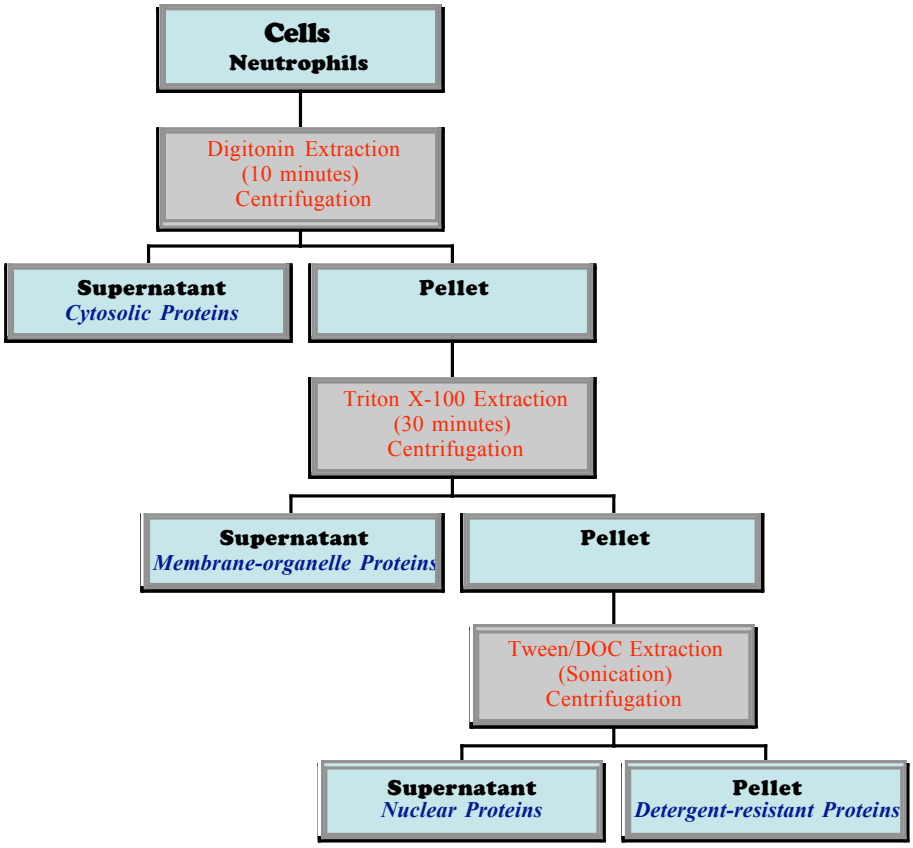


Fig. 5: Schematic for Differential Detergent Fractionation (DDF)

The first step in the DDF procedure makes use of the nonionic detergent ***Digitonin*** (fig. 6), which binds specifically and rapidly to sterols and precipitates them out, allowing the formation of pores in the membranes and release of soluble cytoplasmic proteins (Ramsby M. and Makowski G. 2003; Mackall, J. *et al.,* 1979). The use of digitonin at low concentrations (Fiskum, G. *et al.,* 1980; Weigel, P. *et al.,* 1983; Veitch, N. *et al.,* 2004) and the large differences in sterol contents of the plasma membrane and the cell organelles (Colbeau, A. *et al.,* 1971) and other organelles allow selective lysis of the sterol-rich plasma membrane while keeping more or less intact sterol-poor intracellular membranes such as endoplasmic reticulum, lysosomes (Schulz, I. 1990), and mitochondria (Bijleveld, C. and Geelen, M. J. 1987).

Another considerable advantage of digitonin extraction for proteomics is its capacity to preserve the cytoskeleton of the cell as observed in rat cells and in *Leishmania* (Fiskum, G. *et al.,* 1980; Foucher, A. *et al.,* 2006). This trait can be considered an advantage given the fact that a number of cytoskeleton proteins may be overrepresented in a variety of cells; hence the identification of the low-abundance proteins would be hindered (see the ***dynamic range*** issue in ***Tackling the Proteome Complexity***).

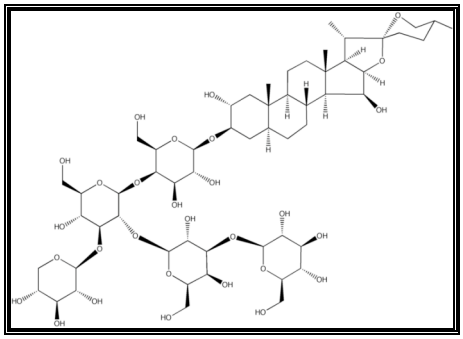


Fig. 6: Chemical structure of digitonin.

Neutrophil Cell Biology – Lessons from a technical perspective

Flow cytometry is one of the most versatile techniques in many clinical and research applications in the fields of immunology, biology, genetics, and, more recently, proteomics (Krutzik, P. *et al.,* 2004). Undoubtedly, flow cytometry has considerably improved our understanding of cell function by providing the possibility to reproducibly measure several parameters in individual cells in a short time using multiple fluorochromes. Although conventional techniques for measuring a given molecule such as spectophotometry, fluorimetry, Western blot, RT-PCR and ELISA have been vastly used to study cell signaling pathways, these methods usually fail to distinguish subsets in heterogeneous populations of cells. In contrast, flow cytometry has gained acceptance in the past years as a powerful multiparameter technique capable of measuring events at the single cell level as contrasted with average measurements of the abovementioned techniques.

Flow cytometry has provided many new insights into the functional status of neutrophils (Rothe, G *et al.,*, 1991; Vowells, S *et al.,*, 1995; Antal, P. *et al.,*, 1995; Santos, J, *et al.,*, 1995; Bassøe, C. *et al.,*, 2000), particularly interesting is the method for assessing oxidative burst (a measure of NAPH oxidase activity) based on detection of rhodamine 123 derived from dihydrorhodamine 123 (DHR 123) oxidation by some ROS including H_2O_2. This method involves the free uptake of DHR 123 by neutrophils. Upon entry in the cell, the nonfluorescent compound DHR 123 is oxidized in presence of H_2O_2 (and possibly other ROS) to its fluorescent derivative, rhodamine 123, in activated neutrophils (O'Gorman, M. and Corrochano, V. 1995). In a comparative study (Vowells, S. *et al.,* 1995) of three fluorescent probes (27-dichlorofluorescein diacetate, 5,6-carboxy-27-dichlorofluorescein diacetate, bis(acetoxymethyl) ester, and DHR), DHR was demonstrated to be the most sensitive probe to detect H_2O_2 production. In addition, flow cytometric assay for neutrophil respiratory burst with DHR was found to be more sensitive and reliable than conventional methods, such as chemiluminescence, cytochrome C reduction, or nitroblue tetrazolium reduction (Emmendorffer, A. *et al.,* 1994).

2. Objectives

The general objective of this Master Dissertation is to build a solid and robust platform to integrate basic proteomics research and clinical research. To this end, a series of sample processing optimization has been proposed to evaluate our capacity to generate high-level biological data from sampling. The main goals are as follows:

1. To implement an effective and low-cost neutrophil purification methodology in the Laboratory of Biochemistry and Protein Chemistry at University of Brasilia.
2. To set up a practical approach to evaluate the functional and morphological characteristics of the samples (neutrophils) being analyzed.
3. To determine the optimal conditions and strategies for proteomics studies of neutrophils. This goal was further divided into two 'specific aims':
    a. To standardize the optimal conditions for 2D gel electrophoresis of whole neutrophil proteome.
    b. To examine the feasibility of subcellular fractionation of neutrophils for proteomics analysis

3. Experimental Methods

[3.1] Blood Sample Collection

Peripheral heparinized blood samples were obtained from healthy adults, and processed immediately after sampling. Blood was collected either into Vacuntainer tubes (Becton Dickson, USA) coated with EDTA as anticoagulant agent, or heparin-coated syringes. The preferred anticoagulant, recommended by the International Council for Standardization in Haematology (ICSH), is K_2EDTA in a final concentration of 1.5–2.2mg/ml.

[3.2] Neutrophil Purification from Blood by Isopycnic Centrifugation in Percoll Discontinuous Gradient.

In this study, isolation of neutrophils from peripheral blood for all cellular and proteomic experiments was carried out by centrifugation on Percoll density gradients. Two different approaches, two- and four-layer Percoll discontinuous gradients, were assessed by flow cytometry for best purity, viability and yield of purified neutrophils. Detailed procedure for the two-layer separation is described bellow. To make a four-layer gradient, one should include the 63% and 72% solutions to the tube, reducing therefore the final volume of each solution to 2 mL.

Procedure

1. Prepare the 57% Percoll solution and the 67% Percoll solution (see table bellow). Each separation tube takes 4 ml of each solution
2. Pipette 4 ml of the 57% solution into a 15-ml Falcon tube.
3. Underlay 4 ml of the 67% solution slowly using a bore-end needle.
4. Collect blood into Vacuntainer tubes and then mix the blood with PBS 1x in a 2:1 ratio.
5. Overlay 4 ml of blood-PBS mixture on top of the gradient.
6. Place the tubes with the same weight in a swing-out rotor and centrifuge for 30 min at 400x*g*.
7. After centrifugation, discard the top layers and collect the neutrophils in the 57%-67% interface.
8. Wash the cells twice with PBS 1x (5 volumes to 1 volume of cell suspension) and pellet the cells for 10 min at 1500 rpm.
9. After the second centrifugation, decant the PBS leaving approximately 400 μL of the buffer in the tube. Then, resuspend the pelleted cells.
10. Lyse the remaining red blood cells by adding 9 mL of MilliQ H_2O and homogenizing the solution gently for 20-25 sec. Then, reconstitute the osmolarity of the medium by adding 1 mL of PBS 10x.
11. Centrifuge the tube for 10 min at 1500 rpm and remove the supernatant.
12. Wash the cells with 5 mL of PBS 1x and centrifuge for 10 minutes at 1500 rpm. Repeat this step one more time.
13. Resuspend neutrophils at the desired concentration with the desired buffer.

Percoll Solutions

67% Percoll	4 mL (1tube)
Percoll	**2.68 mL**
PBS 10x	**0.27 mL**
PBS 1x	**1.05 mL**

57% Percoll	4 mL (1tube)
Percoll	**2.28 mL**
PBS 10x	**0.23 mL**
PBS 1x	**1.49 mL**

Critical. One should take into account the effect of having, in the percoll solution, solid silica particles that obviously occupy a certain volume. The electrolytes in the stock solution have a higher effective concentration than in physiological salt solution, and the percoll solution made in this way will be hyperosmolal (Vincent and Nadeau 1984). Adding 9 parts (v/v) of Percoll to 1 part (v/v) of 10x concentrated phosphate buffered saline (PBS from Sigma) gives a stock hypertonic (354 mOsm) Percoll solution. To obtain a stock solution of ~ 320 mOsm, a quick procedure is to add 1 part of 10X concentrated PBS to 10 parts of Percoll.

[3.3] Neutrophil Priming and Activation

All reagents for neutrophil priming and activation were obtained from Sigma Aldrich (USA) unless otherwise stated. Stock solutions of ***tumor necrosis factor α*** (TNFα), ***lipopolysacharide*** (LPS), ***phorbol 12-myristate 13-acetate*** (PMA), ***platelet-activating factor*** (PAF), ***N-formylmethionine-leucyl-phenylalanine*** (fMLF) were prepared and stored at -20^{o}C. ***Dihydrorhodamine 123*** (Molecular Probes, Eugene, OR, USA) stock solution was also prepared in dimethyl sulphoxide, and stored at -20^{o}C protected from light.

Immediatelly after purification, neutrophils were washed with ice-cold phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, and then separated into seven aliquots of approximately $3x10^{6}$. The cell suspension were pellet again by centrifugation and each aliquot were resuspended with its respective stimulation solution (PBS with Ca^{2+} and Mg^{2+}) containing either one of the following agents - ***TNFα*** at 10 ng/ml, ***LPS*** 10 ng/ml, ***PAF*** 20 ng/ml, ***fMLP*** 100 mM, or ***PMA*** at 100 ng/ml. The remaining two aliquots were resuspended as follows: the ***control sample*** was resuspended in PBS with Ca^{2+} and Mg^{2+} only, and the 'priming sample' was resuspend firstly in PAF solution (as above) and after 5 minutes fMLP was added to a final concetration of 10 ng/ml. Neutrophil priming and activation were induced by incubating each aliquot for 30 minutes. Twenty-five minutes following incubation, a

small sub-aliquot (~$3x10^5$) was taken from each of the 7 aliquots for flow cytometry analysis, and dihydrorhodamine 123 (Molecular Probes, Eugene, OR, USA) was added to each of these subaliquots to a final concentration of 15μM. After 40 min of initiating the resuspension in the agent solution, each aliquot was analysed by flow cytometry as detailed bellow.

[3.4] Flow Cytometry Acquisition and Analysis

All flow cytometry data in this study were collected on a FACSCalibur or FACSAria (Becton Dickinson, San Jose, CA), and analyzed with CellQuest software. The instrument was calibrated according to the manufacturer's instructions. Before acquiring data the instrument was set up using the control sample. Herein, forward and side scatter signals were collected on a linear scale, and adjusted to ensure that the neutrophil population was clearly displayed. Fluorescence was detected on its respective channel using a logarithmic scale, and the photomultiplier gain was adjusted so that the fluorescence of the control sample (no activation) was confined to the first decade of the channel histogram display. Data were then acquired from all resting and stimulated tubes. Sample acquisition was performed at a low–medium rate (150 events/s), was stopped at 10,000-15,000 total events.

The dot plot of forward scatter vs. side scatter was displayed, and neutrophil population was gated for further analysis. The gated region was then plotted to display rhodamine fluorescence FL1 vs. Forward Scatter or PI fluorescence FL3 vs. Forward Scatter. The percentage of activated cells (rhodamine-positive cells) was determined based on the number of ROS-producing cells present above the baseline (region) determined by control sample, whereas percentage of PI-positive cells were considered as the cells above the 'zero' baseline. The level of activation was measured in arbitrary units of the geometric mean fluorescence intensity (GMFI) in FL-1.

[3.4] Cytosolic and Membrane/Organelle Proteins Fractionation

The use of the nonionic detergent digitonin at low concentrations has been reported as highly effective and selective for cytosolic proteins extraction (see ***Using Detergents to Fractionate Cellular Compartments*** in section ***[1.6]***). In this study, digitonin extraction was carried out immediately after neutrophil separation. The procedure was performed at 4°C unless otherwise stated. Approximately $3x10^6$ neutrophils were washed twice in phosphate-buffered saline solution pH 7.4 by centrifugation at 1500 × *g* for 5 min. Digitonin fractions were obtained by resuspending the cells in 5 volumes of freshly prepared ***Digitonin extraction buffer*** (0.015% w/v digitonin, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 10 mM PIPES pH 7.4) containing inhibitors of proteases (1 mM PMSF, 1 μM pepstatin A and 5 mM EDTA) and phosphatases (2 mM of sodium orthovanadate, 10 mM β-glycerophosphate, 10 mM sodium fluoride and 5 mM sodium pyrophosphate). The solution was then incubated for 10 min at 4°C with gently shaking. The soluble fraction (cytosolic proteins) were then recovered by centrifugation 2000 × *g* for 5 min at 4°C. This procedure was repeated three more times, and each collected fraction was labeled 1 through 4. The insoluble fraction (centrifuged pellet after the fourth digitonin extraction) was resuspended in 5 volumes of freshly prepared ***Triton extraction buffer*** (0.5% v/v

triton x-100, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 10 mM PIPES pH 7.4) in the presence of the same set of protease and phosphatase inhibitors described for digitonin extraction buffer. After 30 min of incubation with shaking at 4°C, the suspension was centrifuged (5,000 × *g*, 4°C, 5 min), and the supernatant was recovered and saved as the membrane/organelles fraction.

[3.5] Preparation of Cytosolic Protein Samples for 2DE.

The extracted cytosolic proteins were precipitated overnight with 3 volumes of ice-cold acetone and, after centrifugation at 10,000 rpm for 30 minutes, the pellet were resolubilized in IEF buffer (7M urea, 2 M thiourea, 2% CHAPS, 40 mM DTT, and 0.5% IPG buffer 3-10NL). The protein concentrations on each tube were determined using the 2D Quant Kit (Amersham) as recommended by the manufacturer.

[3.6] Preparation of Cytosolic and Membrane/Organelle Protein Samples for 1DE.

The extracted cytosolic and membrane/organelle proteins were also precipitated overnight with 3 volumes of ice-cold acetone and, after centrifugation at 10,000 rpm for 30 minutes, the pellet were resolubilized in SDS-PAGE sample buffer (Tris-HCl pH, glycerol, SDS, DTT and bromophenol blue). The protein concentrations on each tube were determined using the 2D Quant Kit (Amersham) as recommended by the manufacturer.

[3.7] Preparation of Total Protein Samples for 4-7 gels.

Immediately after purification by Percoll, neutrophils were briefly washed twice with cold PBS to remove debris from the red blood cell lysis process. Two aliquots containing approximately the same number of neutrophils (~$3x10^6$) were treated respectively with TNFα or PBS. The treated neutrophils were then resuspended in 2D lysis buffer containing 7 M urea, 2 M thiourea, 2% Triton X-100, 0.5% IPG buffer 4-7, and protease inhibitor cocktail, and RNase mix. After 20 minutes, EDTA and DTT were added to a final concentration of 5 mM and 40mM, respectively. Lysis was carried out for 1 h at room temperature (~22°C) in a platform mixer with periodic vortexing. The protein concentrations were assayed by Bradford method (Bradford, M. 1976).

[3.8] Preparation of Cytosolic and Membrane/Organelles Protein Fractions for 1DE.

Proteins from the cytosolic (10 and 50 μg) and the membrane/organelle fractions (10 μg) in SDS-PAGE sample buffer were heated at 95°C for 5 minutes and then separated by one dimensional SDS-PAGE. The gel was assembled using the Ettan II system from Amersham Pharmacia (size 20x25 cm^2). The gel was buffered with 375 mM Tris (pH 8.8), and a 4% T, 3.6% C stacking gel (~5 mm tall) were cast on top of 12% T, 3.6% C separating gel. Electrophoresis was carried out at 20°C using tris-glycine-SDS electrode buffer with constant voltage (200V) and amperage around

18mA. After electrophoresis, the gel was stained with Colloidal Coomassie Brilliant Blue.

[3.9] Isoelectric Focusing.

One hundred μg of cytosolic-fractionated protein was made up to a final volume of 450 μL in a 7 M urea, 2 M thiourea, DTT, 0.5% IPG buffer, 2% CHAPS sample buffer. The same protein extract was used to rehydrate the 24 cm IPG strips (3-10NL) for 12 h at 20°C and constant voltage of 30 V. Isoelectric focusing (IEF) was carried out at 200 V for 200 Vh, 500 V for 500 Vh, 1000 V for 1000 Vh and 8000 V for 40,000 Vh at 20°C and a maximum current setting 50 μA/ strip in an IPGphor isoelectric focusing unit (Amersham Pharmacia).

[3.10] Equilibration and 2nd Dimension PAGE.

Following isoelectric focusing, IPG strips were equilibrated (reduced and alkylated) to allow the separated proteins to fully interact with SDS. Firstly, proteins were reduced by immersing IPG strips for 15 minutes in DTT equilibration buffer (50 mM Tris-HCl (pH 8.8), containing 2% w/v SDS, 1% w/v DTT, 6 M urea and 30% w/v glycerol). Urea and glycerol are used to reduce electroendosmotic effects improving protein transfer from the first to the second dimension. Following reduction, IPG strips were incubated for 15 minutes in IAA equilibration buffer (50 mM Tris-HCl (pH 8.8), containing 2% w/v SDS, 4% (w/v) iodoacetamide, 6 M urea and 30% w/v glycerol). Iodoacetamide alkylates sulfhydryl groups and prevents their reoxidation; in addition, it alkylates any free DTT, as otherwise it migrates through the second-dimension SDS-PAGE gel, resulting in an artifact.

Second-dimension of cytosolic protein extracts – The equilibrated IPG strip was laid atop of a 1-mm thick polyacrylamide sodium dodecyl sulfate gel (PAGE-SDS), and separation was carried out using the Ettan DALT*twelve* system from Amersham (gel size 20x25 cm^2). A 0.5% low melting agarose overlay, containing 0.1% SDS and 375 mM Tris (pH 8.8), was warmed to its melting point and used to seal the IPG to the surface of the stacking gel. The gels were buffered with 375 mM Tris (pH 8.8), and 5% T, 3.6% C stacking gels (~5 mm tall) were cast on top of 12.5% T, 3.6% C separating gels. Electrophoresis was carried out at 20°C with constant voltage (200V); to rapidly drive proteins out of the IPG and through the stacking gel, 5 mA per gel was applied, after which the amperage was increased to 20mA for completion of the separation.

Second-dimension of total protein extracts – The equilibrated IPG strip was laid atop of a 1-mm thick polyacrylamide sodium dodecyl sulfate gel (PAGE-SDS), and separation was carried out using the Protean II XL Cell system from Biorad (gel size 18x20 cm^2). A 0.5% low melting agarose overlay, containing 0.1% SDS and 375 mM Tris (pH 8.8), was warmed to its melting point and used to seal the IPG to the surface of the stacking gel. The gels were buffered with 375 mM Tris (pH 8.8), and 5% T, 3.6% C stacking gels (~5 mm tall) were cast on top of 12.5% T, 3.6% C separating gels. Electrophoresis was carried out at 20°C with constant voltage (200V); to rapidly drive proteins out of the IPG and through the stacking gel, 10 mA per gel was applied, after which the amperage was increased to 20mA for completion of the separation.

[3.11] Gel Staining – 1D and 2D gels.

Colloidal Coomassie Staining of 1D gel - This protocol is a modification of the method of Neuhoff *et al.,* (1988), and uses Coomassie Blue G250 that complexes with basic amino acids, such as arginine, tyrosine, lysine, and histidine, producing a low background and a detection range of 8 – 50 ng. After electrophoresis, the gel was fixed for 1h with 10% acetic acid, 40% methanol. Following three wash steps of 10 minutes each, the gel was stained overnight with Staining solution (8% ammonium sulfate, 0.5 % phosphoric acid (at 85%), 16% of Coomassie Blue G250 stock solution (at 5%). To remove the residual Coomassie after overnight staining, the gel was washed 5 times in MilliQ water at 45-50°C. Finally, the gel was stored with 5% glycerol solution until band excision.

Ammoniacal Silver Staining for 2D gels - After electrophoresis, the slab gels were fixed for 1h to overnight with 12% trichoroacetic acid, and 50% methanol. A second fixation step was carried out for 1h in 50% ethanol, 5% acetic acid. The gels were then washed twice in MilliQ water, and stained in ammoniacal silver solution (20mM sodium hydroxide, 0.3% ammonium hydroxide at 25-28%, and 0.89% silver nitrate). Following 20 min of staining, the gels were washed twice in MilliQ water for 5 min to remove the residual silver ions from staining step. Staining development was carried out by immersing the gels in developing solution (0.0175% citric acid and 0.236 formaldehyde), and after approximately 90 sec, the reaction was stoped in 30% ethanol, 7% acetic acid and 2.5% glycerol. Finally, the gels were stored in 5% glycerol solution until imaging and excision.

[3.12] Computer-assisted 2-D gel analysis

Stained gels were imaged on an ImageScanner (Amersham Pharmacia) using the LabScan software (v.3) with 300 DPI of resolution, 16 bits per pixel and in a transmissive light path mode. Gel images were imported as TIFF files into the ImageMaster software (v.5) for automated spot detection under the following conditions: smooth 3 or 4, minimal area 65 to 80, and saliency 1,200 – 1,300. Following detection, the gels were imported into the ImageMaster Platinum version 6 for further analysis. Firstly, 10 user-defined spot landmarks were picked in each set of image to improve automatic matching. The landmarks were selected taking into consideration whether the spot appeared clearly in other gels. Then gels were matched with a reference gel. In the case of the three cytosolic protein gels, the reference gel was choosen the gel_2, while the TNF-activated gels were matched against the normal gel that runs together with the TNF-activated gels in the same 2DE experiment; that is, the same IEF and the same SDS-PAGE runs.

Data analysis was performed using statistical functions from Microsoft Excel software, the ImageMaster Platinum analysis software version 6. Firstly, spot volumes from every match across all the gels were exported to Excel tables. The spot volumes were then normalized by dividing each spot volume '*s'* by the average volume for all spots *'a'* in each gel *'g'*. Alternatively, the spot %volume retrieved from the image analysis software report for each gel was used as the normalized value. The normalized volumes were then used to extract the ratio between two gels. In the case

of TNF vs. normal expression analysis, this ratio was then transformed to Logarithm base 2. The Pearson correlation coefficients (***r***) and standard deviations were also calculated using the following formulas:

Pearson coefficient (r)

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

where *x* and *y* are the sample means AVERAGE (gel_1) and and AVERAGE(gel_2)

Standard Deviation

$$\sqrt{\frac{\sum (x - \bar{x})^2}{(n-1)}}$$

where *x* is the sample mean AVERAGE (%vol1, %vol2,…) and '*n*' is the sample size.

[3.13] In-gel Digestion of Proteins

The in-gel digestion protocol described here was modified from Shevchenko *et al.,* (Shevchenko, A. *et al.,* 1996). Briefly, protein spots of interest were excised from the 2D gel with a cutted pipette tip and washed once with acetonitrile (ACN). Then, proteins were reduced and alkylated, respectively, by treatment for 30 min with 10 mM DTT and 55 mM IAA. After washing with ACN, proteins were digested overnight at 37ºC with 0.27 nM trypsin. Tryptic peptides were extracted from the gel matrix with extraction buffer (1:2 v/v 5% formic acid/ACN) for 15 minutes at 37°C; the extracts were finally dried in a vacuum centrifuge.

[3.14] Protein Identification by Mass Spectrometry

The digests were redissolved in 10 μL 0.1% TFA. Each digest sample was desalted on a homemade POROS R3 microcolumn, which was then washed with 20 μL 0.1% TFA. The peptides were eluted with 1μL of matrix solution (2 mg/mL α-ciano-4-hydroxycinnamic acid (CHCA) (Aldrich) directly onto the MALDI target plate. Tryptic digested peptide samples were analyzed by MALDI-TOF/TOF MS (4700 Proteomics Analyzer, Applied Biosystems). MS spectra were acquired in positive reflector mode (voltage of 20 kV in the source1 and laser intensity ranged from 5200 – 5800). Typically, 500 shots per spectrum were accumulated, and three major peaks were selected for further characterization by MS/MS analysis. MSMS spectra were acquired using CID with atmosferic air as the collision gas. A MS-MS 1kV positive mode was used. MS and MSMS spectra from the same spot were merged in a single mgf file prior to submission for database searching.

The resulting peptide mass maps and the associated fragmentation spectra were collectively used to interrogate sequences present in the Swiss-Prot and National Center for Biotechnology Information non-redundant (NCBInr) databases to generate

statistically significant candidate identifications using GPS Explorer software (Applied Biosystems) running the MASCOT search algorithm (Matrix Science). Searches were performed with complete carbamidomethylation of cysteine, with partial oxidation of methionine residues, and with one missed cleavage also allowed in the search parameters. Mass tolerance was set at 100 ppm for the masses of peptide precursors and at 0.25 Da for the masses of fragment ions.

Significant Molecular Weight Search (MOWSE) scores ($p < 0.05$), number of matched ions, number of matching ions with independent MS/MS matches, percent protein sequence coverage, and correlation of gel region with predicted molecular weight and pI were collectively considered for each protein identification (Table 6).

[3.15] Confocal Laser Scanning Microscopy

Neutrophils were stained with rhodamine 123 and propidium iodide at final concentration of 15μM and 60μM, respectively. Rhodamine 123 was obtained from the oxidation of dihydrorhodamine 123. Cells were washed three times with PBS and mounted onto a coverslip slide, which was then analyzed with a Zeiss LSM510 confocal laser-scanning microscope coupled to an Axiovert 200M inverted microscope (Carl Zeiss, Inc., Thornwood, NY). Images were acquired using a 100 Plan-Apochromat oil immersion objective (1.4 numerical aperture) and a two-channel setup.

4. Results and Discussion

[4.1] Cell Separation by Gradient Centrifugation

In the past decades, various gradient media have been developed for specific applications (e.g. Percoll [see bellow], sucrose, Ficoll [a synthetic polymer of sucrose], dextran, CsCl, K-tartrate and NaBr). Iodinated compounds developed for use as X-ray contrast media are also widely used as centrifugation media, e.g. Hypaque®, Urografin, Metrizoate, Isopaque, Metrizamide, Nycodenz®, Optiprep®, etc (Graham, J. 2003). Discontinuous gradients of Percoll are vastly used to isopycnically separate mononuclear cells, polymorphonuclear cells and erythrocytes. In general, Percoll performs better for isopycnic separations as compared to rate zonal separations. Several conditions were optimized in this study, including blood withdrawn, separation technique, activation conditions, and concentration of the chemicals used in the experiment.

Percoll purification in a two-layer gradient of Percoll gives a highly pure population of neutrophils without affecting its original resting state.

Neutrophils could be clearly separated from the whole blood by a two-layer gradient of Percoll at 57% and 67%. The degree of neutrophil purification using this approach ranges between 96% and 99% as measured by light scatter in flow cytometry in over 30 experiments performed between November/ 2005 and May/ 2006. Figure 7 shows the flow cytometric pattern of one such purification process, in which neutrophils were purified at a 99% level.

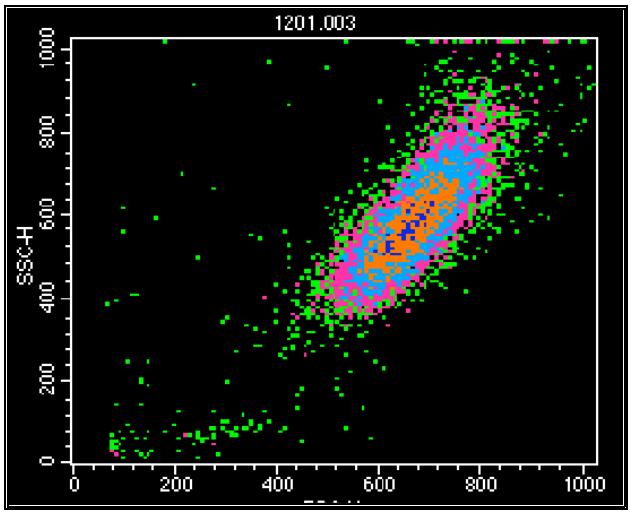


Fig. 7: Example of the flow cytometry analysis of a two-layer gradient Percoll purification of neutrophils. Approximately 99% of the cells appear on the region of the density dot plot assumed to contain polymorphonuclears.

To test whether neutrophil purification in Percoll gradient affects the original state of these cells, the degree of oxidative burst of neutrophils and the integrity of plasma membrane were assessed by flow cytometry assays using DHR and PI, respectively, as probes. To assess the oxidative burst of the purified neutrophils,

phorbol-12-myristate-13-acetate (PMA), an inducer of oxidative burst through activation of protein kinase C, was used as a positive control for the assay. Figure 8 shows that while non-activated purified neutrophils maintain a basal level of ROS production, PMA-activated purified neutrophils present a significant higher production of ROS. The ROS production level was also assessed in whole blood neutrophils and compared with Percoll purified neutrophils (data not shown). Purified neutrophils are extremely fragile cells, highly susceptible to membrane disruption. Purified neutrophil membrane integrity, an indirect measurement of cell viability, was assessed by the capacity to remain impermeable to propidium iodide. Overall, purification of neutrophils in an iso-osmotic Percoll medium maintains the integrity of cell membranes (viability ~98%). Importantly, neutrophil viability decreased with time, 2 h after purification, approximately 23% of the cells had undergone lysis (data not shown).

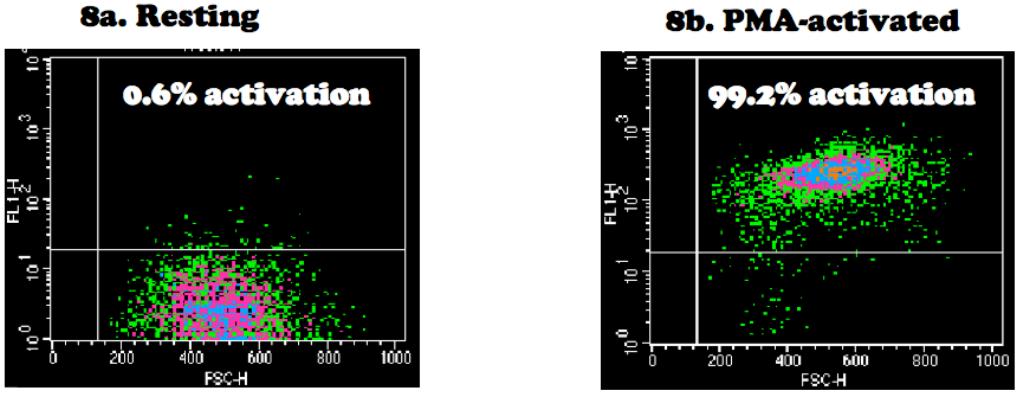


Fig. 8: (a) Rhodamine fluorescence as a measurement of the ***Oxidative Burst*** in Percoll-purified neutrophils before (a) and after (b) PMA activation.

The final step of almost any neutrophil purification technique based on centrifugation gradient is the lysis of remaining erythrocytes. Several techniques have been described in the literature including lysis with formaldehyde-containing reagents (such as Optilyse B from Immunotech, and FACS Lyse from BD bioscience), hypotonic shock lysis with H_2O or lysis with ammonium chloride (NH_4Cl) solutions (Vuorte, J. 2001). In this study, hypotonic lysis with H_2O for ~20 seconds followed by osmolality reconstitution with 10x phosphate-buffered saline (or 10x NaCl) was found to perform better than lysis with ammonium chloride-containing solution in terms of not inducing oxidative burst of the neutrophils (figure 9a). In addition, the use of FACS*Lyse* solution must be avoided since this reagent caused a strong activation of a subpopulation of neutrophils (figure 9b). Other methods or solutions for erythrocyte lysis were not tested in this study.

Gradient centrifugation in a defined medium is arguably the most common method for cell separation (as well as separation of subcellular compartments). Complete separation of components in a mixture can be achieved according to size (rate zonal centrifugation) or density (isopycnic centrifugation). In the former type, the size difference between particles affects the separation along with the density of the

particles. Large particles move faster through the gradient than small particles, and the density range is chosen so that the density of the particles is greater than the density of the medium at all points during the separation. The run is terminated before the separated zones reach the bottom of the tube (or their equilibrium positions). In isopycnic centrifugation, the density range of the gradient medium encompasses all densities of the sample particles. Here, each particle sediments to its equilibrium position in the gradient where the gradient density is equal to the density of the particle (isopycnic position). Thus, in this type of separation, the particles are separated solely on the basis of differences in density, irrespective of size. Separation of cells from biofluids for proteomic analysis requires highly purified preparations. In addition, the isolated cells must be unimpaired regarding viability and biological function after the separation process. It is important that the separation and purification procedure is as harmless as possible and that no interfering substances are introduced during the process.

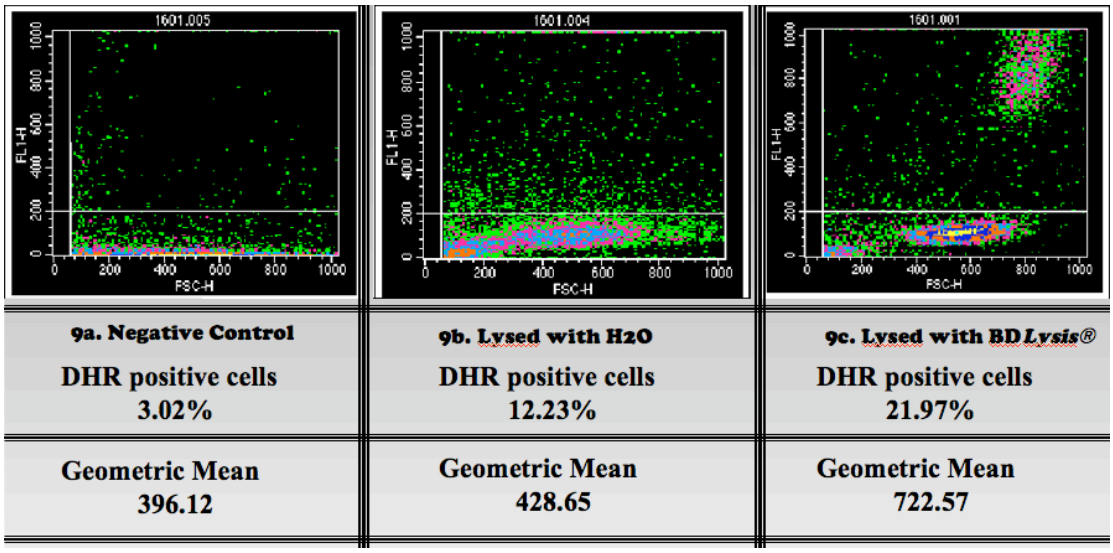


Fig. 9: Comparison of different methods for erythrocytes lysis. Hypotonic lysis with (b) water did not affect the activation state of the neutrophils as measured by rhodamine fluorescence, whereas lysis with (c) BD FACS*Lyse* solution strongly activates a subpopulation of neutrophils. Dot plot (a) represents the negative control (without DHR 123). Of note, the FL1-H parameter (y-axis) was set to a linear scale instead of logarithm for "better visualization" of the activated population.

[4.2] Assessing Neutrophil Oxidative Burst in Primed and Activated Neutrophils by Flow Cytometry

The ability of neutrophil to kill pathogenic microorganisms depends on its microbicidal arsenal of proteases and reactive oxygen species (ROS). The latter plays roles in a myriad of physiological and pathophysiological events (see ***Introduction***). Our understanding of how neutrophils progress from a circulating naïve cell to a fully activated microbicidal cell has greatly evolved during the past decade. Some immunologists have reasoned that neutrophil is not activated in an all-or-none fashion; instead the concept of a two-stage process - ***priming and activation*** - has been applied to explain the activation process of neutrophils. Swain and colleagues

(2002) have conceptualized neutrophil priming as follow: "Priming is when the neutrophil's functional response (e.g., ROS production, chemotaxis) to a stimulus is amplified by previous exposure of the cell to a priming agent. The priming agent itself, however, does not normally cause a noticeable functional response, except possibly at very high concentrations of the priming agent."

Pro-inflamatory agents, in particular fMLP, evoke the generation of ROS in neutrophils. Nevertheless, the effects of priming with TNFα or PAF were not significantly different from the direct activation with a single agent.

To better understand the role of certain inflammatory mediators on neutrophil oxidative burst, neutrophils were activated with either LPS, fMLP, TNF, PAF, PMA, TNF followed by LPS, or PAF followed by fMLP. Then, after incubation with DHR 123, rhodamine fluorescence was read on the FL1 channel of a flow cytometry as a measurement of the oxidative burst; that is, the generation of ROS. The level of activation for each agent is measured by 'geometric mean fluorescence intensity' (GMFI) and the percent of activated cells is calculated from a baseline (threshold) established from control measurements (figure 10). All agents showed a similar capacity to induce oxidative burst; except PMA and fMLP, which presented a significantly higher level of oxidative burst reaching its plateau within the first 15 minutes (data not shown). Interestingly, the priming/activation with PAF and fMLP, respectively, did not induce a high degree of activation comparable to or higher than PMA as has been reported by other authors (Botha, A. *et al.,* 1995).

Neutrophil oxidative burst was also profiled in a time-series fashion; that is, after neutrophil incubation with a given agent, flow cytometry measurements were taken at different time points. In general, after incubation with the activating agent, a small degree of neutrophil activation was observed within the first 5 min, after which the activation level increased steadily within the 60 min to then reach a plateau (data not shown). At this point, the cell viability becomes a problem in this assay since a relatively large amount of neutrophils are lysed after ~60 minutes.

Perhaps, one of the most noteworthy results from these experiments was the relatively low degree of ROS generation of primed/activated neutrophils as compared with the findings from Botha *et al.* (1995). A plausible reason for this difference comes from the intrinsic differences between the methods used in the present study and in Botha's study. Much of the conflicting literature regarding priming and activation in neutrophils results from an ill-defined process of priming and the use of a non-standardized broad range of approaches to measure the priming and activation level (e.g. oxidative burst, degranulation). Not only have several techniques such as cytochrome c reduction, NBT, luminol, flow cytometry been used to measure neutrophil priming, but also no consistency exists regarding of which marker should be measured. In addition to ROS production, other molecules have been also used as a measure to priming including elastase release and L-selectin expression (Kishimoto, T. *et al.,* 1989). Compared to most methods used to measure oxidative burst which are time-consuming and do not allow the measurement of other cell functions simultaneously, the method optimized in the present study was found simple, fast and amenable to multiplexing. In addition, and most notably, flow cytometry measures the intracellular production of ROS at a single cell level allowing the observation of subpopulations of cells in the whole sample.

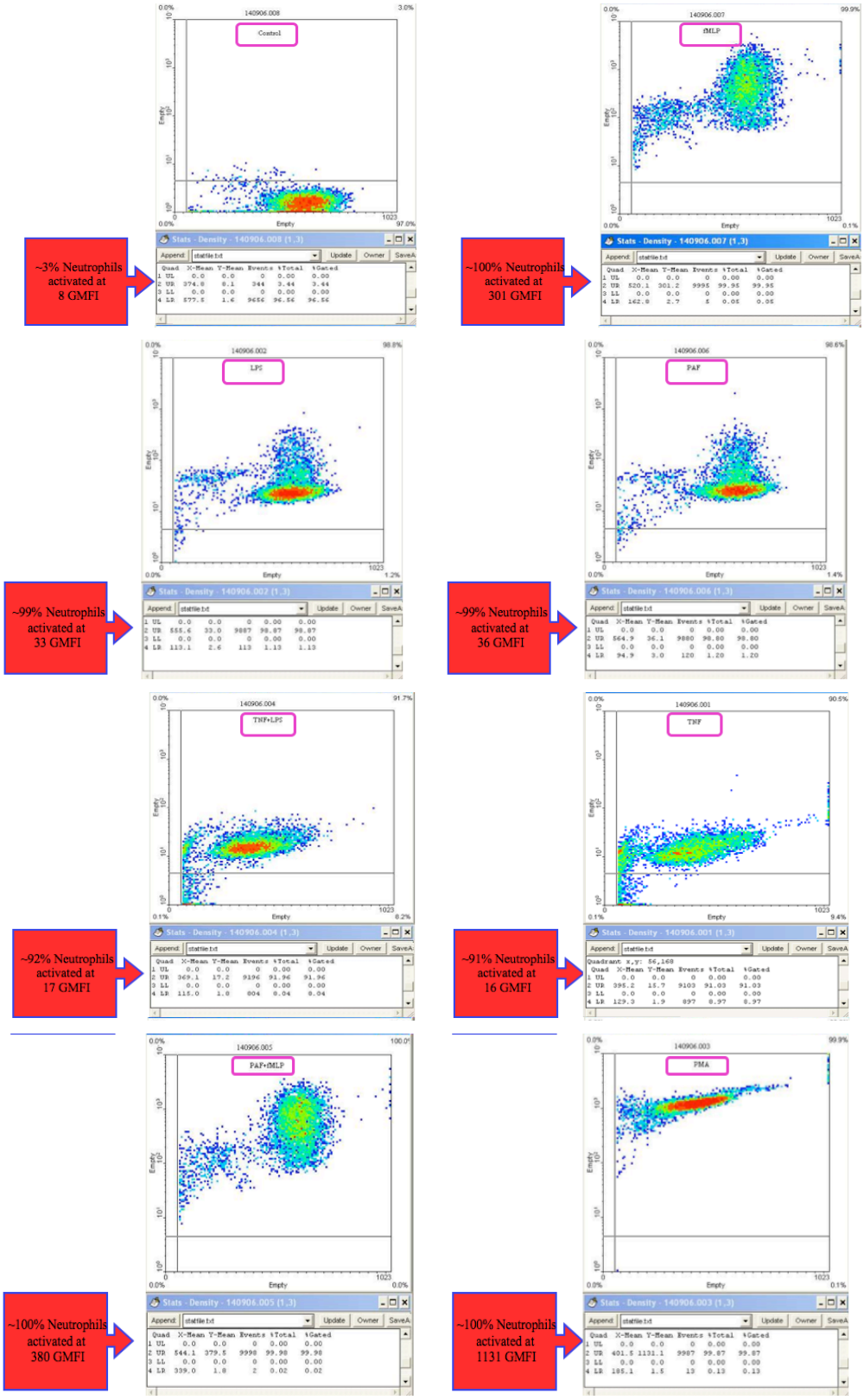


Fig. 10: Degree of activation in GMFI's in resting neutrophils (control), fMLP-, PAF-, LPS-, TNFα-, PMA-, TNFα/LPS-, and PAF/fMLP-activated neutrophils. Stimulation agent concentrations and other parameters are given in Material and methods (sections 3.3 and 3.4)

Conceptually, priming agents should not activate the NADPH oxidase in neutrophils when typical priming doses are used. It is thought that priming agents activate various signal transduction cascades that amplify the NADPH oxidase assembly upon stimulation with a secondary "activating" agent. However, the molecular basis for priming is still unknown, and in the past years a number of signaling pathways have been implicated in the priming process (Cadwallader, K. *et al.,* 2002; McLeish, K. *et al.,* 1998; Nick, J. *et al.,* 1997; Forsberg, M *et al.,* 2001; Yaffe, M. *et al.,* 1999). Based on the aforementioned definition of priming offered by Swain *et al.* one can appreciate the difficulty, not to say unfeasibility, to separate priming from activation so as what is measured under a specific condition is an exclusive representation of that process. In addition, caution in interpreting and comparing laboratory results of neutrophil functional studies must be taken since striking differences arise from whether the neutrophil is in contact with other components such as other cells and extracellular matrix. Therefore, in contrast to cells in suspension, the adherent neutrophils may respond to a variety of stimuli differently and probably in higher order of magnitude (Nathan, C. 1987).

[4.3] Two-dimensional Gel Electrophoresis (2-DE) Analysis – The 'Divide-and-Conquer' Workflow

Two-dimensional gel electrophoresis (2-DE) analysis has remained the bottleneck in gel-based proteomics approaches. Despite the recent improvements, 2-DE analysis is technically challenging and has built-in limitations in terms of reproducibility, resolving power and dynamic range (see ***Introduction***). To gain qualitative and quantitative information about protein expression in 2-DE experiments for different conditions (e.g., healthy versus diseased state), gels have to be matched. Nevertheless, quantitative changes that refer to the variations in the observed volume, optical density or area of individual spots on 2-DE gels pose a major limitation in 2-DE reproducibility. A high degree of gel-to-gel variation in spot patterns can make comparative 2-DE analysis difficult to achieve in order to glean any true biological variation from experimental variation. Slight technical variability from gel to gel often yields deviations in the number of spots. Thus, some spots may appear in one gel that are not observed in a parallel gel of the same sample.

To overcome some of the aforementioned limitations, investigators often choose to run each sample multiple times (replicates) and assume that any spot that appears in the majority of the resulting 2-DGE patterns is 'real.' Replicates permit the estimation of the non-systematic error associated with a measurement and the assessment of the significance of results by the assignment of a 'confidence score'. In general, there are two types of replicates: ***technical replicates***, which can be used to assess the experiment noise, and ***biological replicates***, which are mostly directed towards assessing the natural variability in the system. To assess natural biological variability, one should take into consideration the technical variability of the experiment and minimize it as much as possible. Notwithstanding, the application of replicates to 2-DE experiments exposes the variability issue of this technique with a dramatic decrease in matching efficiency when large numbers of gels are to be matched. In a recently reported 2-DE analysis, Voss and Haberl (2000) found that 20% of all spots differed by a factor of 2- to 5-fold and 2.3% of spots by 5- to 10-fold when using silver staining for parallel gels.

Gel heterogeneities are caused by many factors including sample preparation,

staining, unequal mobility in the different regions of gels, and variations in electrophoretic conditions. The level of variability in staining intensity depends very significantly on the intensity itself, which makes the identification of error models critically dependent on a suitable normalization procedure. Other well-known source of variability is associated with incorrect matching (automatic by the image analysis software or manual by the operator).

To surmount the issue of gel-to-gel technical variability, a novel approach has been taken in the present study to glean qualitative and quantitative data from 2-DE experiments, namely ***'Divide-and-Conquer' workflow***. Instead of matching all gels one to another or all gels to one unique reference gel as recommended in most of image analysis software (e.g., ImageMaster, Progenesis), gels were matched, grouped in a given "*MatchSet*", and then analyzed in 'batches'. A '**batch**' is comprised of a given number of gels that, ***together*** (at the same time), underwent the same sample preparation procedures, run in the same conditions (first and second dimensions) and are stained in the same batch. This strategy can be carried out for as many batches as one desires in an experiment; that is, several batches can make part of the whole experiment and be matched afterwards. Data generated from each "Match Set" analysis (from each batch) were exported to Excel tables and differential protein expression was statistically analyzed. The workflow of this strategy is schematized on figure 11, and comprises of the following steps: (1) ***match*** the gels the run together (batch), (2) create a ***NODE*** to link all gels in the experiment by matching the control gels from each batch, (3) generate match and spot ***reports***, and (4) link information from each spot to generate a ***common experiment report***. The data is then ready to be statistically analyzed.

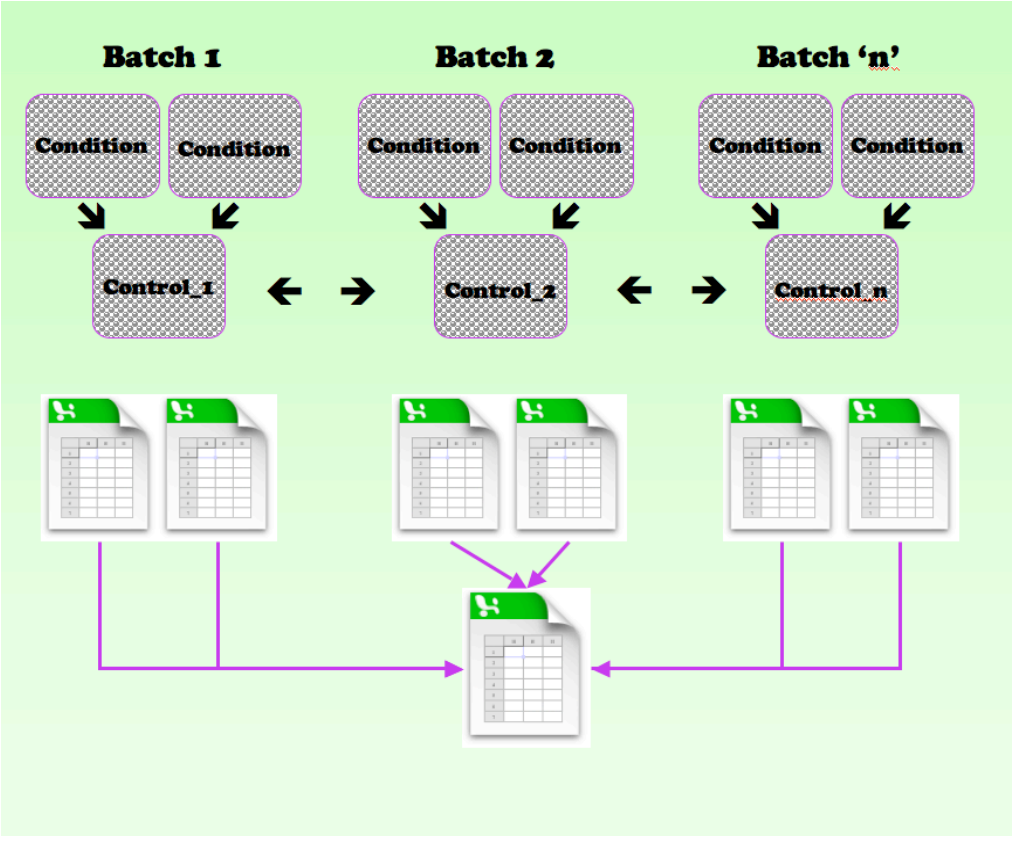


Fig. 11: Schematic of the 'Divide-and-Conquer' strategy for 2DE analysis

Image analysis workflow

A generic workflow for gel image analysis with ImageMaster using the 'Divide-and-Conquer' strategy is illustrated in figure 12. The steps are spot detection and editing, spot landmarking, alignment and matching of gels in a given batch, and finally, data analysis including normalization, ratio transformation, and analysis of variance using methods such as MANOVA (multivariate analysis of variance; see ***Introduction***). In the present study, TNFα were used to activate neutrophils from healthy donors. The total neutrophil proteome was extracted after incubation with TNFα for 30-min, and two technical replicates (two samples from the same subject; samples '1' and '2') and two biological replicates (two samples from two different subjects, samples 'D' and 'E') were run in two 'batches' as shown in the workflow depicted on figure 11. For these samples, 12% 2D-PAGE (pI 4-7) was used to separate the neutrophil proteins. In each 'batch', the control gel (nonactivated sample) was used as the reference gel to matching the other two TNF-activated sample gels. The link between the two 'batches' was then made by matching the reference gels (controls) from batch 1 to batch 2.

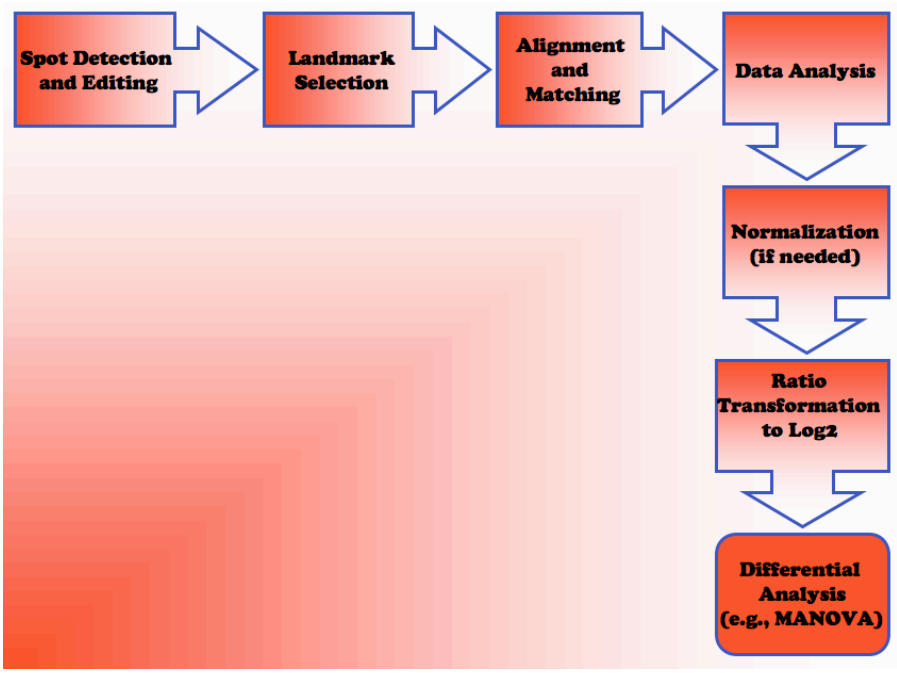
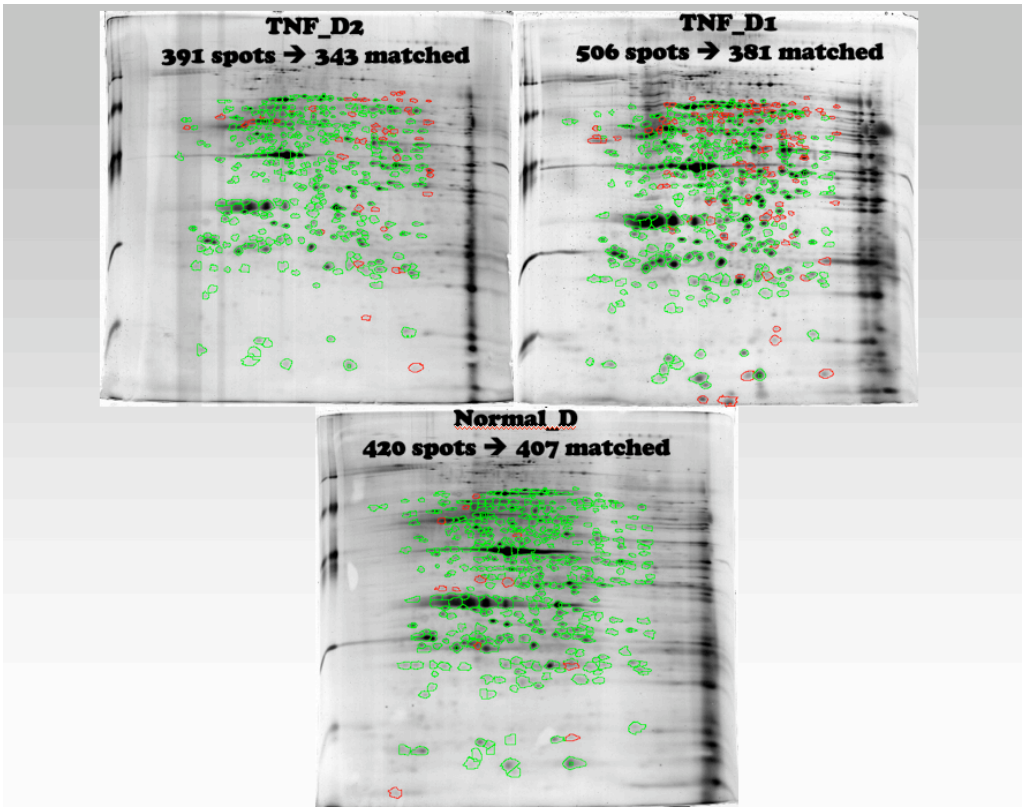


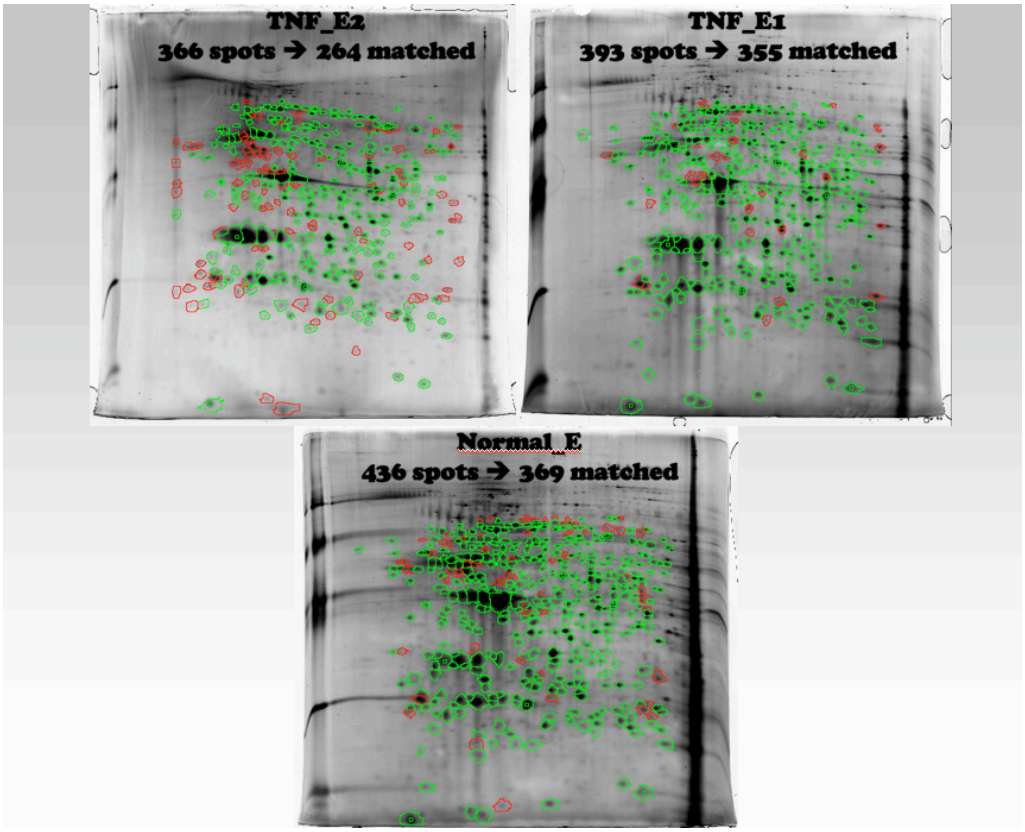
Fig. 12: The workflow of ImageMaster analysis for 'Divide-and-Conquer' strategy.

Overall, the gels that run together in the same experiment, i.e. the same isoelectric focusing run and the same electrophoresis run, showed better reproducibility as shown by the number of matched spots among gels from the same batch compared to a lower number of matches between gels from different batches (or experiments) (figures 13 a, b and c). The same lack of reproducibility across batches was also observed upon differential spot volume analysis of replicates (data not shown).

(a)



(b)



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(c)

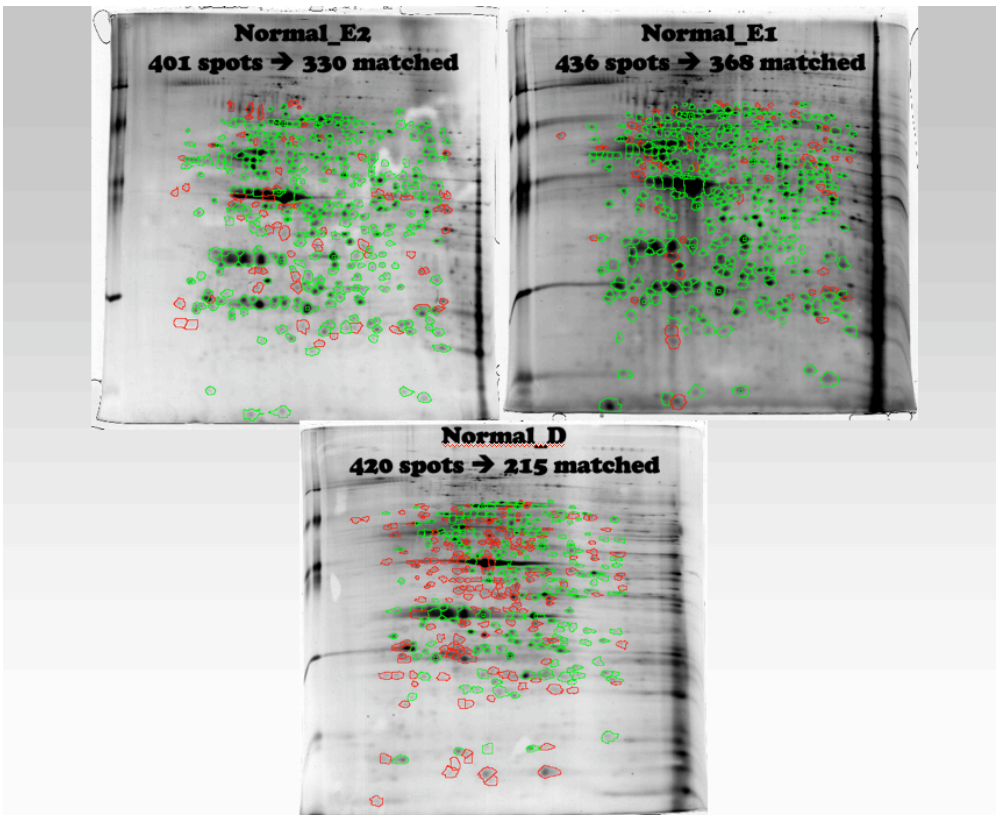


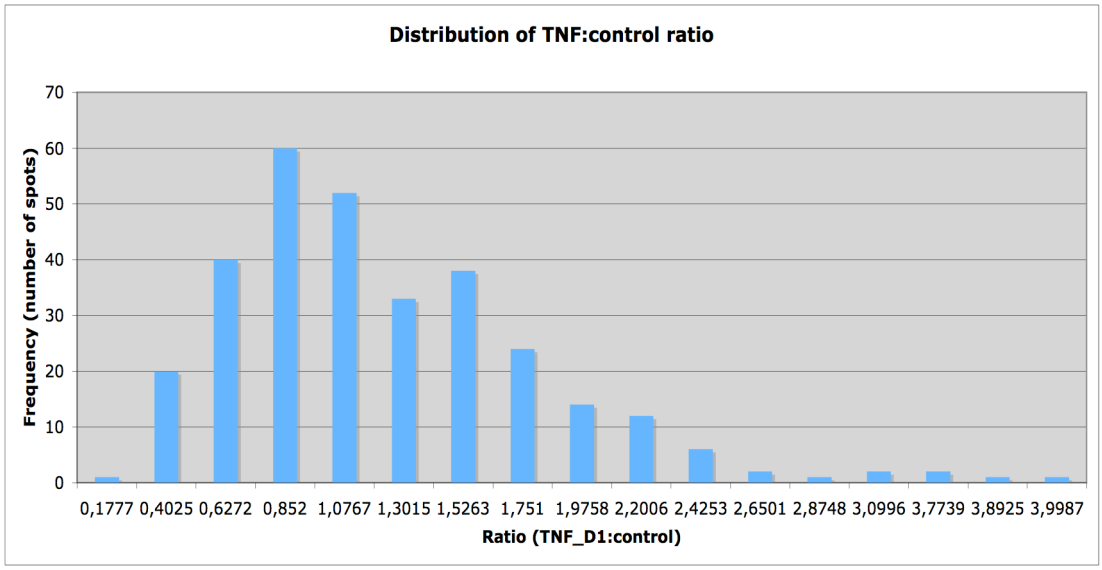
Fig. 13: Gels making up batch '1' (a) from donor '**D**' include the control (nonactivated neutrophils), ***TNF_D1*** and its replicate ***TNF_D2***; whereas batch '2' (b) was made up by control, ***TNF_E1*** and its replicate ***TNF_E2***, all from donor '**E**'. The node (c) was generated by matching the control gels from both batches (Normal_D and Normal_E1 plus a technical replicate from the latter (Normal_E2).

Log_2 transformation produces a continuous spectrum of values, whereas ratio measurements compresses the data below 1 and expands it above 1.

In the present study, the gels in a given batch were normalized by dividing the volume in each spot by the arithmetic mean volume of all spots of that given gel. The purpose of normalization was to remove, as much as possible, the effects of any systematic sources of variation such as differences in isoelectric focalization, small differences in protein load or stain development time. To measure expression differences between a TNF-treated neutrophils and the control, a ***logarithmic transformation*** was applied to the ratio. Although ratios are commonly used to measure expression differences between two proteins (that is, spot volume or intensity), this simple statistical analysis treats up- and down-expressed proteins differently. That is, proteins up-expressed by a factor of 2 have an expression ratio of 2, whereas those down-expressed by the same factor have an expression ratio of ½ (0.5). Consequently, down-expressed proteins are compressed between 1 and 0 while up-expressed proteins ranges from 0 to ∝. Transformation of the ratios to a logarithmic scale (logarithm base 2) minimizes this problem by providing a similar

representation of down- or up-expressed proteins and producing a continuous spectrum of values (Figure 14 and 15).

(a)



(b)

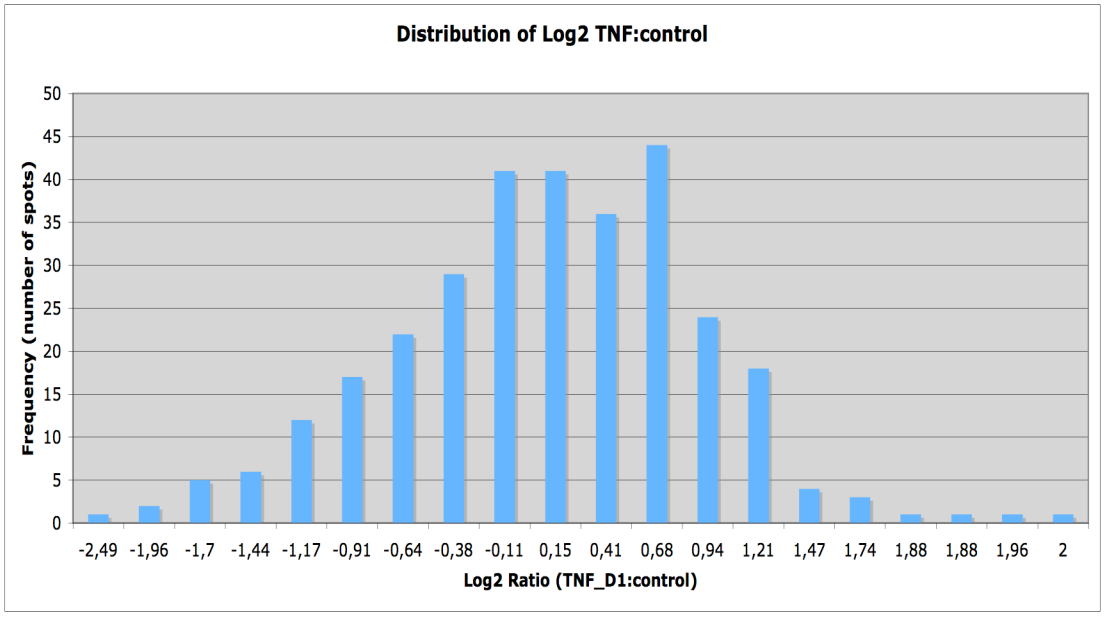
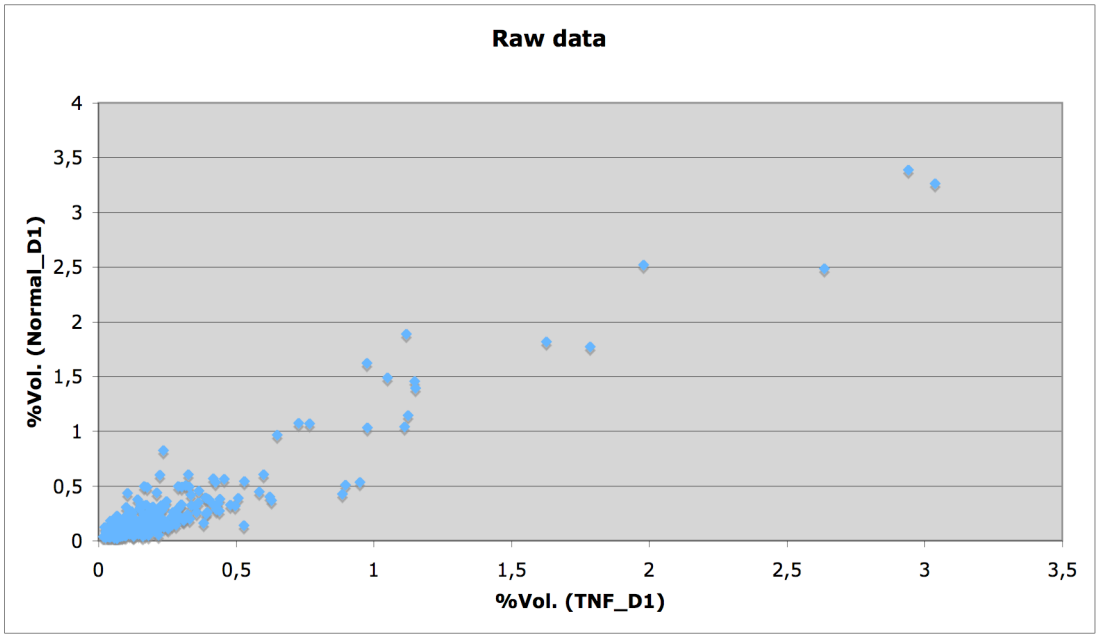


Fig. 14: Histogram of protein expression ratios for 309 spots matched between TNF-treated (TNF_D1) and control gels before (a) and after (b) Log2 transformation showing a more normalized distribution pattern after logarithmic transformation.

(a)



(b)

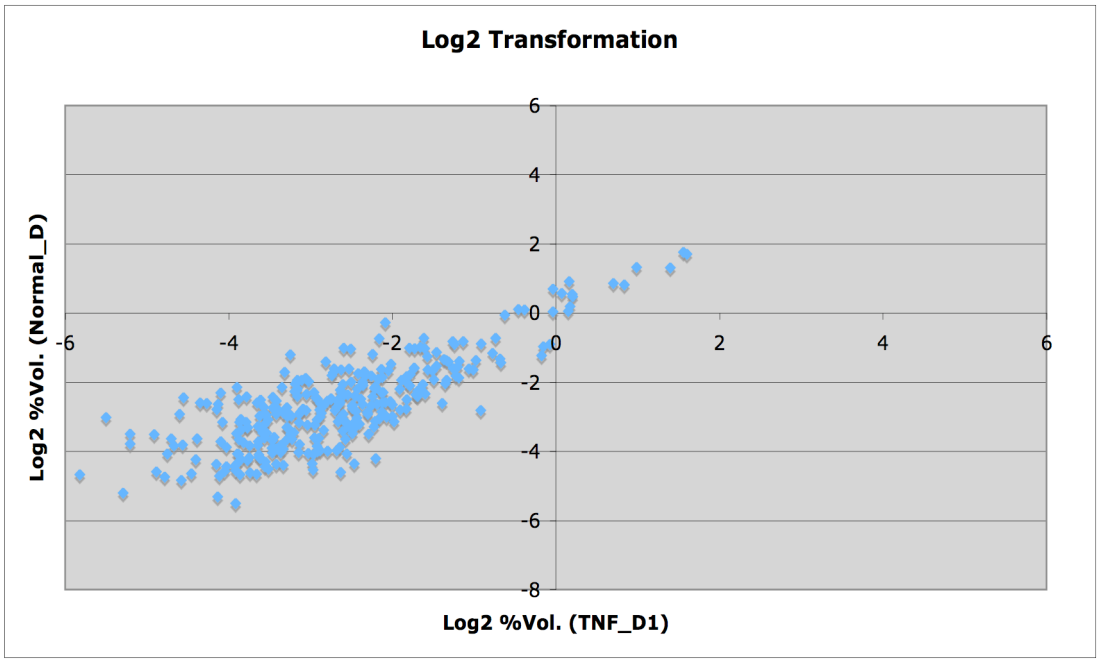


Fig. 15: Scatterplots for 309 spots matched between TNF (TNF_D1) and control gels showing the effect of Log2 transformation. (a) raw and (b) log-transformed %volume values obtained from TNF-treated vs. control gels. Protein expression patterns are generally represented by ratios (a) comparing measured spot volumes between two samples. Ratios, however, have the disadvantage of compressing measurements below 1 and expanding them above; consequently, the logarithm transformation (b) of the ratio was used to surmount such problem.

Differential Expression Analysis of 2D Gels.

Differential analysis of proteins by spot volume, intensity, or area is generally handled by using commercial software packages that propose statistical tools to help to conclude on the significance of variation. Unfortunately, most of image analysis packages available still require manual editing to improve detection and matching. Thus, there is some room for user interpretation variability and errors. ***The 'two-fold' change 'rule' to determine statistically significant differential protein expression does not conclusively implicate in a biological significance.***

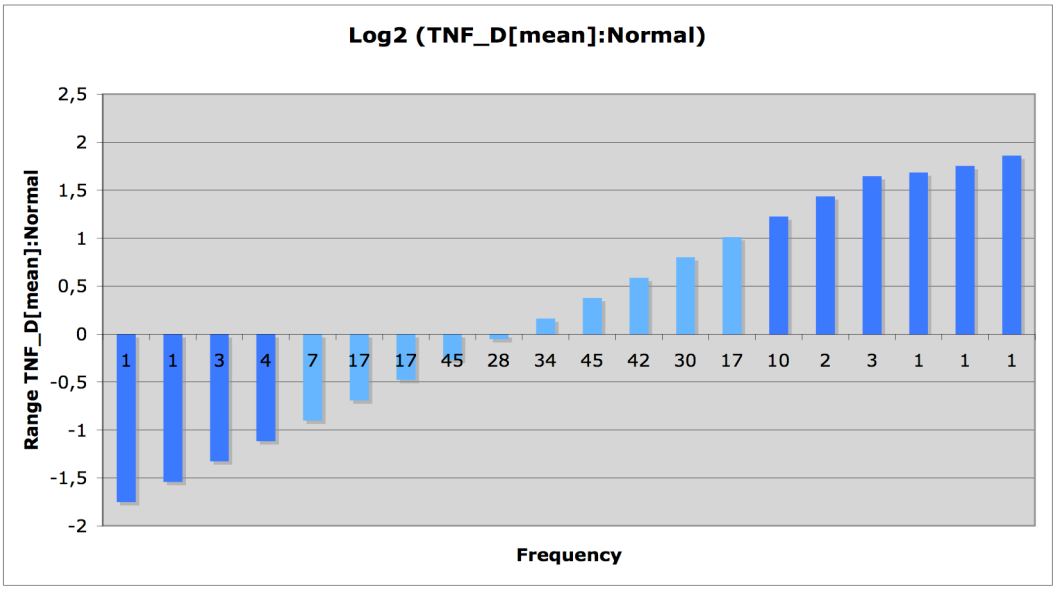
In the present study, a simple differential expression gel analysis was performed to show the potentiality of differential protein expression analysis using the 'Divide-and-Conquer' workflow. Although ImageMaster software offers a package for such statistical analyses which includes two-sample t test, Mann-Whitney U test and the Kolmogorov-Smirnov test, these statistic tests are limited to univariate analyses of a very strict software workflow. To carry out further statistical analysis, the raw data containing information on the every spot volume was exported into Excel tables. As shown on the workflow on figure 11 and 12, after exporting data into Excel tables, each spot was normalized to mean value of all spots in the gel. Then, the normalized mean for each spot was obtained by taking the average between a given spot and a its replicate in a technical 'replicate' gel. The differential expression between TNF and control (normal) gels were determined by calculating the Log_2 of the ratio TNF[mean]:Normal[mean]. An eminent issue in 2D gel analysis is related to the choice of arbitrary cut-offs (e.g., two-fold up- or down-regulation as significant, or equivalently, log_2 (ratio) values greater than 1 or less than -1). Such arbitrary, fixed cut-offs may or may not be supported by the underlying biology. By taking into consideration the commonly used 'two-fold change' (equivalent to one-fold change in log2 ratio) in expression of proteins as significant, 18 and 9 proteins (outlined in figure 17) were found to be upregulated and downregulated, respectively, between TNF_D spots (mean of a technical duplicate) and their matched spots in the control gel (figure 16a). Importantly, the analysis of a biological replicate showed great variability such that most down- and up-regulated proteins from one biological replicate did not overlap with the other replicate (figure 16b).

In conclusion, despite the development of techniques for accurate identification of differentially expressed proteins and their statistical significance, extracting clear and coherent hypotheses from 2DE data remains a daunting task. Statistics cannot decide what is biologically relevant in a 2D gel experiment, but can propose objective methods based on the data, to suggest both what could be interesting, and what should be moved aside or corrected. In fact, the difficulty in 2DE analysis lies mainly in the interpretation of the biological relevance of the data. In order to assess differential expression in a way that controls both false positives and false negatives, the standard approach should be based on statistical significance and hypothesis testing, with careful attention paid to multiple comparisons issues.

The use of fold change between spot volume in different conditions has been commonly used in 2D gel analysis in an attempt to determine what is 'biologically' relevant (Marengo, E. *et al.,* 2005). Nevertheless, a two-fold change in expression of a given protein between two conditions does not conclusively implicate in a biological significance of such protein in terms of differentiation between the two conditions. Transcriptomics studies have shown that stress-induced genes have a more variable expression, albeit other less induced genes may present a greater biological relevancy

(Dudoit, S. and Speed, T. *et al.*, 2000). A statistically more powerful approach is to calculate the ***mean*** μ and ***standard deviation*** σ for the distribution of Log_2 (ratio) values. Differential expression at the 95% confidence level can then be identified as Log_2 (ratio) values more than 1.96 standard deviations from the mean. In fact, a handful statistic tests have emerged mainly from the microarray community for analysis of long and lean datasets such as 2D gel data.

(a)



(b)

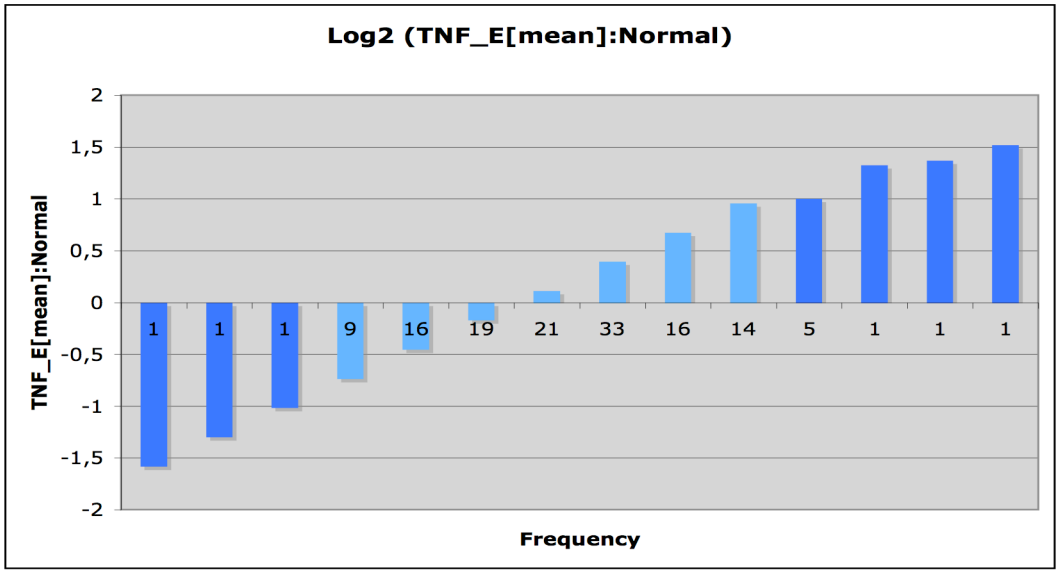


Fig. 16: Histogram of protein expression (**a**) log_2 ratio (TNF_D[mean]:Normal) and (**b**) log_2 ratio (TNF_E[mean]:Normal) showing the number of up- and down-regulated (bars in dark blue) proteins considering a cut-off of one-fold in log2 ratio. Values for spot %Vol in this analysis were obtained after averaging two technical replicate for each, TNF_D and TNF_E gels. Most proteins found up- or down-regulated in one of the biological replicates (e.g., TNF_D) do not follow up in the other replicate (e.g., TNF_E) in terms of number and match.

The transformation of the ratio to Log2 and application of more statistic-oriented tests for differential expression are promising for gel analysis. In summary, there is still a need for more powerful tools that can help to rationalize the decisions on significance and can draw attention on the imperfections of gels, spot mismatches and other artifacts of 2-DE gels. This study was not meant to find appropriate approaches for statistical treatment of 2D gel data, yet to demonstrate the potentiality of the 'Divide-and-Conquer' strategy in terms of simplicity.

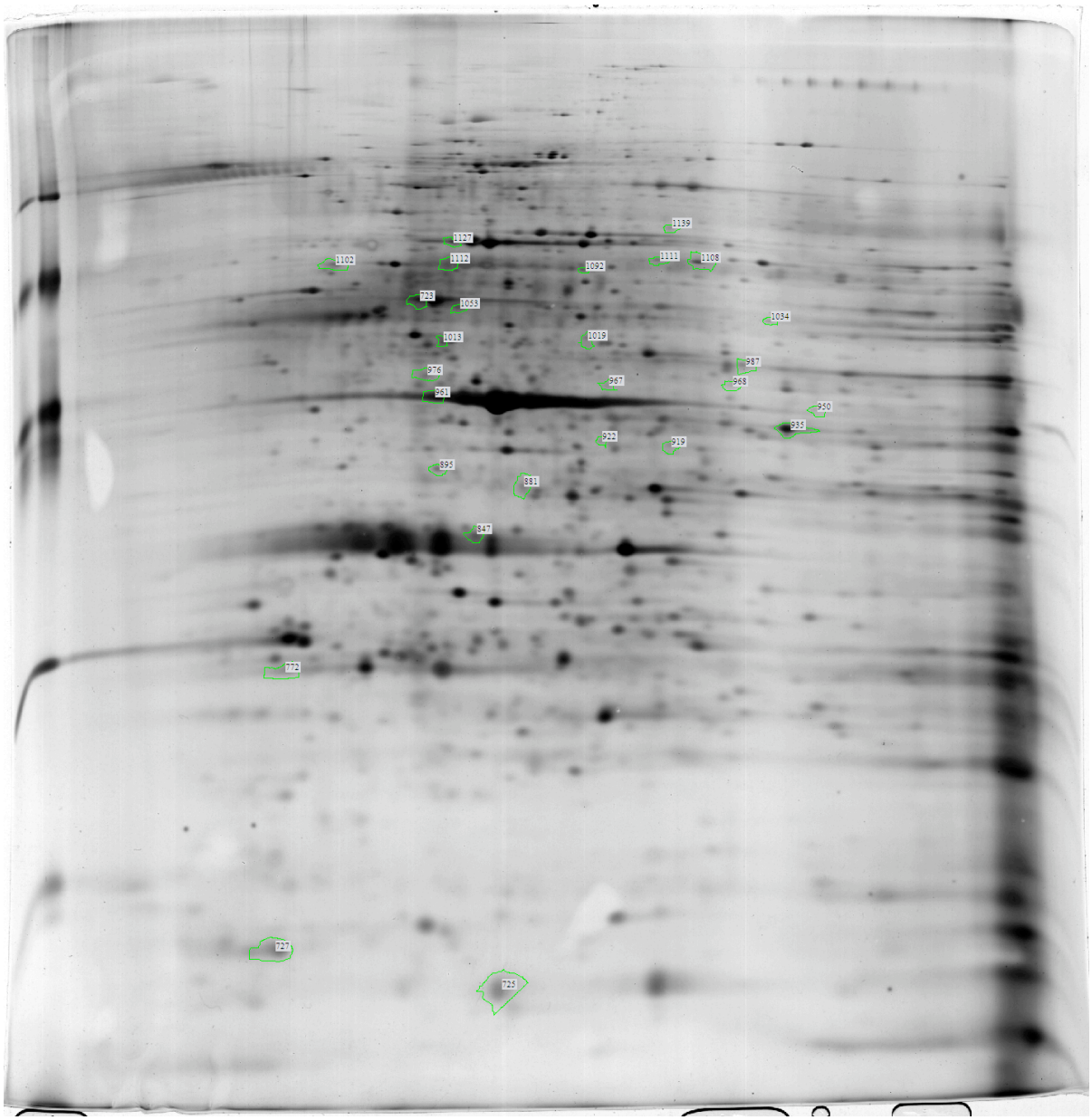


Fig.17: Differentially expressed spots obtained from figure 16a. The proteins with log2 ratio (TNF_D[mean]:Normal_D) greater or lower than one-fold are outlined on Normal_D gel.

[4.4] Subcellular Fractionation of Cytosolic and Plasma Membrane Compartments

Rationalizing Subcellular Fractionation in Neutrophils – an In Silico Analysis - Neutrophils contain effector proteins of the innate immune system, which are stored in granules and mobilized in response to various immunoregulatory mediators (Faurschou, M. and Borregaard, N. 2003; Borregaard N. and Cowland J. 1997; Lominadze, G. *et al.,* 2005). Synthesis of granule and secretory vesicle proteins is initiated in early promyelocytes, and ends in segmented neutrophils. During neutrophil circulation as a naïve cell, synthesis of granule and vesicle proteins comed to a halt, and if no activation stimulus is given to the neutrophil during circulation, programmed cell death is ensued. Upon activation, neutrophils discharge their microbicidal arsenal stored in granules and vesicles to phagosomes or the extracellular environment in a stepwise and highly orchestrated fashion. Granule membrane proteins can be added to the phagosome or plasma membrane membranes, whereas granule luminal contents are released into the extracellular space or phagocytic vacuole. Therefore, granule content accrued during cell development is thought to remain unchanged in terms of new synthesis of proteins. In this regard, granule and vesicle proteins subcellular localization can be seen as the functional proteomics of neutrophils; that is, the mobilization of these proteins from one compartment to another will determine the phenotype of the neutrophil. Secretory vesicles for example fuse with the plasma membrane of neutrophils in response to a wide variety of inflammatory stimuli (Faurschou, M. and Borregaard, N. 2003). This fusion results in the incorporation of a spectrum of membrane-associated receptors including the β2-integrin CD11b/CD18 (Mac-1, CR3) (Segelov, H. *et al.,* 1993), and the metalloprotease leukolysin (Kang, T. *et al.,* 2001), both of which are essential for neutrophil exocytosis.

At the cell signaling transduction level, neutrophil phenotype (e.g., granule discharge, NAPH oxidase assembly and chemotaxis) is finely regulated by signals received by membrane receptors that are then relayed into the cell interior by a combination of small intracellular signaling molecules such as diacylglycerol, cyclic AMP and Ca^{2+}, and ***intracellular signaling proteins,*** most of which are located primarily in the cytosol. The resulting chain of intracellular signaling events ultimately alters target proteins, resulting in a specific cellular behavior. For future work in neutrophil signaling transduction, we have hypothesized that most of proteins involved in signaling transduction leading to neutrophil migration, extravasation, phagocytosis, degranulation and apoptosis reside in cytosol or plasma membrane. To corroborate with this hypothesis, DNA microarray data from neutrophils of septic patients (Coldren, C *et al.,* 2006) were re-analyzed *in silico* in order to (1) ***determine the subcellular localization of the most upregulated genes*** and compare this list with the proteins found by Lominadze *et al.,* in neutrophil granules; and (2) find ***potential protein phosphorylation*** in each subcompartment. The findings of this analysis are summarized on table 3. It is noteworthy that the 357 genes (almost 40%) out of 912 of most upregulated genes pos-endotoxin stimulation have their protein cognate localized on the cytoplasm (268 primarily localized on cytoplasm and 89 primarily localize on plasma membrane or nucleus and with alternative cytoplasmic localization). Similar analysis for plasma membrane proteins reveals that a total number of 267 genes (approximately 30%) out of the same 912 most upregulated genes have their protein cognate localized on the plasma membrane (183 primary to the plasma membrane and 84 alternatively localized on the membrane).

***Table 3**: In silico* re-analysis summary of the 912 most upregulated genes reported by Coldren *et al.,* The table contains the primary and alternative localization for the 912 most upregulated genes from the microarray analysis of lung-infiltrating neutrophils (compared to circulating neutrophils) pos-endotoxin administration to healthy human subjects.

Cellular Compartment	Primary Localization	Alternative Localization	Proteins Potentially Phosphorylated
Cytoplasm & Cytoskeleton	**268**	**89**	149
Plasma membrane	**183**	**84**	6

Source of Protein Localization and potential phosphorylation: Human Protein Reference Database (www.hrpd.org) and Phospho Site (www.phosphosite.org).

In another comparative analysis, the list of proteins found in the three granule subsets of neutrophils (Lominadze, G. *et al.,* 2005) was cross-examined against the list of proteins found during phagosome maturation in neutrophils (Burlak, C. *et al.,* 2006). Notably, nineteen proteins from a list of 29 host defense proteins found in neutrophil phagosomes (Burlak, C. *et al.,* 2006) have also been identified by proteomics in at least one of the three granule subsets (Table 4). This indicates that the key role that granule proteins play in delivering its microbicidal arsenal to the phagosomes by membrane fusion. Other proteins typically found in the ER (e.g., calnexin, ERp29, GRP58/ERp57, GRP78/BiP, and PDI) and mitochondria (e.g., F_1 ATPase subunits, peroxiredoxin 3 [PRDX3)] and prohibitin) were also found associated with neutrophil phagosomes. The finding that neutrophil phagosomes contain components of the three subsets of granules, endoplasmic reticulum and mitochondria evokes a complex picture of the phagosome formation and maturation. Although the application of high-scale proteomic technologies has accelerated the discovery of many proteins that play important roles in neutrophil function such as phagocytosis, a central challenge now will be to integrate these proteins into pathways and networks that account for the diversity of neutrophil function.

High-scale gene expression profiling has become an important tool for investigating transcriptional activity in a variety of biological samples. DNA microarray-based gene expression profiling relies on nucleic acid hybridization and the use of nucleic acid polymers, immobilized on a solid surface, as probes for complementary gene sequences. This prominent experimental tool offers a snapshot of the gene expression in a given sample, permitting the measurement of the level of expression of thousands of genes simultaneously. Despite the ***technical*** (robustness, cost and throughput) advantages of using DNA microarrays over proteomics technologies, not all transcripts generate protein, and alternate translation efficiency and posttranslational turnover may result in differential protein accumulation. These differences may underlie at least in part the modest correspondence reported between quantitative measurements of gene expression and cognate protein levels (Gygi, S. *et*

al., 1999). Therefore, caution should be applied when comparing genes and their protein cognate.

Table 4: Comparison between two subcellular proteomic studies. The first column corresponds to the 29 host defense proteins from Burlak *et al.* and the second column whether the protein was found in one of the three granule subsets in Lominadze *et al.* Abbreviations: GE – gelatinase granules, SP – specific granules and AZ – azurophil granules. "Rejected protein" means that the protein was identified but did not pass the trustworthy criteria.

Phagosome host defense proteins	***Granule proteins***
Anti-inflammatory factor 1 (AIF-1)	Not found
Arginase 1 (ARG1)	Not found
BPI	GE, SP, AZ
Calgizzarin	Gelatinase granule rejected protein
Calgranulin A	Not found
Calgranulin B	Not found
Calgranulin C	SP
Calprotectin L1L subunit	Not found
Cathepsin G	GE, SP, AZ
Cathepsin G-like	Not found
Cathepsin S	Not found
Azurocidin 1; CAP37	GE, SP, AZ
Chitinase 1; chitotriosidase	GE, SP, AZ
Defesin α1 preproprotein	GE, SP
Defesin α3 precursor	Not found
Elastase 2	SP, AZ
Eosinophil preperoxidase	GE, SP, AZ
Cathelicidin antimicrobial peptide; CAP18	Not found
Ficolin	GE, SP
Lactoferrin	GE, SP, AZ
Lipocalin; NGAL	GE, SP, AZ
Lysozyme C	GE, SP, AZ
MMP8; neutrophil collagenase	SP
Myeloperoxidase (MPO)	GE, SP, AZ
Myeloperoxidase (pre-pro-MPO)	Not found
Myeloperoxidase, chain B (light chain)	GE, SP, AZ
Myeloperoxidase, chain D (heavy chain)	GE, SP, AZ
Proteinase-3	GE, SP, AZ
Ribonuclease, mase A family 3; eosinophil cationic protein	Specific granule rejected protein

Abbreviations: GE – gelatinase granules, SP – specific granules and AZ – azurophil granules. "Rejected protein" means that the protein was identified but did not pass the trustworthy criteria determined by the authors of the article.

From the abovementioned comparisons, it is obvious that proper biological

activity and cellular homeostasis depend on spatially and temporally restricted partitioning of functionally related sets of proteins. In fact, many specialized functions are compartmentalized within organelles. Nevertheless, there is an important caveat in analyzing a given protein at one time point, namely the ***dynamic nature of proteins***. Cell compartments are composed of proteins that are present almost all of the time ('resident' proteins), proteins that are in transit and proteins that transiently interact with the organelle to carry out defined functions. In fact, many proteins seem to have an alternative subcellular localization to its primary one. Hence, whether a given protein will be associated with a specific organelle may depend on very tightly regulated temporal window during the cell signaling process.

[4.5] Optimizing Cytosolic Protein Enrichment in Neutrophils

Sample preparation is one of the most crucial stages in proteomics analysis. Proper experimental model and careful sample preparation is vital to obtain significant and trustworthy results. The power of most of proteomic platforms is such that minimal contamination with proteins that are not bona fide components of the enriched compartment will be equivocally identified as being a part of that proteome.

The success of proteomic analysis depends very much on the complexity of samples, sample preparation methods and analytical methods. Currently, the proteomic approaches using many strategies and technologies show certain limitations of detectability. In the past years, efforts in reducing these limitations and enhancing detectability have been a major focus of several proteomics groups worldwide. The technical development in the fields of sample preparation, protein separation and identification, and bioinformatics can be integrated to solve the limitations in proteome studies and to improve the effective proteomic approaches for determining the differentially expressed proteins or novel biomarkers.

Digitonin extraction is highly effective for cytosolic proteins

Cytosolic proteins from neutrophil were fractionated by repeated washes in digitonin buffer containing sucrose, PIPES, $MgCl_2$, protease and phosphatase inhibitors. To confirm the effectiveness of the method, four goals were established here. The first method based on the ***quantitative measurement of proteins*** from the digitonin fractions. On average, the first digitonin wash generated approximately 210 μg of protein per $3x10^6$ neutrophils. Since the subsequent digitonin washes did not generate a satisfactory amount of proteins to perform 2DE analysis, only the first wash was processed further downstream this point. In the second validation approach, the neutrophils were imaged by ***confocal microscopy*** to confirm that, after digitonin treatment, the cells retain their morphology, but become impermeable. It was hypothesized that if the digitonin-treated cells were incubated with the DNA-intercalating dye propidium iodide, which normally does not permeate neutrophils, the nuclei would appear stained only if the digitonin form ‘holes’ on the cell membrane. To create a contrast between the nucleus and the cell cytoplasm, the neutrophils were also incubated with the membrane permeable rhodamine 123 (DHR 123 pre-oxidized, see material and methods for oxidative burst flow cytometry analysis). Figure 18 shows that after digitonin treatment the neutrophil nucleus stained with the propidium iodide dye forming a multibolular shape as commonly described in the literature. In addition, the rhodamine was nicely scattered throughout the cytoplasm. The third method to evaluate effectiveness of the extraction method

based on the comparison of the ***1D gel electrophoresis patterns*** between cytosolic fraction and the remaining of the neutrophil proteins. Clearly, the 1D gel pattern of the cytosolic proteins differs from that of the remaining proteins (figure 19).

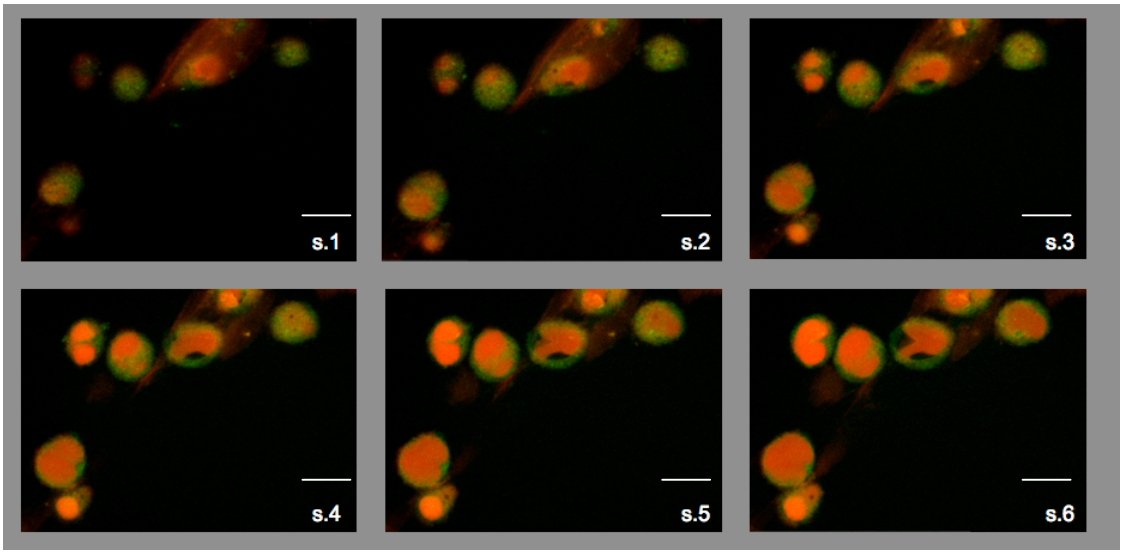


Fig. 18: Confocal optical sections (s.1-s.6) of digitonin-treated neutrophils incubated with rhodamine 123 and propidium iodide. White bar = 10μm.

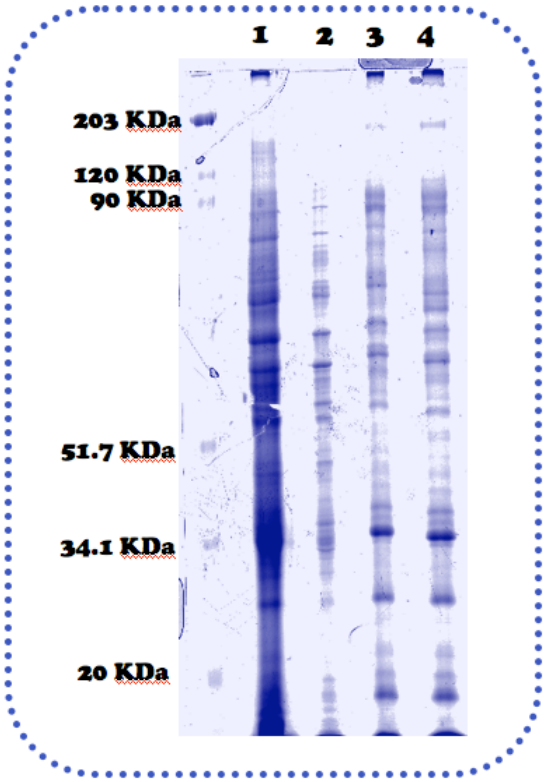


Fig. 19: Coomassie-stained 1D gel of cytosolic fraction (lanes 1 and 2) and membrane/organelle fraction (lanes 3 and 4). Protein load: lane 1 (50μg), lane 2 (10 μg), lane 3 (10 μg) and lane 4 (10 μg).

Although highly useful, the abovementioned experiments lack a conclusive assertiveness for the enrichment strategy. The ultimate test is, in fact, a work in progress, and consists in the identification of the enriched proteins followed by literature and/or experimental confirmation of the subcellular localization of that protein. To this end, enriched proteins were fractionated by 2D gel electrophoresis, the spots were excised digested and identified by mass spectrometry (MALDI-TOF/TOF). To date, 15 proteins have been identified (figure 20) with a high degree of confidence (table 5). Most notably, all identified proteins have been reported to be primarily located in the cytoplasm.

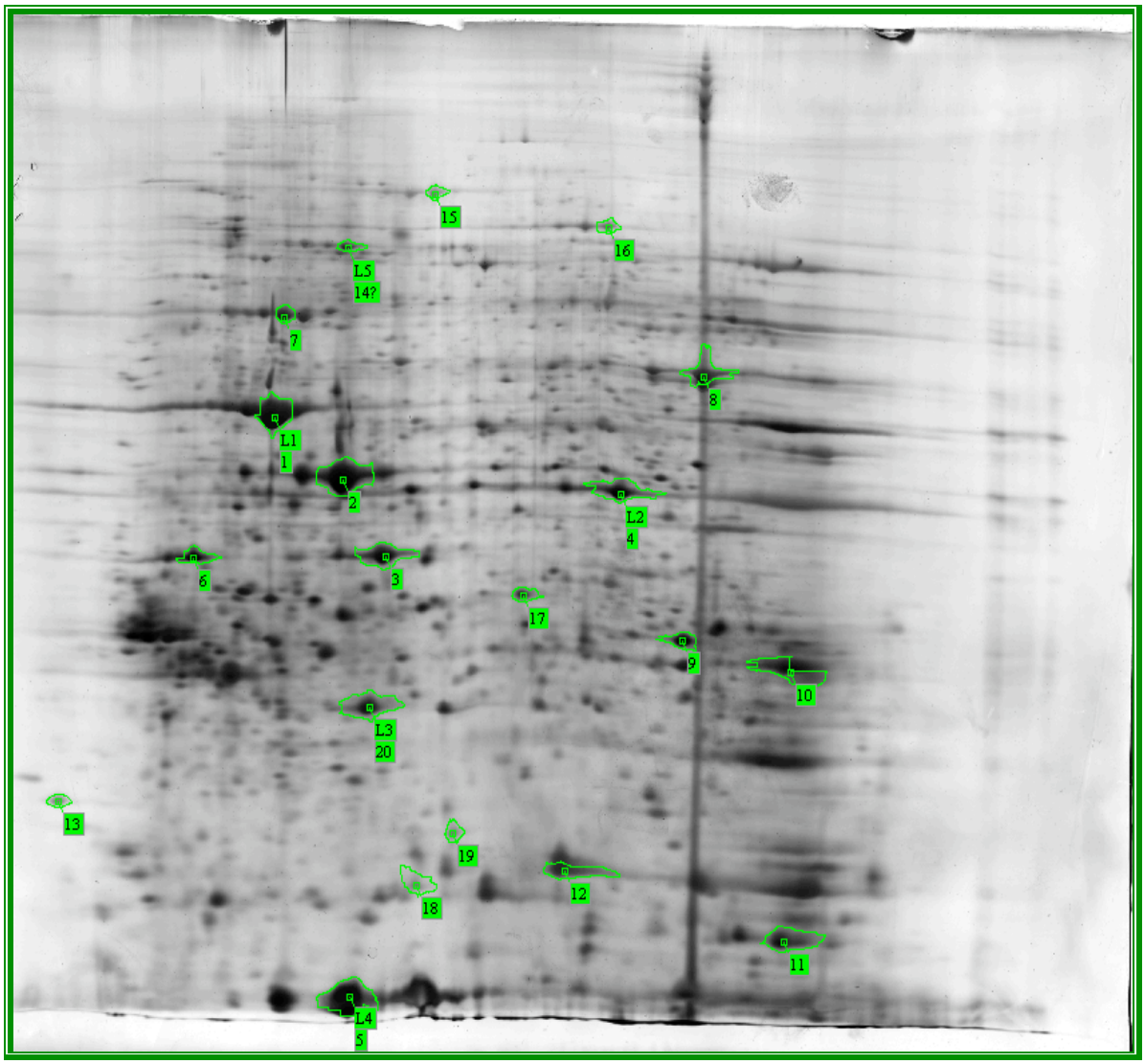


Fig. 20: Spots outlined were excised for further charaterization by mass spectrometry (MALDI-TOF/TOF).

Table 5: Proteins identified by mass spectrometry in this study. Out of 20 spots excised from the 2D gel, 15 were identified with a high degree of confidence. Spot number corresponds to the spots highlighted in figure 20. For spots number 4, 6 and 15, the information gathered from MS and MSMS were not sufficient to distinguished between different isoforms. Detailed information on each identification is given on Appendix 1.

Protein Name	Mascot Score	PMF Coverage (%)	Number of Peptides Sequenced / Coverage (%)	Cellular Compartment
1. β-Actin	311	64%	3/16%	Cytosol
2. γ-Actin	280	66%	3/16%	Cytosol
3. Annexin A3	153	56%	2/5%	Cytosol
4. Annexin A1 isoform B 4. Annexin A1 isoform C	297 289	44 % 40 %	2/10% 2/10%	Cytosol
5. S100-A9	209	67 %	3/37%	Cytosol
6. Annexin A5 isoform C 6. Annexin A5	164 164	84 % 82 %	2/6% 2/5%	Cytosol
7. L-plastin isoform 2	342	57 %	2/4%	Cytosol
8. Enolase 1	147	49 %	3/10%	Cytosol
9. Phosphoglycerato mutase	188	47 %	2/11%	Cytosol
10. Proteinase 3	151	48 %	2/7%	Cytosol
11. Peptidyl isomerase A	224	69 %	3/28%	Cytosol
12. Cofilin 1	128	67 %	1/10%	Cytosol
14. Annexin A6	126	19 %	2/3%	Cytosol
15. Gelsolin isoform A 15. Gelsolin isoform B	108 111	22 % 23 %	1/2% 1/3%	Cytosol
20. Gluthatione-S transferase P	195	54 %	3/21%	Cytosol

Digitonin extraction of cytosolic proteins confers a high degree of reproducibility

A high degree of gel-to-gel variation in spot patterns can make comparative 2-DE analysis difficult to achieve, and any true biological variation could be masked or amplified by experimental variation. Slight technical variability from gel to gel inherent to the sample preparation often yields deviations in the number and volume

of spots. Gel heterogeneities caused by sample preparation must be minimized and methods that lack reproducibility are in general not suitable for proteomics analysis. To assess the reproducibility of the proposed method for cytosolic proteins enrichment, sample preparation procedure (including blood withdrawn, neutrophil purification and cytosolic protein extraction) were carried out separately (in different days). To minimize the influence of the biological variability as much as possible, the same sample donor for the three replicates was used. Then, every step in the preparation procedure for 2DE and staining were carried our in parallel to reduce 2D gel experimental variability.

A high degree of reproducibility was reached among the three sample preparations. As shown in figure 21, gels '1' and '3' have respectively 70% and 75% of their spots matched with gel '2'. Importantly, this analysis was executed uniquely with the 'automatic matching' features of the ImageMaster Platinum software, with no manual editing (operator editing) carried out. Manual matching can significantly increase the degree of matching among the gels. Moreover, spot volumes among the three gels showed excellent Pearson correlation coefficient (table 6).

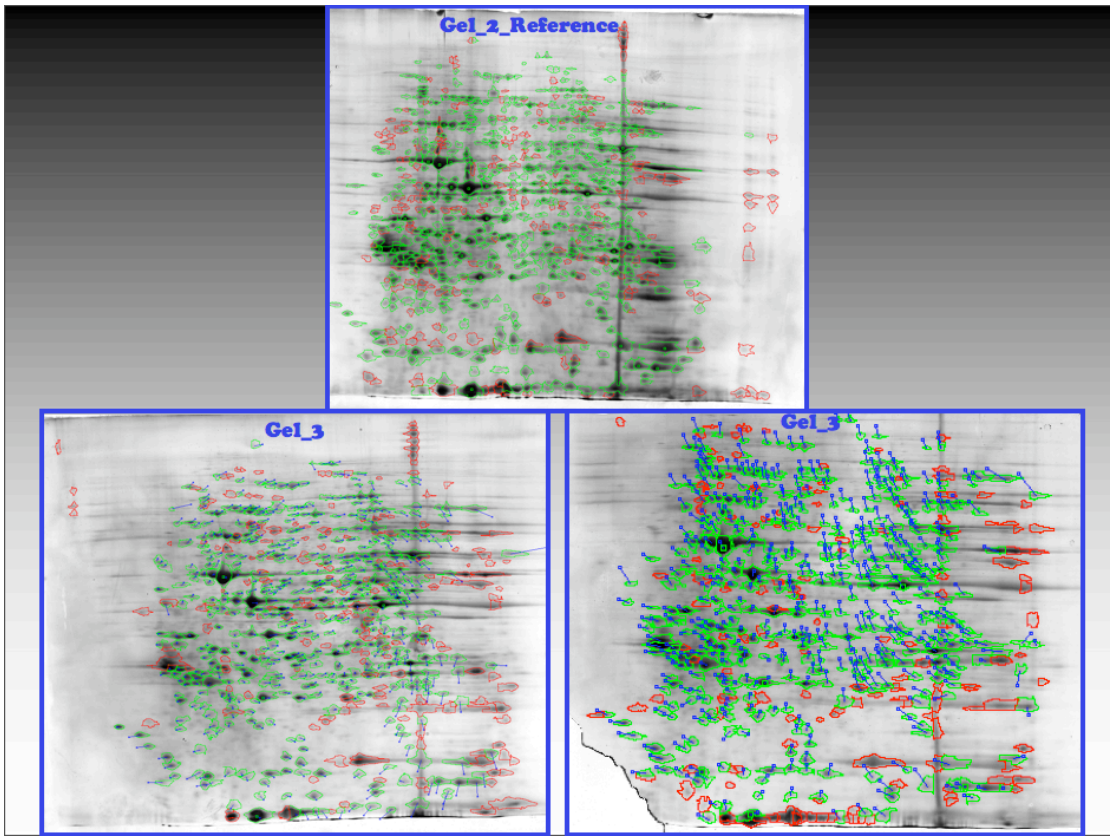


Fig. 21: Digitonin extraction provides a high degree of reproducibility. Automatic matching generated by ImageMaster Platinum software (v.6) assigned matches in 70 and 75% of the spots present in gels '1' and '3', respectively. Spots outlined in green represent the matches in each gel, while spots in red are unmatched. Blue vectors represent the direction of the match between the reference and the matched gel.

In the present Thesis, a simple, fast subcellular fractionation method for cytosolic protein extraction of neutrophils was developed taking as the optimal

parameters the number of cytosolic proteins identified, the reproducibility of separation, and the sensitivity of staining detection. The results presented here suggest that this method offers a great efficiency for enriching cytosolic protein from neutrophils, and excellent reproducibility. The percentage of cytosolic proteins identified using the digitonin extraction method was 100%; that is, thus far, all 15 proteins identified by mass spectrometry are cytoplasmic resident proteins. The other parameter taken into consideration during the optimization process, namely reproducibility in spot volumes among the 2D gels, was also quite satisfactory. However, the small variability shown for some of the spots among the replicates could not be discerned whether it came from sample preparation or the staining procedure.

Table 6 Pearson correlation coefficient between any two digitonin gels. Pairwise comparisons of 389 spots (%vol) present in all three gels.

	Gel_1	*Gel_2*	*Gel_3*
Gel_1	**1**		
Gel_2	**0,856424824**	**1**	
Gel_3	**0,904034608**	**0,749812798**	**1**

The optimal concentration of 100 μM digitonin affects selectively the cytoplasmic membranes of neutrophils, releasing the cytosolic proteins with little contamination from the organelles or plasma membrane. Extraction of the remaining cellular extracts with sequential washes with the same concentration of digitonin was ineffective for releasing more proteins as shown by protein quantification of the different washing fractions. Treating the left over pellet with 0.5% of Triton X-100 allowed the recovery of a different set of proteins, which has not been identified **yet**. As the extraction using digitonin neither denature nor alter the conformation of proteins, cytosolic protein complexes would be expected to be recovered, which would enable future studies of protein-protein interactions.

Other clear advantage this method is that it provides some clues on protein subcellular localization. That is, proteins identified from the digitonin-extracted fraction are likely to reside or transitorily reside in the cell cytoplasm. Overall, the results presented here clearly suggest that subcellular fractionation by digitonin extraction offers great efficiency and reproducibility, least time consuption, and least cost. This protocol is robust and may be well-suited for analyses of other complex specimens.

5. Conclusions

The study of signaling transduction in neutrophils and current proteomics approaches for biomarker discovery pose a significant technical challenge. Although a variety of proteomic approaches have been attempted so far, the number of protein identifications has been quite modest in many studies. The selection of an appropriate sample preparation method is important for confident results. Moreover, clinical proteomics studies have special needs, namely practicability and feasibility of the sample preparation methods in the clinical context. To this end, we have implemented a sample preparation platform comprising of a simple neutrophil purification method (Percoll gradient purification) and a highly robust and practical technology for cellular characterization at the cellular and molecular levels (flow cytometry). Overall, both methods meet the sample preparation requirements for clinical and proteomics studies, namely simplicity, reproducibility, yield and purity of the preparation.

In the recent years, a general trend towards subcellular proteomics analysis in many different cell types, particularly in neutrophils, has emerged as a powerful and promising alternative to the whole cell proteomics studies. In the present study, we have demonstrated the feasibility of cytosolic protein enrichment from human neutrophils. By reducing sample complexity, we were able to achieve highly reproducible 2D gel patterns. Overall, digitonin extraction is simple, reproducible and highly specific for cytosolic proteins. In addition, a novel workflow for 2D gel analysis has been proposed in this thesis, namely the 'Divide-and-conquer' workflow. Perhaps, the major advantages of this novel approach are the improved matching capacity, the reduced gel variability, and the significant reduction in time dedicated for gel analysis. The small degree of variability still observed after the application of the 'Divide-and-conquer' workflow may be caused by either technical 2D electrophoresis limitations or gel staining issues. In the present study, ammoniacal silver staining protocol was used to increase sensitivity of detection. Generally speaking, ammonical silver protocols use fast staining development steps (2 min or less), which are difficult to make very reproducible. This may be a problem when staining consistency is a critical parameter since the gel-to-gel reproducibility may be reduced.

6. Perspectives

The clinical utility of proteomics is a promising new field that has the potential to have many applications, including the identification of biomarker for early diagnosis and prognosis, disease monitoring, and prediction of response to therapy. Further advancement of proteomics technologies could usher in an era of personalized molecular medicine, where diseases are diagnosed at earlier stages using robust molecular markers. Then, the clinicians would use the patient's specific proteomic profile to rationally tailor and monitor molecular therapy. In clinical proteomics studies, samples from normal and disease subjects must be compared to identify the protein (or a panel of them) differentially expressed between the two conditions. Moreover, expression proteomics may shed some light into our understanding of the disease progression. Although the challenges are great for global analysis of proteomes, the proteomic approaches for reduction of limitations and enhancement of proteomic detectability exposed in the present Thesis have the potential to be translated into the clinical settings to study neutrophil-related diseases in the proteomic context.

The importance of a better understanding of the mechanisms of neutrophil signaling transduction during sepsis development has been highlighted by several experts in the field over the past decades (see ***Introduction***). In this regard, flow cytometry and proteomics have emerged as suitable tools designed to address this need from the level of signal transduction, biomarker discovery and validation, and phenotype analysis. Currently, a multicenter study proposed to profile cytosolic and membrane proteins differentially expressed among healthy subjects, patients with severe sepsis, and patients with shock septic is under development. This study was projected in a subcellular strategy that divides the samples into subproteomes to be further analyzed by proteomics and immulogical approaches.

Resumo da dissertação em português.

Pesquisas biomédica comumente começa com a elaboração de uma hipotese para resolver um problema. Nesse contexto, cientistas selecionam o(s) metodo(s) mais apropriado(s) para responder a questão e solucionar um dilema. Nos últimas anos, nós temos testemunhado uma revolução de novas tecnologias em biologia molecular – a ciência ***-Omica***. Tecnologias de alta-escala tais como metabolômica, citômica, genômica e proteômica estão mudando o modo que estudamos sistemas biológicos complexos. Métodos contemporâneos para o entendimento da diversidade funcional em um dado organismo preferencialmente abordam uma visão de biologia de sistemas onde primeiro a classificação fenótipa de um citoma é alcançado antes de uma tentativa de caracterizar o proteoma de tal célula. Dentro desse contexto, e para proporcionar um melhor entendimento dos componentes envolvidos na ativação e morte celular programada dos neutrófilos nos estados patológicos e sano, esse estudo propõe a integração de métodos em biologia celular tal como citometria de fluxo com uma robusta plataforma proteômica em uma tentativa de integrar dados a nível molecular com dados fenótipicos de neutrófilos. Fracionamento subcelular usando um método de extração e enriquecimento de proteínas citosólicas com o detergente digitonina foi otimizado nesse trabalho, e encontrado ser altamente reprodutível, fácil de realizar, e de baixo custo.

Objetivo

O objetivo geral dessa dissertação de Máster é a construção de uma plataforma sólida e robusta para integração de pesquisa básica em proteômica e pesquisa médica. Para tal objetivo, uma série de otimizações para processamento de amostras foram propostas para avaliar nossa capacidade de gerar dados biológicos de alta qualidade das amostras trabalhadas. As metas principais são as seguintes:

1. Implementar um método efetivo e de baixo custo para purificação de neutrofilos humano no Laboratório de Bioquímica e Química de Proteínas na Universidade de Brasília.
2. Desenhar e provar uma abordagem prática para avaliar características funcionais e morfológicas dos neutrofilos sob análize.
3. Determinar as condições apropriadas e estratégias para estudos proteomicos de neutrofilos. Esta meta foi subdividida em outras duas "metas específicas":
    a. Padronizar as condições optimas para eletroforese bidimensional do proteoma total do neutrofilo
    b. Examinar a viabilidade de realizar fracionamento subcelular do proteoma dos neutrofilos.

Resultados

Citômica

Purificação com Percoll em gradientes de duas camadas proporciona uma população altamente pura de neutrofilos sem afetar o estado original de repouso dos neutrofilos. No geral, nosso protocolo de separação de neutrofilos humano direto do sangue é altamente reproduzível, e produz uma alta quantidade de células. Utilizando apenas duas camadas de Percoll (a 57% and 67%), neutrofilos foram separados com um grau de pureza superior a 96%, e uma viabilidade de aproximadamente 98%.

Agentes pro-inflamatorios, em particular fMLP, evocam a geração de espécies reativas do oxigênio (EROs) em neutrofilos. Em comparação com outros autores (Botha, A et al. 1995) que hipotetizam um efeito estimulante ('priming effect') de certos agentes pro-inflamatórios, nossos resultados e testes *in vitro* com citometria de fluxo desafia esta hipótese já que não encontramos um aumento significativo quando os neutrofilos foram pré-ativados ('primed') com TNFα ou PAF seguido de ativação com LPS ou fMLP, respectivamente. Esses resultados estão representados na figura 10 da dissertação.

Proteômica

Devido a variabilidade técnica comumente observada em experimentos de géis bidimensionais, nesse estudo, uma nova metodologia/fluxograma para analise de gel bidimensional foi empregada, sendo nomidada 'Divide-e-Conquiste'. Ao contrario de métodos mais tradicionais utilizados em programas de análise de imagem de géis bidimensionais, nosso método consiste no pareamento de géis dentro de um "grupo", que tem em comum o fato que correram juntos; isto é, focalização isoelétrica, separação electroforética em gel de poliacrilamida e coloração foram realizadas concomitantemente. Essa estratégia pode ser utilizada com vários grupos de géis. Dois fluxogramas dessa estratégia estão representado na figura 11 e 12 da dissertação. No geral, os géis que fazem parte de um grupo, ou seja, que correram juntos, apresentaram melhor reproducibilidade em termos de pareamento (fig. 13a e 13b) em comparação a géis pareados de dois grupos distintos (fig. 13c).

Tratamento estatístico dos dados para análise de géis bidimensionais é uma das etapas mais importantes para extração de resultados significantes. Dois passos foram encontrados serem altamente relevantes para análise estatística nesse trabalho. O primeiro, a normalização do volumes das manchas (spots) selecionadas para análise é capaz de remover os efeitos de erros sistemáticos devidos a problemas técnicos tais como diferenças em focalização isoelétrica, em coloração, entre outros problemas. Outro passo extremamente vantajoso é a transformação das taxas de expressão diferencial (relação volume da mancha gel A:volume da mancha gel B) de cada pareamento em uma função logarítmica na base 2. Embora taxas de expressão sejam largamente utilizadas em análise de géis bidimensionais, esse tipo de análise trata dados de sub- e sobre-expressão diferencial diferentemente. Proteínas duas vezes mais sobre-expressas apresentam uma taxa de 2, enquanto proteínas duas vezes sub-expressas apresentam uma taxa de ½ (0.5). Consequentemente, proteínas sub-expressas estarão comprimidas entre um valor 1e 0, enquanto proteínas subre-expressas apresentarão um valor entre 0 e $\propto$. Transformação das taxas de expressão para uma escala logarítmica, tal como Log2, minimiza esse problema gerando uma representação similar do que esta sub- e sobre-expresso, e com um espectro de valores em forma contínua (ver figuras 14 e 15).

A manutenção homoestática celular depende em grande parte de uma funcionalidade protéica regulada em tempo e espaço. Nesse contexto, uma grande maioria das funções celulares especializadas são compartimentalizadas; isto é, acontecem dentro de um especifico compartimento celular. Uma importante limitação dos estudos proteômicos portanto refere-se a natureza dinâmica das proteínas. Em outras palavras, caracterização de uma dada proteína a um especifico ponto em tempo é uma análise incompleta que não revela com exatidão a função dessa proteína dentro de um processo. No geral, proteínas apresentam uma alto dinamismo em termos de localização subcelular que em muitos casos esta diretamente relacionado com a

função de uma proteína. Do ponto de vista técnico, o sucesso de separação de proteínas em géis bidimensionais esta diretamente relacionado, entre outros fatores, com a complexidade da amostra. Uma abordagem que tem ganhado especial atenção na comunidade proteômica nos últimos anos para redução da complexidade da amostra é a ***Análise Proteômica Subcelular***. Nesse tipo de análise, compartimentos subcelulares são separados com um alto grau de pureza (mínimo de contaminação), e então o proteoma de cada fração é analisado separadamente. Uma vantagem adicional desse tipo de análise, além da redução da complexidade, é a obtenção de informação sobre a dinâmica das proteínas em termos de sub-localização.

Em termos de sinalização celular, neutrófilos, assim como a maioria das células vivas, são regulados através de sinais recebidos por proteínas de membranas, esses sinais são traduzidos em funções por uma rede de proteínas presentes principalmente na fração citosólica das células. Nesse trabalho de dissertação e para trabalhos futuro nesse campo, nós hipotetizamos que a maioria das proteínas com um aumento ou diminuição significativa nos níveis de expressão estariam co-localizadas nas frações citosólicas e membranicas. Para corroborar tal hipótese, dados provenientes de microarranjos de ADN de neutrófilos de indivíduos após administração de endotoxina (Coldren, C. *et al*., 2006) foram re-analisados *in silico* com o intuito de caracterizar a localização teórica das proteínas referentes aos genes mais expressos (tabela 3). Interessantemente, cerca de 40% dessas proteínas tem localização primária ou secundária no citoplasma. Com esse fato em mente, um método para extração e caracterização de proteínas citosólicas foi desenhado e otimizado. Dentre os diversos métodos existentes para fracionamento subcelular, nós elegemos o método com digitonina para purificar proteínas citosólicas devido a simplicidade, baixo custo desse método. Digitonina é um detergente não-ionico que se liga especificamente e rapidamente a esteróis, precipitando-os, e permitindo portanto a formação de poros na membrana e a liberação do proteínas citosólicas.

Para confirmar a efetividade do nosso método de fracionamento para proteínas citosólicas, quatro metas foram estabelecidas. Primeiramente, através de uma medida da quantidade de proteínas gerada após o fracionamento com digitonina, a concentração ideal e o número de lavagens necessários foram determinados. Em resumo, uma concentração de digitonina de 100 μM e apenas uma única incubação de 10 minutos foram suficientes para extração de proteínas de citosol. Com o segundo método de verificação, microscopia confocal, verificou-se que após tratamento com digitonina, a integridade do núcleo e o 'contorno' celular do neutrófilo são mantidos (fig. 18). O padrão do proteoma da fração citosólica foi também perfilado por separação em gel de poliacrilamida (1D gel) e comparado com a fração restante (membranas e organelas) (fig. 19). E finalmente, o teste mais conclusivo foi realizado com identificação de um número de proteínas presentes na fração citosólica. Esse experimento foi realizado inicialmente com a separação das proteínas da suposta fração citosólicas por electroforese bidimensional, seguido por excisão de 20 manchas aleatoriamente escolhidas (fig. 20), e identificação das proteínas por espectrometria de massa (MALDI-TOF/TOF). De 20 manchas digeridas e analisadas, 15 proteinas foram identificadas com um alto grau de confiabilidade (MASCOT > 100, e com 1 a 3 peptideos sequenciados) (tabela 5). De suma importância, todas as proteínas identificadas são primariamente, e algumas secundariamente, presentes no citosol segundo análise literária.

Como já mencionado acima, uma das principais e mais conhecidas limitações em análise de géis bidimensionais esta vinculada a problemas de reproducibilidade.

Heterogenidade causada durante preparação da amostra deve ser minimizadas, e métodos que violam essa regra devem ser considerados inapropriados para estudos proteômicos. Para avaliar reproducibilidade do nosso método de enriquecimento de proteínas citosólicas, o procedimento de purificação de neutrófilos e extração de proteínas citosólicas foi repetido em três vezes cada um em dias diferentes. Para minimizar a influência causada pela variabilidade biológica, as amostras para cada preparação foram obtidas do mesmo individuo. E também, para minimizar a variabilidade oriunda da electroforese bidimensional, todas as etapas de electroforese e coloração foram realizadas em um único experimento. Em resumo, um alto grau de reproducibilidade foi alcançado em termos de padrão apresentados pela triplicata de géis. Mais de 70% de manchas entre os três géis foram pareadas utilizando unicamente recursos automáticos do programa de análise de imagem conhecido como ImageMaster Platinum (Amersham Bioscience, parte da GE Healthcare) (fig. 21). Obviamente, edição manual do pareamento produziria um aumento significativo no número de pares entre os três géis. Outro resultado significativo em termos de reproducibilidade é a correlação positiva (calculada como o coeficiente de correlação de Pearson) entre o volume normalizado de cada mancha em um gel e seu par correspondente em outros géis (tabela 6).

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Mascot Search Results

Protein View

Match to: **gi|119582950** Score: **297** Expect: **3.9e-25**
annexin A1, isoform CRA_b [Homo sapiens]

Nominal mass (M_r): **40475**; Calculated pI value: **6.57**
NCBI BLAST search of gi|119582950 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **44%**

Matched peptides shown in **Bold Red**

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   1 MSPRMECSDT FSKMAMVSEF LKQAWFIENE EQEYVQTVKS SKGGPGSAVS
  51 PYPTFNPSSD VAALHKAIMV KGVDEATIID ILTKRNNAQR QQIKAAYLQE
 101 TGKPLDETLK KALTGHLEEV VLALLKTPAQ FDADELRAAM KGLGTDEDTL
 151 IEILASRTNK EIRDINRVYR EELKRDLAKD ITSDTSGDFR NALLSLAKGD
 201 RSEDFGVNED LADSDARALY EAGERRKGTD VNVFNTILTT RSYPQLRRVF
 251 QKYTKYSKHD MNKVLDLELK GDIEKCLTAI VKCATSKPAF FAEKLHQAMK
 301 GVGTRHKALI RIMVSRSEID MNDIKAFYQK MYGISLCQAI LDETKGDYEK
 351 ILVALCGGN
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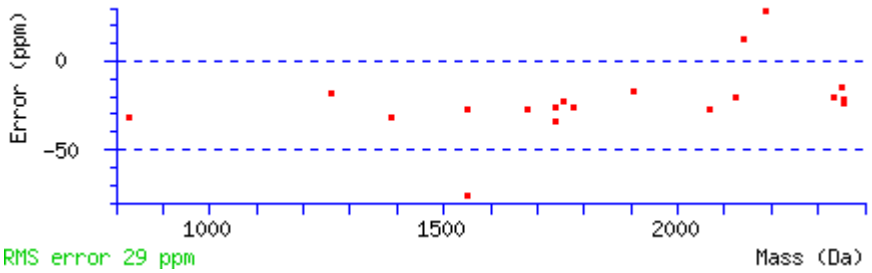
Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
5 - 22	2140.9690	2139.9617	2139.9349	13	1	R.MECSDTFSKMAMVSEFLK.Q (No mat
5 - 22	2188.9890	2187.9817	2187.9196	28	1	R.MECSDTFSKMAMVSEFLK.Q 3 Oxida
23 - 39	2123.9485	2122.9412	2122.9847	-21	0	K.QAWFIENEEQEYVQTVK.S Gln->pyr
43 - 66	2356.1000	2355.0927	2355.1495	-24	0	K.GGPGSAVSPYPTFNPSSDVAALHK.A (
43 - 66	2356.1057	2355.0984	2355.1495	-22	0	K.GGPGSAVSPYPTFNPSSDVAALHK.A (
72 - 84	1387.7245	1386.7172	1386.7606	-31	0	K.GVDEATIIDILTK.R (No match)
95 - 110	1776.8908	1775.8835	1775.9305	-26	0	K.AAYLQETGKPLDETLK.K (No match
95 - 111	1904.9998	1903.9925	1904.0255	-17	1	K.AAYLQETGKPLDETLKK.A (No matc
127 - 137	1262.5789	1261.5716	1261.5939	-18	0	K.TPAQFDADELR.A (No match)
199 - 217	2067.8284	2066.8211	2066.8777	-27	1	K.GDRSEDFGVNEDLADSDAR.A (No ma
202 - 217	1739.6904	1738.6832	1738.7282	-26	0	R.SEDFGVNEDLADSDAR.A (No match
227 - 241	1678.8667	1677.8594	1677.9050	-27	1	R.KGTDVNVFNTILTTR.S (No match)
228 - 241	1550.7000	1549.6927	1549.8100	-76	0	K.GTDVNVFNTILTTR.S (Ions score
228 - 241	1550.7755	1549.7682	1549.8100	-27	0	K.GTDVNVFNTILTTR.S (No match)
264 - 270	829.4769	828.4696	828.4957	-31	0	K.VLDLELK.G (No match)
331 - 345	1741.7902	1740.7829	1740.8426	-34	0	K.MYGISLCQAILDETK.G (No match)
331 - 345	1757.8043	1756.7970	1756.8375	-23	0	K.MYGISLCQAILDETK.G Oxidation
331 - 350	2334.0515	2333.0442	2333.0919	-20	1	K.MYGISLCQAILDETKGDYEK.I (No m
331 - 350	2350.0606	2349.0533	2349.0868	-14	1	K.MYGISLCQAILDETKGDYEK.I Oxida



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LOCUS       EAW62546                 359 aa            linear   PRI 18-DEC-2006
DEFINITION  annexin A1, isoform CRA_b [Homo sapiens].
ACCESSION   EAW62546
VERSION     EAW62546.1  GI:119582950
DBSOURCE    accession CH471089.1
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 359)
  AUTHORS   Venter,J.C., Adams,M.D., Myers,E.W., Li,P.W., Mural,R.J.,
            Sutton,G.G., Smith,H.O., Yandell,M., Evans,C.A., Holt,R.A.,
            Gocayne,J.D., Amanatides,P., Ballew,R.M., Huson,D.H., Wortman,J.R.,
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            Thomas,R., Tint,N.N., Tse,S., Vech,C., Wang,G., Wetter,J.,
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            Mays,A., Dombroski,M., Donnelly,M., Ely,D., Esparham,S., Fosler,C.,
            Gire,H., Glanowski,S., Glasser,K., Glodek,A., Gorokhov,M.,
```

Mascot Search Results

Protein View

Match to: **gi|119582952** Score: **289** Expect: **2.4e-24**
annexin A1, isoform CRA_c [Homo sapiens]

Nominal mass (M_r): **40285**; Calculated pI value: **7.60**
NCBI BLAST search of gi|119582952 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **40%**

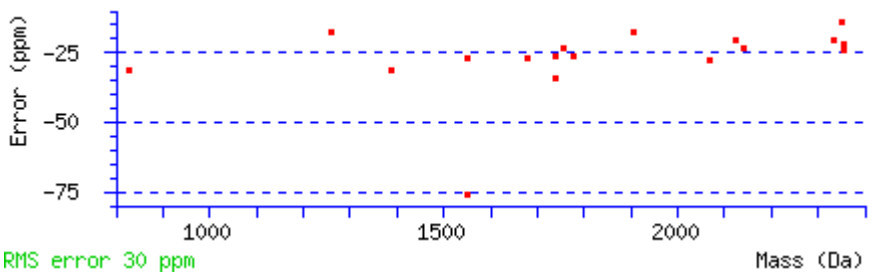
Matched peptides shown in **Bold Red**

```
   1 MNLILRYTFS KMAMVSEFLK QAWFIENEEQ EYVQTVKSSK GGPGSAVSPY
  51 PTFNPSSDVA ALHKAIMVKG VDEATIIDIL TKRNNAQRQQ IKAAYLQETG
 101 KPLDETLKKA LTGHLEEVVL ALLKTPAQFD ADELRAAMKG LGTDEDTLIE
 151 ILASRTNKEI RDINRVYREE LKRDLAKDIT SDTSGDFRNA LLSLAKGDRS
 201 EDFGVNEDLA DSDARALYEA GERRKGTDVN VFNTILTTRS YPQLRRVFQK
 251 YTKYSKHDMN KVLDLELKGD IEKCLTAIVK CATSKPAFFA EKLHQAMKGV
 301 GTRHKALIRI MVSRSEIDMN DIKAFYQKMY GISLCQAILD ETKGDYEKIL
 351 VALCGGN
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
21 - 37	2123.9485	2122.9412	2122.9847	-21	0	K.QAWFIENEEQEYVQTVK.S Gln->pyr
21 - 37	2140.9690	2139.9617	2140.0113	-23	0	K.QAWFIENEEQEYVQTVK.S (No matc
41 - 64	2356.1000	2355.0927	2355.1495	-24	0	K.GGPGSAVSPYPTFNPSSDVAALHK.A (
41 - 64	2356.1057	2355.0984	2355.1495	-22	0	K.GGPGSAVSPYPTFNPSSDVAALHK.A (
70 - 82	1387.7245	1386.7172	1386.7606	-31	0	K.GVDEATIIDILTK.R (No match)
93 - 108	1776.8908	1775.8835	1775.9305	-26	0	K.AAYLQETGKPLDETLK.K (No match
93 - 109	1904.9998	1903.9925	1904.0255	-17	1	K.AAYLQETGKPLDETLKK.A (No matc
125 - 135	1262.5789	1261.5716	1261.5939	-18	0	K.TPAQFDADELR.A (No match)
197 - 215	2067.8284	2066.8211	2066.8777	-27	1	K.GDRSEDFGVNEDLADSDAR.A (No ma
200 - 215	1739.6904	1738.6832	1738.7282	-26	0	R.SEDFGVNEDLADSDAR.A (No match
225 - 239	1678.8667	1677.8594	1677.9050	-27	1	R.KGTDVNVFNTILTTR.S (No match)
226 - 239	1550.7000	1549.6927	1549.8100	-76	0	K.GTDVNVFNTILTTR.S (Ions score
226 - 239	1550.7755	1549.7682	1549.8100	-27	0	K.GTDVNVFNTILTTR.S (No match)
262 - 268	829.4769	828.4696	828.4957	-31	0	K.VLDLELK.G (No match)
329 - 343	1741.7902	1740.7829	1740.8426	-34	0	K.MYGISLCQAILDETK.G (No match)
329 - 343	1757.8043	1756.7970	1756.8375	-23	0	K.MYGISLCQAILDETK.G Oxidation
329 - 348	2334.0515	2333.0442	2333.0919	-20	1	K.MYGISLCQAILDETKGDYEK.I (No m
329 - 348	2350.0606	2349.0533	2349.0868	-14	1	K.MYGISLCQAILDETKGDYEK.I Oxida



Mascot Search Results

Protein View

Match to: **gi|4826643** Score: **153** Expect: **9.7e-11**
annexin A3 [Homo sapiens]

Nominal mass (M_r): **36524**; Calculated pI value: **5.63**
NCBI BLAST search of gi|4826643 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|113954 from Homo sapiens
gi|1942126 from Homo sapiens
gi|178697 from Homo sapiens
gi|307115 from Homo sapiens
gi|410202 from Homo sapiens
gi|12654115 from Homo sapiens
gi|119626227 from Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Methyl (C-term),Methyl (DE),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **56%**

Matched peptides shown in **Bold Red**

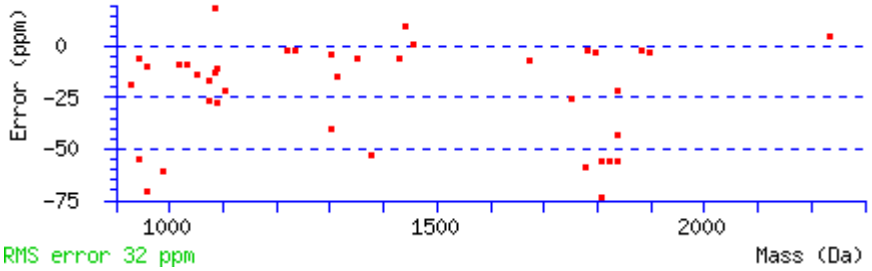
```
    1 MASIWVGHRG TVRDYPDFSP SVDAEAIQKA IRGIGTDEKM LISILTERSN
   51 AQRQLIVKEY QAAYGKELKD DLKGDLSGHF EHLMVALVTP PAVFDAKQLK
  101 KSMKGAGTNE DALIEILTTR TSRQMKDISQ AYYTVYKKSL GDDISSETSG
  151 DFRKALLTLA DGRRDESLKV DEHLAKQDAQ ILYKAGENRW GTDEDKFTEI
  201 LCLRSFPQLK LTFDEYRNIS QKDIVDSIKG ELSGHFEDLL LAIVNCVRNT
  251 PAFLAERLHR ALKGIGTDEF TLNRIMVSRS EIDLLDIRTE FKKHYGYSLY
  301 SAIKSDTSGD YEITLLKICG GDD
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
1 - 9	1086.5714	1085.5641	1085.5440	19	0	-.MASIWVGHR.G Methyl (C-term);
10 - 29	2237.1201	2236.1128	2236.1012	5	1	R.GTVRDYPDFSPSVDAEAIQK.A 3 Met
14 - 29	1781.8187	1780.8115	1780.8156	-2	0	R.DYPDFSPSVDAEAIQK.A (No match
14 - 29	1795.8325	1794.8252	1794.8312	-3	0	R.DYPDFSPSVDAEAIQK.A Methyl (C
14 - 29	1837.8462	1836.8389	1836.8782	-21	0	R.DYPDFSPSVDAEAIQK.A Methyl (C
40 - 48	1075.5896	1074.5823	1074.6107	-26	0	K.MLISILTER.S (No match)
40 - 48	1089.6038	1088.5965	1088.6263	-27	0	K.MLISILTER.S Methyl (C-term)
40 - 48	1091.6007	1090.5934	1090.6056	-11	0	K.MLISILTER.S Oxidation (M) (N
40 - 48	1105.6051	1104.5978	1104.6212	-21	0	K.MLISILTER.S Methyl (C-term);
59 - 66	943.4460	942.4388	942.4447	-6	0	K.EYQAAYGK.E Methyl (C-term) (
59 - 66	957.4581	956.4508	956.4603	-10	0	K.EYQAAYGK.E Methyl (C-term);
59 - 69	1313.6543	1312.6470	1312.6663	-15	1	K.EYQAAYGKELK.D Methyl (C-term
105 - 120	1673.8596	1672.8523	1672.8632	-6	0	K.GAGTNEDALIEILTTR.T (No match
124 - 137	1751.8220	1750.8147	1750.8600	-26	1	R.QMKDISQAYYTVYK.K Methyl (C-t
127 - 137	1350.6501	1349.6429	1349.6503	-6	0	K.DISQAYYTVYK.K (No match)
127 - 137	1378.6162	1377.6089	1377.6816	-53	0	K.DISQAYYTVYK.K Methyl (C-term
155 - 163	929.5243	928.5170	928.5342	-18	0	K.ALLTLADGR.R (No match)
177 - 184	989.4698	988.4625	988.5229	-61	0	K.QDAQILYK.A Gln->pyro-Glu (N-
190 - 204	1882.8972	1881.8899	1881.8931	-2	1	R.WGTDEDKFTEILCLR.S (No match)
190 - 204	1896.9103	1895.9030	1895.9087	-3	1	R.WGTDEDKFTEILCLR.S Methyl (C-
197 - 204	1051.5464	1050.5391	1050.5532	-13	0	K.FTEILCLR.S (No match)

197 - 210	1779.8778	1778.8705	1778.9753	-59	1	K.FTEILCLRSFPQLK.L	Methyl (C-t
211 - 217	943.4000	942.3927	942.4447	-55	0	K.LTFDEYR.N	(Ions score 38)
211 - 217	957.4000	956.3927	956.4603	-71	0	K.LTFDEYR.N	Methyl (DE) (Ions
249 - 257	1018.5225	1017.5152	1017.5243	-9	0	R.NTPAFLAER.L	(No match)
249 - 257	1032.5386	1031.5313	1031.5400	-8	0	R.NTPAFLAER.L	Methyl (C-term)
264 - 274	1222.6043	1221.5970	1221.5990	-2	0	K.GIGTDEFTLNR.I	(No match)
264 - 274	1236.6189	1235.6116	1235.6146	-2	0	K.GIGTDEFTLNR.I	Methyl (C-term
264 - 279	1808.8000	1807.7927	1807.9251	-73	1	K.GIGTDEFTLNRIMVSR.S	(No match
264 - 279	1808.8314	1807.8241	1807.9251	-56	1	K.GIGTDEFTLNRIMVSR.S	(No match
264 - 279	1822.8467	1821.8394	1821.9407	-56	1	K.GIGTDEFTLNRIMVSR.S	Methyl (C
264 - 279	1836.8611	1835.8538	1835.9564	-56	1	K.GIGTDEFTLNRIMVSR.S	Methyl (C
264 - 279	1838.8628	1837.8555	1837.9356	-44	1	K.GIGTDEFTLNRIMVSR.S	Methyl (C
280 - 288	1073.5658	1072.5585	1072.5764	-17	0	R.SEIDLLDIR.T	(No match)
280 - 288	1087.5850	1086.5777	1086.5921	-13	0	R.SEIDLLDIR.T	Methyl (C-term)
293 - 304	1429.7387	1428.7314	1428.7401	-6	1	K.KHYGYSLYSAIK.S	(No match)
294 - 304	1301.6000	1300.5927	1300.6452	-40	0	K.HYGYSLYSAIK.S	(Ions score 80
294 - 304	1301.6470	1300.6397	1300.6452	-4	0	K.HYGYSLYSAIK.S	(No match)
305 - 317	1441.7202	1440.7129	1440.6984	10	0	K.SDTSGDYEITLLK.I	(No match)
305 - 317	1455.7221	1454.7148	1454.7141	0	0	K.SDTSGDYEITLLK.I	Methyl (DE)



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LOCUS       NP_005130                323 aa            linear   PRI 03-SEP-2007
DEFINITION  annexin A3 [Homo sapiens].
ACCESSION   NP_005130
VERSION     NP_005130.1  GI:4826643
DBSOURCE    REFSEQ: accession NM_005139.2
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 323)
  AUTHORS   Park,J.E., Lee,D.H., Lee,J.A., Park,S.G., Kim,N.S., Park,B.C. and
            Cho,S.
  TITLE     Annexin A3 is a potential angiogenic mediator
  JOURNAL   Biochem. Biophys. Res. Commun. 337 (4), 1283-1287 (2005)
   PUBMED   16236264
  REMARK    GeneRIF: ANXA3 is a novel angiogenic factor that induces vascular
            endothelial growth factor production through the hypoxia-inducible
            factor-1 pathway
REFERENCE   2  (residues 1 to 323)
  AUTHORS   Bruneel,A., Labas,V., Mailloux,A., Sharma,S., Royer,N., Vinh,J.,
            Pernet,P., Vaubourdolle,M. and Baudin,B.
  TITLE     Proteomics of human umbilical vein endothelial cells applied to
            etoposide-induced apoptosis
  JOURNAL   Proteomics 5 (15), 3876-3884 (2005)
   PUBMED   16130169
REFERENCE   3  (residues 1 to 323)
  AUTHORS   Stelzl,U., Worm,U., Lalowski,M., Haenig,C., Brembeck,F.H.,
            Goehler,H., Stroedicke,M., Zenkner,M., Schoenherr,A., Koeppen,S.,
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            Goedde,A., Toksoz,E., Droege,A., Krobitsch,S., Korn,B.,
            Birchmeier,W., Lehrach,H. and Wanker,E.E.
  TITLE     A human protein-protein interaction network: a resource for
            annotating the proteome
```

Mascot Search Results

Protein View

Match to: **gi|119625664** Score: **164** Expect: **7.7e-12**
annexin A5, isoform CRA_c [Homo sapiens]

Nominal mass (M_r): **32996**; Calculated pI value: **4.95**
NCBI BLAST search of gi|119625664 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Glu->pyro-Glu (N-term E),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **84%**

Matched peptides shown in **Bold Red**

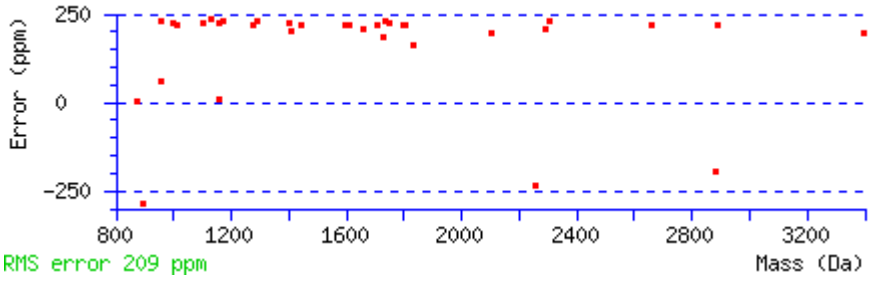
```
     1 MKGLGTDEES ILTLLTSRSN AQRQEISAAF KTLFGRDLLD DLKSELTGKF
    51 EKLIVALMKP SRLYDAYELK HALKGAGTNE KVLTEIIASR TPEELRAIKQ
   101 VYEEEYGSSL EDDVVGDTSG YYQRMLVVLL QANRDPDAGI DEAQVEQDAQ
   151 ALFQAGELKW GTDEEKFITI FGTRSVSHLR KVFDKYMTIS GFQIEETIDR
   201 ETSGNLEQLL LAVVKSIRSI PAYLAETLYY AMKGAGTDDH TLIRVMVSRS
   251 EIDLFNIRKE FRKNFATSLY SMIKGDTSGD YKKALLLLCG EDD
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
2 - 18	1833.2894	1832.2821	1831.9891	160	1	M.KGLGTDEESILTLLTSR.S (No matc
3 - 18	1705.2751	1704.2678	1703.8941	219	0	K.GLGTDEESILTLLTSR.S (No match
3 - 23	2260.6448	2259.6375	2260.1659	-234	1	K.GLGTDEESILTLLTSRSNAQR.Q (No
24 - 31	876.4473	875.4400	875.4389	1	0	R.QEISAAFK.T Gln->pyro-Glu (N-
24 - 31	893.2166	892.2093	892.4654	-287	0	R.QEISAAFK.T (No match)
32 - 43	1406.0509	1405.0436	1404.7613	201	1	K.TLFGRDLLDDLK.S (No match)
37 - 49	1447.0825	1446.0752	1445.7613	217	1	R.DLLDDLKSELTGK.F (No match)
53 - 62	1127.9645	1126.9572	1126.6896	238	0	K.LIVALMKPSR.L (No match)
63 - 70	1014.7349	1013.7276	1013.5069	218	0	R.LYDAYELK.H (No match)
75 - 90	1659.2500	1658.2427	1657.8999	207	1	K.GAGTNEKVLTEIIASR.T (No match
82 - 90	1001.8231	1000.8158	1000.5917	224	0	K.VLTEIIASR.T (No match)
82 - 96	1727.2902	1726.2829	1725.9625	186	1	K.VLTEIIASRTPEELR.A (No match)
100 - 124	2888.8721	2887.8648	2887.2308	220	0	K.QVYEEEYGSSLEDDVVGDTSGYYQR.M
125 - 134	1156.7000	1155.6927	1155.6798	11	0	R.MLVVLLQANR.D (Ions score 25)
125 - 134	1156.9492	1155.9419	1155.6798	227	0	R.MLVVLLQANR.D (No match)
125 - 134	1172.9490	1171.9417	1171.6747	228	0	R.MLVVLLQANR.D Oxidation (M) (
135 - 159	2658.8389	2657.8316	2657.2457	221	0	R.DPDAGIDEAQVEQDAQALFQAGELK.W
160 - 174	1800.2906	1799.2833	1798.8890	219	1	K.WGTDEEKFITIFGTR.S (No match)
167 - 174	954.6000	953.5927	953.5335	62	0	K.FITIFGTR.S (Ions score 31)
167 - 174	954.7597	953.7524	953.5335	230	0	K.FITIFGTR.S (No match)
182 - 200	2292.5974	2291.5901	2291.1144	208	1	K.VFDKYMTISGFQIEETIDR.E (No ma
182 - 200	2308.6533	2307.6460	2307.1093	233	1	K.VFDKYMTISGFQIEETIDR.E Oxidat
186 - 200	1803.2620	1802.2547	1801.8556	221	0	K.YMTISGFQIEETIDR.E (No match)
186 - 215	3398.4246	3397.4173	3396.7486	197	1	K.YMTISGFQIEETIDRETSGNLEQLLLAV
201 - 215	1596.2495	1595.2422	1594.8930	219	0	R.ETSGNLEQLLLAVVK.S Glu->pyro-
201 - 215	1614.2596	1613.2523	1612.9036	216	0	R.ETSGNLEQLLLAVVK.S (No match)
216 - 233	2106.5098	2105.5025	2105.0867	198	1	K.SIRSIPAYLAETLYYAMK.G Oxidati
219 - 233	1734.2820	1733.2747	1732.8745	231	0	R.SIPAYLAETLYYAMK.G (No match)
219 - 233	1750.2719	1749.2646	1748.8695	226	0	R.SIPAYLAETLYYAMK.G Oxidation
219 - 244	2885.8726	2884.8653	2885.4269	-195	1	R.SIPAYLAETLYYAMKGAGTDDHTLIR.V

250 - 258	1106.8314	1105.8241	1105.5768	224	0	R.SEIDLFNIR.K (No match)
263 - 274	1403.0558	1402.0485	1401.7326	225	1	R.KNFATSLYSMIK.G (No match)
264 - 274	1274.9224	1273.9151	1273.6376	218	0	K.NFATSLYSMIK.G (No match)
264 - 274	1290.9353	1289.9280	1289.6326	229	0	K.NFATSLYSMIK.G Oxidation (M)



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LOCUS       EAX05259                 293 aa            linear   PRI 18-DEC-2006
DEFINITION  annexin A5, isoform CRA_c [Homo sapiens].
ACCESSION   EAX05259
VERSION     EAX05259.1  GI:119625664
DBSOURCE    accession CH471056.2
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 293)
  AUTHORS   Venter,J.C., Adams,M.D., Myers,E.W., Li,P.W., Mural,R.J.,
            Sutton,G.G., Smith,H.O., Yandell,M., Evans,C.A., Holt,R.A.,
            Gocayne,J.D., Amanatides,P., Ballew,R.M., Huson,D.H., Wortman,J.R.,
            Zhang,Q., Kodira,C.D., Zheng,X.H., Chen,L., Skupski,M.,
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            Flanigan,M., Florea,L., Halpern,A., Hannenhalli,S., Kravitz,S.,
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            Chandramouliswaran,I., Charlab,R., Chaturvedi,K., Deng,Z., Di
            Francesco,V., Dunn,P., Eilbeck,K., Evangelista,C., Gabrielian,A.E.,
            Gan,W., Ge,W., Gong,F., Gu,Z., Guan,P., Heiman,T.J., Higgins,M.E.,
            Ji,R.R., Ke,Z., Ketchum,K.A., Lai,Z., Lei,Y., Li,Z., Li,J.,
            Liang,Y., Lin,X., Lu,F., Merkulov,G.V., Milshina,N., Moore,H.M.,
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            Sjolander,K.V., Karlak,B., Kejariwal,A., Mi,H., Lazareva,B.,
            Hatton,T., Narechania,A., Diemer,K., Muruganujan,A., Guo,N.,
            Sato,S., Bafna,V., Istrail,S., Lippert,R., Schwartz,R., Walenz,B.,
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Mascot Search Results

Protein View

Match to: **gi|71773329** Score: **126** Expect: **4.9e-08**
annexin VI isoform 1 [Homo sapiens]

Nominal mass (M_r): **76168**; Calculated pI value: **5.42**
NCBI BLAST search of gi|71773329 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|113962 from Homo sapiens
gi|219551 from Homo sapiens
gi|16877589 from Homo sapiens
gi|119582088 from Homo sapiens
gi|119582090 from Homo sapiens
gi|123981414 from synthetic construct
gi|364966 from Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **19%**

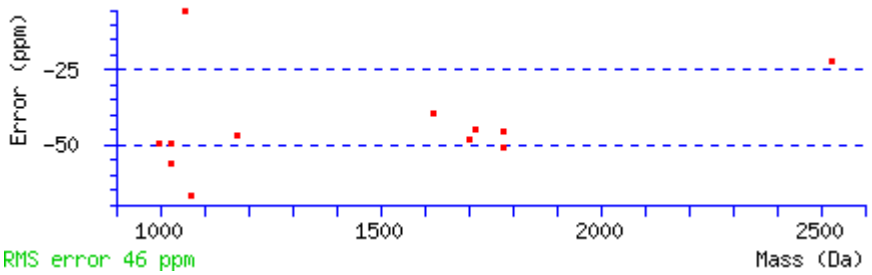
Matched peptides shown in **Bold Red**

```
     1 MAKPAQGAKY RGSIHDFPGF DPNQDAEALY TAMKGFGSDK EAILDIITSR
    51 SNRQRQEVCQ SYKSLYGKDL IADLKYELTG KFERLIVGLM RPPAYCDAKE
   101 IKDAISGIGT DEKCLIEILA SRTNEQMHQL VAAYKDAYER DLEADIIGDT
   151 SGHFQKMLVV LLQGTREEDD VVSEDLVQQD VQDLYEAGEL KWGTDEAQFI
   201 YILGNRSKQH LRLVFDEYLK TTGKPIEASI RGELSGDFEK LMLAVVKCIR
   251 STPEYFAERL FKAMKGLGTR DNTLIRIMVS RSELDMLDIR EIFRTKYEKS
   301 LYSMIKNDTS GEYKKTLLKL SGGDDDAAGQ FFPEAAQVAY QMWELSAVAR
   351 VELKGTVRPA NDFNPDADAK ALRKAMKGLG TDEDTIIDII THRSNVQRQQ
   401 IRQTFKSHFG RDLMTDLKSE ISGDLARLIL GLMMPPAHYD AKQLKKAMEG
   451 AGTDEKALIE ILATRTNAEI RAINEAYKED YHKSLEDALS SDTSGHFRRI
   501 LISLATGHRE EGGENLDQAR EDAQVAAEIL EIADTPSGDK TSLETRFMTI
   551 LCTRSYPHLR RVFQEFIKMT NYDVEHTIKK EMSGDVRDAF VAIVQSVKNK
   601 PLFFADKLYK SMKGAGTDEK TLTRIMVSRS EIDLLNIRRE FIEKYDKSLH
   651 QAIEGDTSGD FLKALLALCG GED
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
12 - 34	2524.0896	2523.0823	2523.1376	-22	0	R.GSIHDFPGFDPNQDAEALYTAMK.G (N
85 - 99	1703.8146	1702.8073	1702.8898	-48	0	R.LIVGLMRPPAYCDAK.E (No match)
192 - 206	1782.7905	1781.7833	1781.8737	-51	0	K.WGTDEAQFIYILGNR.S (No match)
192 - 206	1782.8000	1781.7927	1781.8737	-45	0	K.WGTDEAQFIYILGNR.S (Ions scor
213 - 220	1026.4928	1025.4855	1025.5433	-56	0	R.LVFDEYLK.T (No match)
213 - 220	1026.5000	1025.4927	1025.5433	-49	0	R.LVFDEYLK.T (Ions score 43)
457 - 465	999.5699	998.5626	998.6124	-50	0	K.ALIEILATR.T (No match)
484 - 498	1621.6815	1620.6742	1620.7380	-39	0	K.SLEDALSSDTSGHFR.R (No match)
547 - 554	1057.5113	1056.5041	1056.5096	-5	0	R.FMTILCTR.S Oxidation (M) (Nc
588 - 598	1176.6069	1175.5997	1175.6550	-47	0	R.DAFVAIVQSVK.N (No match)
630 - 638	1072.5276	1071.5203	1071.5924	-67	0	R.SEIDLLNIR.R (No match)
648 - 663	1717.7621	1716.7548	1716.8319	-45	0	K.SLHQAIEGDTSGDFLK.A (No match



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LOCUS       NP_001146                673 aa            linear   PRI 27-JUN-2007
DEFINITION  annexin VI isoform 1 [Homo sapiens].
ACCESSION   NP_001146
VERSION     NP_001146.2  GI:71773329
DBSOURCE    REFSEQ: accession NM_001155.3
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 673)
  AUTHORS   Chi,A., Valencia,J.C., Hu,Z.Z., Watabe,H., Yamaguchi,H.,
            Mangini,N.J., Huang,H., Canfield,V.A., Cheng,K.C., Yang,F., Abe,R.,
            Yamagishi,S., Shabanowitz,J., Hearing,V.J., Wu,C., Appella,E. and
            Hunt,D.F.
  TITLE     Proteomic and bioinformatic characterization of the biogenesis and
            function of melanosomes
  JOURNAL   J. Proteome Res. 5 (11), 3135-3144 (2006)
   PUBMED   17081065
REFERENCE   2  (residues 1 to 673)
  AUTHORS   Balcerzak,M., Bandorowicz-Pikula,J., Buchet,R. and Pikula,S.
  TITLE     A novel retinoid binding property of human annexin A6
  JOURNAL   FEBS Lett. 580 (13), 3065-3069 (2006)
   PUBMED   16674946
  REMARK    GeneRIF: It is a first report supporting the hypothesis of a direct
            implication of AnxA6 in vitamin A-dependent tissue mineralization.
REFERENCE   3  (residues 1 to 673)
  AUTHORS   Cuschieri,J., Bulger,E., Garcia,I. and Maier,R.V.
  TITLE     Oxidative-induced calcium mobilization is dependent on annexin VI
            release from lipid rafts
  JOURNAL   Surgery 138 (2), 158-164 (2005)
   PUBMED   16153422
  REMARK    GeneRIF: Macrophage reprogramming during oxidative stress occurs
            through the cytosolic mobilization of annexin VI from lipid rafts.
REFERENCE   4  (residues 1 to 673)
  AUTHORS   Tiacci,E., Orvietani,P.L., Bigerna,B., Pucciarini,A.,
            Corthals,G.L., Pettirossi,V., Martelli,M.P., Liso,A., Benedetti,R.,
            Pacini,R., Bolli,N., Pileri,S., Pulford,K., Gambacorta,M.,
            Carbone,A., Pasquarello,C., Scherl,A., Robertson,H., Sciurpi,M.T.,
            Alunni-Bistocchi,G., Binaglia,L., Byrne,J.A. and Falini,B.
  TITLE     Tumor protein D52 (TPD52): a novel B-cell/plasma-cell molecule with
            unique expression pattern and Ca(2+)-dependent association with
            annexin VI
  JOURNAL   Blood 105 (7), 2812-2820 (2005)
   PUBMED   15576473
  REMARK    GeneRIF: TPD52 bound to annexin VI in a Ca(2+)-dependent manner
            suggesting that these molecules may act in concert to regulate
            secretory processes in plasma cells
REFERENCE   5  (residues 1 to 673)
  AUTHORS   Riquelme,G., Llanos,P., Tischner,E., Neil,J. and Campos,B.
  TITLE     Annexin 6 modulates the maxi-chloride channel of the apical
            membrane of syncytiotrophoblast isolated from human placenta
  JOURNAL   J. Biol. Chem. 279 (48), 50601-50608 (2004)
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Mascot Search Results

Protein View

Match to: **gi|62897671** Score: **311** Expect: **1.5e-26**
beta actin variant [Homo sapiens]

Nominal mass (M_r): **42036**; Calculated pI value: **5.29**
NCBI BLAST search of gi|62897671 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **64%**

Matched peptides shown in **Bold Red**

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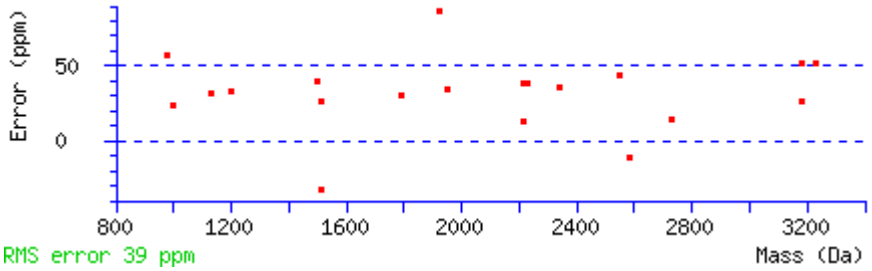
    1 MDDDIAALVV DNGSGMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK
   51 DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEKIWHHTF YNELRVAPEE
  101 HPVLLTEAPL NPKANREKMT QIMFETFNTP AMYVAIQAVL SLYASGRTTG
  151 IVMDSGDGVT HTVPIYEGYA LPHAILRLDL AGRDLTDYLM KILTERGYSF
  201 TTTAERFIVR DIKEKLCYVA LDFEQEMATA ASSSSLEKSY ELPDGQVITI
  251 GNERFRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR KDLYANTVLS
  301 GGTTMYPGIA DRMQKEITAL APSTMKIKII APPERKYSVW IGGSIPASLS
  351 TFQQMWISKQ EYDESGPSIV HRKCF
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
1 - 18	1926.9918	1925.9845	1925.8169	87	0	-.MDDDIAALVVDNGSGMCK.A Oxidati
19 - 28	976.5035	975.4963	975.4410	57	0	K.AGFAGDDAPR.A (No match)
29 - 39	1198.7454	1197.7381	1197.6982	33	0	R.AVFPSIVGRPR.H (No match)
85 - 95	1515.7000	1514.6927	1514.7419	-32	0	K.IWHHTFYNELR.V (Ions score 73
85 - 95	1515.7898	1514.7825	1514.7419	27	0	K.IWHHTFYNELR.V (No match)
96 - 113	1954.1309	1953.1236	1953.0571	34	0	R.VAPEEHPVLLTEAPLNPK.A (No mat
148 - 177	3183.7000	3182.6927	3182.6071	27	0	R.TTGIVMDSGDGVTHTVPIYEGYALPHAIL
148 - 177	3183.7778	3182.7706	3182.6071	51	0	R.TTGIVMDSGDGVTHTVPIYEGYALPHAIL
184 - 191	998.5098	997.5025	997.4790	24	0	R.DLTDYLMK.I (No match)
197 - 206	1132.5631	1131.5558	1131.5197	32	0	R.GYSFTTTAER.E (No match)
216 - 238	2550.2844	2549.2771	2549.1665	43	0	K.LCYVALDFEQEMATAASSSSLEK.S (N
239 - 254	1790.9457	1789.9384	1789.8846	30	0	K.SYELPDGQVITIGNER.F (No match
257 - 284	3231.6306	3230.6233	3230.4545	52	0	R.CPEALFQPSFLGMESCGIHETTFNSIMK.
291 - 312	2343.2488	2342.2415	2342.1576	36	1	R.KDLYANTVLSGGTTMYPGIADR.M (No
292 - 312	2215.1000	2214.0927	2214.0627	14	0	K.DLYANTVLSGGTTMYPGIADR.M (Ion
292 - 312	2215.1550	2214.1478	2214.0627	38	0	K.DLYANTVLSGGTTMYPGIADR.M (No
292 - 312	2231.1494	2230.1421	2230.0576	38	0	K.DLYANTVLSGGTTMYPGIADR.M Oxid
336 - 359	2730.4353	2729.4280	2729.3887	14	1	R.KYSVWIGGSIPASLSTFQQMWISK.Q C
337 - 359	2586.2788	2585.2715	2585.2988	-11	0	K.YSVWIGGSIPASLSTFQQMWISK.Q (N
360 - 372	1499.7358	1498.7286	1498.6688	40	0	K.QEYDESGPSIVHR.K Gln->pyro-Gl



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DEFINITION  beta actin variant [Homo sapiens].
ACCESSION   BAD96775
VERSION     BAD96775.1  GI:62897671
DBSOURCE    accession AK223055.1
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1
  AUTHORS   Maruyama,K. and Sugano,S.
  TITLE     Oligo-capping: a simple method to replace the cap structure of
            eukaryotic mRNAs with oligoribonucleotides
  JOURNAL   Gene 138 (1-2), 171-174 (1994)
   PUBMED   8125298
REFERENCE   2
  AUTHORS   Suzuki,Y., Yoshitomo-Nakagawa,K., Maruyama,K., Suyama,A. and
            Sugano,S.
  TITLE     Construction and characterization of a full length-enriched and a
            5'-end-enriched cDNA library
  JOURNAL   Gene 200 (1-2), 149-156 (1997)
   PUBMED   9373149
REFERENCE   3  (residues 1 to 375)
  AUTHORS   Suzuki,Y., Sugano,S., Totoki,Y., Toyoda,A., Takeda,T., Sakaki,Y.,
            Tanaka,A. and Yokoyama,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-APR-2005) Akiko Tanaka, RIKEN Yokohama Institute,
            Protein Research Group; 1-7-22 Suehiro, Tsurumi, Yokohama, Kanagawa
            230-0045, Japan (E-mail:aktanaka@riken.jp,
            URL:http://protein.gsc.riken.jp/, Tel:81-45-503-9452,
            Fax:81-45-503-9450)
COMMENT     This work was supported in part by the National Project on Protein
            Structural and Functional Analysis, Ministry of Education, Culture,
            Sports, Science and Technology of Japan.
            Sumio Sugano, Yutaka Suzuki
            Laboratory of Functional Genomics Department of Medical Genome
            Sciences Graduate School of Frontier Sciences The University of
            Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639 Japan email:
            ssugano@k.u-tokyo.ac.jp
            URL: http://www.k.u-tokyo.ac.jp/index.html.en.
FEATURES             Location/Qualifiers
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                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
                     /clone="KAT00430"
                     /cell_line="KATO III"
                     /clone_lib="KAT"
                     /note="cloning vector: pME18SFL3
                     this clone is also named as hss001002538"
     Protein         1..375
                     /product="beta actin variant"
     Region          3..375
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Mascot Search Results

Protein View

Match to: **gi|5031635** Score: **128** Expect: **3.1e-08**
cofilin 1 (non-muscle) [Homo sapiens]

Nominal mass (M_r): **18719**; Calculated pI value: **8.22**
NCBI BLAST search of gi|5031635 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|57099669 from Canis lupus familiaris
gi|114638608 from Pan troglodytes
gi|149725588 from Equus caballus
gi|116848 from Homo sapiens
gi|50513339 from Homo sapiens
gi|50513340 from Homo sapiens
gi|219545 from Homo sapiens
gi|736400 from Homo sapiens
gi|1177471 from Homo sapiens
gi|15012201 from Homo sapiens
gi|15126676 from Homo sapiens
gi|15147369 from Homo sapiens
gi|17390594 from Homo sapiens
gi|30582531 from Homo sapiens
gi|61360378 from synthetic construct
gi|119594855 from Homo sapiens
gi|119594858 from Homo sapiens
gi|123981732 from synthetic construct

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Methyl (C-term),Methyl (DE),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **67%**

Matched peptides shown in **Bold Red**

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     1 MASGVAVSDG VIKVFNDMKV RKSSTPEEVK KRKKAVLFCL SEDKKNIILE
    51 EGKEILVGDV GQTVDDPYAT FVKMLPDKDC RYALYDATYE TKESKKEDLV
   101 FIFWAPESAP LKSKMIYASS KDAIKKKLTG IKHELQANCY EEVKDRCTLA
   151 EKLGGSAVIS LEGKPL
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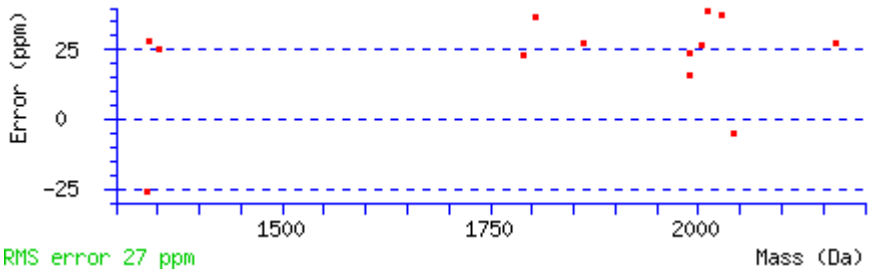
Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
1 - 19	2012.0975	2011.0903	2011.0119	39	1	-.MASGVAVSDGVIKVFNDMK.V 2 Meth
1 - 19	2028.0907	2027.0834	2027.0068	38	1	-.MASGVAVSDGVIKVFNDMK.V 2 Meth
35 - 45	1337.6782	1336.6709	1336.7061	-26	1	K.AVLFCLSEDKK.N Methyl (C-term
54 - 73	2166.1555	2165.1482	2165.0892	27	0	K.EILVGDVGQTVDDPYATFVK.M (No m
82 - 92	1351.6759	1350.6686	1350.6343	25	0	R.YALYDATYETK.E Methyl (DE) (N
96 - 112	1990.1000	1989.0927	1989.0611	16	1	K.KEDLVFIFWAPESAPLK.S (Ions sc
96 - 112	1990.1151	1989.1078	1989.0611	23	1	K.KEDLVFIFWAPESAPLK.S (No matc
96 - 112	2004.1371	2003.1298	2003.0768	26	1	K.KEDLVFIFWAPESAPLK.S Methyl (
97 - 112	1862.0243	1861.0170	1860.9662	27	0	K.EDLVFIFWAPESAPLK.S (No match
133 - 146	1790.8539	1789.8466	1789.8053	23	1	K.HELQANCYEEVKDR.C (No match)
133 - 146	1804.8946	1803.8874	1803.8210	37	1	K.HELQANCYEEVKDR.C Methyl (DE)
147 - 166	2043.1051	2042.0978	2042.1082	-5	1	R.CTLAEKLGGSAVISLEGKPL.- (No m
153 - 166	1340.8156	1339.8083	1339.7711	28	0	K.LGGSAVISLEGKPL.- (No match)



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LOCUS       NP_005498                166 aa            linear   PRI 03-SEP-2007
DEFINITION  cofilin 1 (non-muscle) [Homo sapiens].
ACCESSION   NP_005498
VERSION     NP_005498.1  GI:5031635
DBSOURCE    REFSEQ: accession NM_005507.2
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 166)
  AUTHORS   Mannherz,H.G., Ballweber,E., Galla,M., Villard,S., Granier,C.,
            Steegborn,C., Schmidtmann,A., Jaquet,K., Pope,B. and Weeds,A.G.
  TITLE     Mapping the ADF/cofilin binding site on monomeric actin by
            competitive cross-linking and peptide array: evidence for a second
            binding site on monomeric actin
  JOURNAL   J. Mol. Biol. 366 (3), 745-755 (2007)
   PUBMED   17196218
  REMARK    GeneRIF: In the absence of any crystal structures of ADF or cofilin
            in complex with actin, these studies provide further information
            about the binding sites on F-actin for these important actin
            regulatory proteins.
REFERENCE   2  (residues 1 to 166)
  AUTHORS   Ewing,R.M., Chu,P., Elisma,F., Li,H., Taylor,P., Climie,S.,
            McBroom-Cerajewski,L., Robinson,M.D., O'Connor,L., Li,M.,
            Taylor,R., Dharsee,M., Ho,Y., Heilbut,A., Moore,L., Zhang,S.,
            Ornatsky,O., Bukhman,Y.V., Ethier,M., Sheng,Y., Vasilescu,J.,
            Abu-Farha,M., Lambert,J.P., Duewel,H.S., Stewart,I.I., Kuehl,B.,
            Hogue,K., Colwill,K., Gladwish,K., Muskat,B., Kinach,R.,
            Adams,S.L., Moran,M.F., Morin,G.B., Topaloglou,T. and Figeys,D.
  TITLE     Large-scale mapping of human protein-protein interactions by mass
            spectrometry
  JOURNAL   Mol. Syst. Biol. 3, 89 (2007)
   PUBMED   17353931
REFERENCE   3  (residues 1 to 166)
  AUTHORS   Zhu,H., Enaw,J.O., Ma,C., Shaw,G.M., Lammer,E.J. and Finnell,R.H.
  TITLE     Association between CFL1 gene polymorphisms and spina bifida risk
            in a California population
  JOURNAL   (er) BMC Med. Genet. 8, 12 (2007)
   PUBMED   17352815
  REMARK    GeneRIF: The sequence variation of human CFL1 gene is a genetic
            modifier for spina bifida risk in California population
REFERENCE   4  (residues 1 to 166)
  AUTHORS   Olsen,J.V., Blagoev,B., Gnad,F., Macek,B., Kumar,C., Mortensen,P.
            and Mann,M.
  TITLE     Global, in vivo, and site-specific phosphorylation dynamics in
            signaling networks
  JOURNAL   Cell 127 (3), 635-648 (2006)
   PUBMED   17081983
REFERENCE   5  (residues 1 to 166)
  AUTHORS   Vasavda,N., Eichholtz,T., Takahashi,A., Affleck,K., Matthews,J.G.,
            Barnes,P.J. and Adcock,I.M.
  TITLE     Expression of nonmuscle cofilin-1 and steroid responsiveness in
```

Mascot Search Results

Protein View

Match to: **gi|4503571** Score: **147** Expect: **3.9e-10**
enolase 1 [Homo sapiens]

Nominal mass (M_r): **47481**; Calculated pI value: **7.01**
NCBI BLAST search of gi|4503571 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|114552694 from Pan troglodytes
gi|119339 from Homo sapiens
gi|182114 from Homo sapiens
gi|1167843 from Homo sapiens
gi|12804749 from Homo sapiens
gi|13325287 from Homo sapiens
gi|14530765 from Homo sapiens
gi|14602814 from Homo sapiens
gi|15029814 from Homo sapiens
gi|15990505 from Homo sapiens
gi|18490320 from Homo sapiens
gi|20379496 from Homo sapiens
gi|29792061 from Homo sapiens
gi|30583165 from Homo sapiens
gi|60655645 from synthetic construct
gi|60655647 from synthetic construct
gi|60812591 from synthetic construct
gi|61354812 from synthetic construct
gi|66268795 from Homo sapiens
gi|119592010 from Homo sapiens
gi|119592011 from Homo sapiens
gi|123981076 from synthetic construct
gi|123995887 from synthetic construct

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **49%**

Matched peptides shown in **Bold Red**

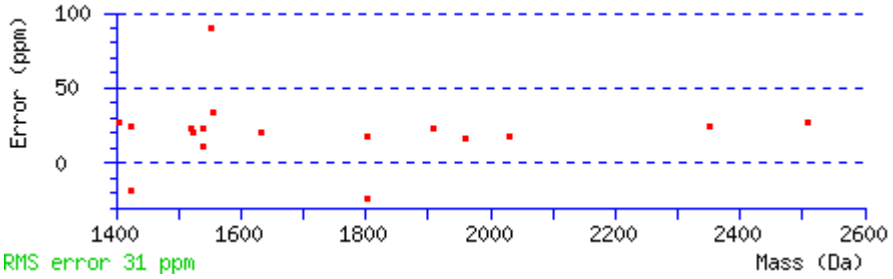
```
     1 MSILKIHARE IFDSRGNPTV EVDLFTSKGL FRAAVPSGAS TGIYEALELR
    51 DNDKTRYMGK GVSKAVEHIN KTIAPALVSK KLNVTEQEKI DKLMIEMDGT
   101 ENKSKFGANA ILGVSLAVCK AGAVEKGVPL YRHIADLAGN SEVILPVPAF
   151 NVINGGSHAG NKLAMQEFMI LPVGAANFRE AMRIGAEVYH NLKNVIKEKY
   201 GKDATNVGDE GGFAPNILEN KEGLELLKTA IGKAGYTDKV VIGMDVAASE
   251 FFRSGKYDLD FKSPDDPSRY ISPDQLADLY KSFIKDYPVV SIEDPFDQDD
   301 WGAWQKFTAS AGIQVVGDDL TVTNPKRIAK AVNEKSCNCL LLKVNQIGSV
   351 TESLQACKLA QANGWGVMVS HRSGETEDTF IADLVVGLCT GQIKTGAPCR
   401 SERLAKYNQL LRIEEELGSK AKFAGRNFRN PLAK
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence	
16 - 28	1406.7549	1405.7476	1405.7089	28	0	R.GNPTVEVDLFTSK.G	(No match)
33 - 50	1804.9000	1803.8927	1803.9366	-24	0	R.AAVPSGASTGIYEALELR.D	(Ions s
33 - 50	1804.9761	1803.9688	1803.9366	18	0	R.AAVPSGASTGIYEALELR.D	(No mat

106 - 120	1519.8656	1518.8583	1518.8228	23	0	K.FGANAILGVSLAVCK.A	(No match)
163 - 179	1908.0308	1907.0235	1906.9797	23	0	K.LAMQEFMILPVGAANFR.E	(No matc
203 - 221	1960.9574	1959.9501	1959.9174	17	0	K.DATNVGDEGGFAPNILENK.E	(No ma
240 - 253	1540.8000	1539.7927	1539.7756	11	0	K.VVIGMDVAASEFFR.S	(Ions score
240 - 253	1540.8185	1539.8112	1539.7756	23	0	K.VVIGMDVAASEFFR.S	(No match)
240 - 253	1556.8298	1555.8226	1555.7705	33	0	K.VVIGMDVAASEFFR.S	Oxidation (
257 - 269	1554.8475	1553.8403	1553.6998	90	1	K.YDLDFKSPDDPSR.Y	(No match)
270 - 281	1425.7000	1424.6927	1424.7187	-18	0	R.YISPDQLADLYK.S	(Ions score 2
270 - 281	1425.7615	1424.7542	1424.7187	25	0	R.YISPDQLADLYK.S	(No match)
286 - 306	2510.1809	2509.1736	2509.1074	26	0	K.DYPVVSIEDPFDQDDWGAWQK.F	(No
307 - 326	2033.0896	2032.0823	2032.0477	17	0	K.FTASAGIQVVGDDLTVTNPK.R	(No m
344 - 358	1633.8552	1632.8479	1632.8141	21	0	K.VNQIGSVTESLQACK.L	(No match)
359 - 372	1525.8003	1524.7930	1524.7620	20	0	K.LAQANGWGVMVSHR.S	(No match)
373 - 394	2353.2168	2352.2095	2352.1519	24	0	R.SGETEDTFIADLVVGLCTGQIK.T	(No



```
LOCUS       NP_001419                434 aa            linear   PRI 29-JUL-2007
DEFINITION  enolase 1 [Homo sapiens].
ACCESSION   NP_001419 NP_005936
VERSION     NP_001419.1  GI:4503571
DBSOURCE    REFSEQ: accession NM_001428.2
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 434)
  AUTHORS   Newman,S.F., Sultana,R., Perluigi,M., Coccia,R., Cai,J.,
            Pierce,W.M., Klein,J.B., Turner,D.M. and Butterfield,D.A.
  TITLE     An increase in S-glutathionylated proteins in the Alzheimer's
            disease inferior parietal lobule, a proteomics approach
  JOURNAL   J. Neurosci. Res. 85 (7), 1506-1514 (2007)
   PUBMED   17387692
  REMARK    GeneRIF: alpha-crystallin B, glyceraldehyde phosphate dehydrogenase
            (GAPDH), and alpha-enolase identified as significantly
            S-glutathionylated in Alzheimer's disease infeior parietal lobule
REFERENCE   2  (residues 1 to 434)
  AUTHORS   Ito,S., Honma,T., Ishida,K., Wada,N., Sasaoka,S., Hosoda,M. and
            Nohno,T.
  TITLE     Differential expression of the human alpha-enolase gene in oral
            epithelium and squamous cell carcinoma
  JOURNAL   Cancer Sci. 98 (4), 499-505 (2007)
   PUBMED   17284257
  REMARK    GeneRIF: Our results indicate that differential subcellular
            localization of ENO1 products may be closely related to
            carcinogenesis of the oral epithelium
REFERENCE   3  (residues 1 to 434)
  AUTHORS   Ghosh,A.K., Steele,R. and Ray,R.B.
  TITLE     Knockdown of MBP-1 in human prostate cancer cells delays cell cycle
            progression
  JOURNAL   J. Biol. Chem. 281 (33), 23652-23657 (2006)
   PUBMED   16762917
  REMARK    GeneRIF: Depletion of MBP-1 in prostate cancer cells perturbs cell
            proliferation by inhibiting cyclin A and cyclin B1 expression.
REFERENCE   4  (residues 1 to 434)
```

Mascot Search Results

Protein View

Match to: **gi|809561** Score: **66** Expect: **0.18**
gamma-actin [Mus musculus]

Nominal mass (M_r): **41335**; Calculated pI value: **5.56**
NCBI BLAST search of gi|809561 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Mus musculus

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **66%**

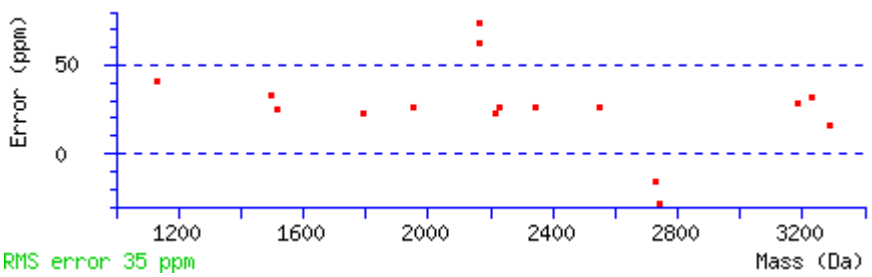
Matched peptides shown in **Bold Red**

```
     1 LVIDNGSGMC KAGFAGDDAP RAVFPSIVGR PRHQGVMVGM GQKDSYVGDE
    51 AQSKRGILTL KYPIEHGIVT NWDDMEKIWH HTFYNELRVA PEEHPVLLTE
   101 APLNPKANRE KMTQIMFETF NTPAMYVAIQ AVLSLYASGR TTGIVMDSGD
   151 GVTHTVPIYE GYALPHAILR LDLAGRDLTD YLMKILTERG YSFTTTAERE
   201 IVRDIKEKLC YVALDFEQEM ATAASSSSLE KSYELPDGQV ITIGNERFRC
   251 PEALFQPSFL GMESCGIHET TFNSIMKCDV DIRKDLYANT VLSGGTTMYP
   301 GIADRMQKEI TALAPSTMKI KIIAPPERKY SVWIGGSILA SLSTFQQMWI
   351 SKQEYDESGP SIVHRKCF
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
1 - 21	2167.1250	2166.1177	2165.9834	62	1	-.LVIDNGSGMCKAGFAGDDAPR.A Oxic
1 - 21	2167.1500	2166.1427	2165.9834	74	1	-.LVIDNGSGMCKAGFAGDDAPR.A Oxic
78 - 88	1515.7874	1514.7801	1514.7419	25	0	K.IWHHTFYNELR.V (No match)
89 - 106	1954.1155	1953.1082	1953.0571	26	0	R.VAPEEHPVLLTEAPLNPK.A (No mat
112 - 140	3285.6526	3284.6453	3284.5920	16	0	K.MTQIMFETFNTPAMYVAIQAVLSLYASGF
141 - 170	3183.7036	3182.6963	3182.6071	28	0	R.TTGIVMDSGDGVTHTVPIYEGYALPHAIL
190 - 199	1132.5737	1131.5665	1131.5197	41	0	R.GYSFTTTAER.E (No match)
209 - 231	2550.2407	2549.2334	2549.1665	26	0	K.LCYVALDFEQEMATAASSSSLEK.S (N
232 - 247	1790.9336	1789.9263	1789.8846	23	0	K.SYELPDGQVITIGNER.F (No match
250 - 277	3231.5635	3230.5562	3230.4545	31	0	R.CPEALFQPSFLGMESCGIHETTFNSIMK.
284 - 305	2343.2270	2342.2198	2342.1576	27	1	R.KDLYANTVLSGGTTMYPGIADR.M (No
285 - 305	2215.1201	2214.1128	2214.0627	23	0	K.DLYANTVLSGGTTMYPGIADR.M (No
285 - 305	2231.1235	2230.1163	2230.0576	26	0	K.DLYANTVLSGGTTMYPGIADR.M Oxic
329 - 352	2730.3877	2729.3804	2729.4251	-16	1	R.KYSVWIGGSILASLSTFQQMWISK.Q (
329 - 352	2746.3499	2745.3426	2745.4200	-28	1	R.KYSVWIGGSILASLSTFQQMWISK.Q C
353 - 365	1499.7263	1498.7190	1498.6688	33	0	K.QEYDESGPSIVHR.K Gln->pyro-Gl



Mascot Search Results

Protein View

Match to: **gi|4504165** Score: **108** Expect: **3.1e-06**
gelsolin isoform a precursor [Homo sapiens]

Nominal mass (M_r): **86043**; Calculated pI value: **5.90**
NCBI BLAST search of gi|4504165 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|121116 from Homo sapiens
gi|736249 from Homo sapiens
gi|19684181 from Homo sapiens
gi|55960299 from Homo sapiens
gi|61364370 from synthetic construct
gi|119607897 from Homo sapiens
gi|123982744 from synthetic construct
gi|225304 from Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **22%**

Matched peptides shown in **Bold Red**

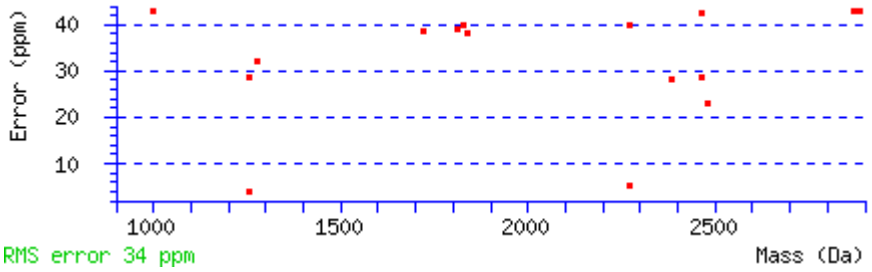
```
     1 MAPHRPAPAL LCALSLALCA LSLPVRAATA SRGASQAGAP QGRVPEARPN
    51 SMVVEHPEFL KAGKEPGLQI WRVEKFDLVP VPTNLYGDFF TGDAYVILKT
   101 VQLRNGNLQY DLHYWLGNEC SQDESGAAAI FTVQLDDYLN GRAVQHREVQ
   151 GFESATFLGY FKSGLKYKKG GVASGFKHVV PNEVVVQRLF QVKGRRVVRA
   201 TEVPVSWESF NNGDCFILDL GNNIHQWCGS NSNRYERLKA TQVSKGIRDN
   251 ERSGRARVHV SEEGTEPEAM LQVLGPKPAL PAGTEDTAKE DAANRKLAKL
   301 YKVSNGAGTM SVSLVADENP FAQGALKSED CFILDHGKDG KIFVWKGKQA
   351 NTEERKAALK TASDFITKMD YPKQTQVSVL PEGGETPLFK QFFKNWRDPD
   401 QTDGLGLSYL SSHIANVERV PFDAATLHTS TAMAAQHGMD DDGTGQKQIW
   451 RIEGSNKVPV DPATYGQFYG GDSYIILYNY RHGGRQGQII YNWQGAQSTQ
   501 DEVAASAILT AQLDEELGGT PVQSRVVQGK EPAHLMSLFG GKPMIIYKGG
   551 TSREGGQTAP ASTRLFQVRA NSAGATRAVE VLPKAGALNS NDAFVLKTPS
   601 AAYLWVGTGA SEAEKTGAQE LLRVLRAQPV QVAEGSEPDG FWEALGGKAA
   651 YRTSPRLKDK KMDAHPPRLF ACSNKIGRFV IEEVPGELMQ EDLATDDVML
   701 LDTWDQVFVW VGKDSQEEEK TEALTSAKRY IETDPANRDR RTPITVVKQG
   751 FEPPSFVGWF LGWDDDYWSV DPLDRAMAEL AA
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
62 - 72	1254.7000	1253.6927	1253.6880	4	1	K.AGKEPGLQIWR.V (No match)
62 - 72	1254.7311	1253.7238	1253.6880	29	1	K.AGKEPGLQIWR.V (No match)
65 - 72	998.5847	997.5774	997.5345	43	0	K.EPGLQIWR.V (No match)
148 - 162	1722.9037	1721.8964	1721.8301	39	0	R.EVQGFESATFLGYFK.S (No match)
178 - 188	1275.7577	1274.7504	1274.7095	32	0	K.HVVPNEVVVQR.L (No match)
303 - 327	2463.3230	2462.3157	2462.2111	42	0	K.VSNGAGTMSVSLVADENPFAQGALK.S
369 - 390	2464.3137	2463.3064	2463.2356	29	1	K.MDYPKQTQVSVLPEGGETPLFK.Q (No
369 - 390	2480.2944	2479.2872	2479.2305	23	1	K.MDYPKQTQVSVLPEGGETPLFK.Q Oxi
374 - 390	1813.0083	1812.0010	1811.9306	39	0	K.QTQVSVLPEGGETPLFK.Q Gln->pyr
374 - 390	1830.0370	1829.0297	1828.9571	40	0	K.QTQVSVLPEGGETPLFK.Q (No matc
398 - 419	2387.2151	2386.2078	2386.1401	28	0	R.DPDQTDGLGLSYLSSHIANVER.V (No

420 - 447	2873.4060	2872.3987	2872.2756	43	0	R.VPFDAATLHTSTAMAAQHGMDDDGTGQK.
420 - 447	2889.4016	2888.3943	2888.2706	43	0	R.VPFDAATLHTSTAMAAQHGMDDDGTGQK.
598 - 615	1837.9666	1836.9593	1836.8894	38	0	K.TPSAAYLWVGTGASEAEK.T (No mat
627 - 648	2272.1000	2271.0927	2271.0808	5	0	R.AQPVQVAEGSEPDGFWEALGGK.A (Ic
627 - 648	2272.1790	2271.1717	2271.0808	40	0	R.AQPVQVAEGSEPDGFWEALGGK.A (Nc



```
LOCUS       NP_000168                782 aa            linear   PRI 30-JUL-2007
DEFINITION  gelsolin isoform a precursor [Homo sapiens].
ACCESSION   NP_000168
VERSION     NP_000168.1  GI:4504165
DBSOURCE    REFSEQ: accession NM_000177.4
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 782)
  AUTHORS   Huerva,V., Velasco,A., Sanchez,M.C., Mateo,A.J. and Matias-Guiu,X.
  TITLE     Lattice corneal dystrophy type II: clinical, pathologic, and
            molecular study in a Spanish family
  JOURNAL   Eur J Ophthalmol 17 (3), 424-429 (2007)
   PUBMED   17534828
  REMARK    GeneRIF: A new family with Meretoja syndrome is reported. This is
            the first documented family with Meretoja syndrome in Spain and in
            the Mediterranean countries. The molecular study shows the same
            mutation of reported families.
REFERENCE   2  (residues 1 to 782)
  AUTHORS   Frum,R., Busby,S.A., Ramamoorthy,M., Deb,S., Shabanowitz,J.,
            Hunt,D.F. and Deb,S.P.
  TITLE     HDM2-binding partners: interaction with translation elongation
            factor EF1alpha
  JOURNAL   J. Proteome Res. 6 (4), 1410-1417 (2007)
   PUBMED   17373842
REFERENCE   3  (residues 1 to 782)
  AUTHORS   Roustan,C., Ferjani,I., Maciver,S.K., Fattoum,A., Rebiere,B. and
            Benyamin,Y.
  TITLE     Calcium-induced conformational changes in the amino-terminal half
            of gelsolin
  JOURNAL   FEBS Lett. 581 (4), 681-686 (2007)
   PUBMED   17258204
  REMARK    GeneRIF: Calcium induced conformational changes in the G1-2 and
            G1-3 sub-domains of gelsolin, and the binding affinities for the
            three type II sites, are reported.
REFERENCE   4  (residues 1 to 782)
  AUTHORS   Qiao,H. and McMillan,J.R.
  TITLE     Gelsolin segment 5 inhibits HIV-induced T-cell apoptosis via
            Vpr-binding to VDAC
  JOURNAL   FEBS Lett. 581 (3), 535-540 (2007)
   PUBMED   17254575
  REMARK    GeneRIF: gelsolin G5 domain inhibits HIV-Vpr-induced T-cell
            apoptosis by blocking the interaction between Vpr and VDAC
REFERENCE   5  (residues 1 to 782)
  AUTHORS   Ewing,R.M., Chu,P., Elisma,F., Li,H., Taylor,P., Climie,S.,
            McBroom-Cerajewski,L., Robinson,M.D., O'Connor,L., Li,M.,
```

Mascot Search Results

Protein View

Match to: **gi|38044288** Score: **111** Expect: **1.5e-06**
gelsolin isoform b [Homo sapiens]

Nominal mass (M_r): **80876**; Calculated pI value: **5.58**
NCBI BLAST search of gi|38044288 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|119607895 from Homo sapiens
gi|123232805 from Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **23%**

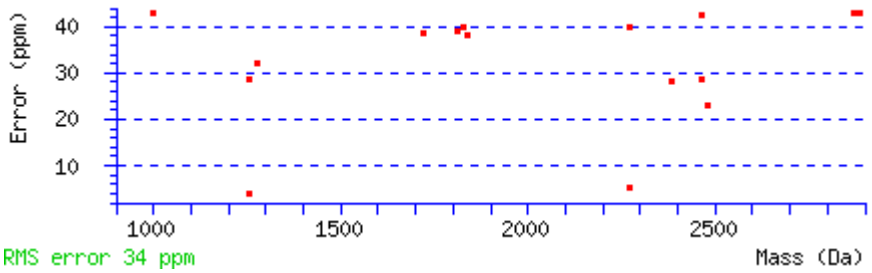
Matched peptides shown in **Bold Red**

```
   1 MVVEHPEFLK AGKEPGLQIW RVEKFDLVPV PTNLYGDFFT GDAYVILKTV
  51 QLRNGNLQYD LHYWLGNECS QDESGAAAIF TVQLDDYLNG RAVQHREVQG
 101 FESATFLGYF KSGLKYKKGG VASGFKHVVP NEVVVQRLFQ VKGRRVVRAT
 151 EVPVSWESFN NGDCFILDLG NNIHQWCGSN SNRYERLKAT QVSKGIRDNE
 201 RSGRARVHVS EEGTEPEAML QVLGPKPALP AGTEDTAKED AANRKLAKLY
 251 KVSNGAGTMS VSLVADENPF AQGALKSEDC FILDHGKDGK IFVWKGKQAN
 301 TEERKAALKT ASDFITKMDY PKQTQVSVLP EGGETPLFKQ FFKNWRDPDQ
 351 TDGLGLSYLS SHIANVERVP FDAATLHTST AMAAQHGMDD DGTGQKQIWR
 401 IEGSNKVPVD PATYGQFYGG DSYIILYNYR HGGRQGQIIY NWQGAQSTQD
 451 EVAASAILTA QLDEELGGTP VQSRVVQGKE PAHLMSLFGG KPMIIYKGGT
 501 SREGGQTAPA STRLFQVRAN SAGATRAVEV LPKAGALNSN DAFVLKTPSA
 551 AYLWVGTGAS EAEKTGAQEL LRVLRAQPVQ VAEGSEPDGF WEALGGKAAY
 601 RTSPRLKDKK MDAHPPRLFA CSNKIGRFVI EEVPGELMQE DLATDDVMLL
 651 DTWDQVFVWV GKDSQEEEKT EALTSAKRYI ETDPANRDRR TPITVVKQGF
 701 EPPSFVGWFL GWDDDYWSVD PLDRAMAELA A
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
11 - 21	1254.7000	1253.6927	1253.6880	4	1	K.AGKEPGLQIWR.V (No match)
11 - 21	1254.7311	1253.7238	1253.6880	29	1	K.AGKEPGLQIWR.V (No match)
14 - 21	998.5847	997.5774	997.5345	43	0	K.EPGLQIWR.V (No match)
97 - 111	1722.9037	1721.8964	1721.8301	39	0	R.EVQGFESATFLGYFK.S (No match)
127 - 137	1275.7577	1274.7504	1274.7095	32	0	K.HVVPNEVVVQR.L (No match)
252 - 276	2463.3230	2462.3157	2462.2111	42	0	K.VSNGAGTMSVSLVADENPFAQGALK.S
318 - 339	2464.3137	2463.3064	2463.2356	29	1	K.MDYPKQTQVSVLPEGGETPLFK.Q (No
318 - 339	2480.2944	2479.2872	2479.2305	23	1	K.MDYPKQTQVSVLPEGGETPLFK.Q Oxi
323 - 339	1813.0083	1812.0010	1811.9306	39	0	K.QTQVSVLPEGGETPLFK.Q Gln->pyr
323 - 339	1830.0370	1829.0297	1828.9571	40	0	K.QTQVSVLPEGGETPLFK.Q (No matc
347 - 368	2387.2151	2386.2078	2386.1401	28	0	R.DPDQTDGLGLSYLSSHIANVER.V (No
369 - 396	2873.4060	2872.3987	2872.2756	43	0	R.VPFDAATLHTSTAMAAQHGMDDDGTGQK.
369 - 396	2889.4016	2888.3943	2888.2706	43	0	R.VPFDAATLHTSTAMAAQHGMDDDGTGQK.
547 - 564	1837.9666	1836.9593	1836.8894	38	0	K.TPSAAYLWVGTGASEAEK.T (No mat
576 - 597	2272.1000	2271.0927	2271.0808	5	0	R.AQPVQVAEGSEPDGFWEALGGK.A (Ic
576 - 597	2272.1790	2271.1717	2271.0808	40	0	R.AQPVQVAEGSEPDGFWEALGGK.A (No



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LOCUS       NP_937895                731 aa            linear   PRI 03-SEP-2007
DEFINITION  gelsolin isoform b [Homo sapiens].
ACCESSION   NP_937895
VERSION     NP_937895.1  GI:38044288
DBSOURCE    REFSEQ: accession NM_198252.2
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 731)
  AUTHORS   Huerva,V., Velasco,A., Sanchez,M.C., Mateo,A.J. and Matias-Guiu,X.
  TITLE     Lattice corneal dystrophy type II: clinical, pathologic, and
            molecular study in a Spanish family
  JOURNAL   Eur J Ophthalmol 17 (3), 424-429 (2007)
   PUBMED   17534828
  REMARK    GeneRIF: A new family with Meretoja syndrome is reported. This is
            the first documented family with Meretoja syndrome in Spain and in
            the Mediterranean countries. The molecular study shows the same
            mutation of reported families.
REFERENCE   2  (residues 1 to 731)
  AUTHORS   Frum,R., Busby,S.A., Ramamoorthy,M., Deb,S., Shabanowitz,J.,
            Hunt,D.F. and Deb,S.P.
  TITLE     HDM2-binding partners: interaction with translation elongation
            factor EF1alpha
  JOURNAL   J. Proteome Res. 6 (4), 1410-1417 (2007)
   PUBMED   17373842
REFERENCE   3  (residues 1 to 731)
  AUTHORS   Roustan,C., Ferjani,I., Maciver,S.K., Fattoum,A., Rebiere,B. and
            Benyamin,Y.
  TITLE     Calcium-induced conformational changes in the amino-terminal half
            of gelsolin
  JOURNAL   FEBS Lett. 581 (4), 681-686 (2007)
   PUBMED   17258204
  REMARK    GeneRIF: Calcium induced conformational changes in the G1-2 and
            G1-3 sub-domains of gelsolin, and the binding affinities for the
            three type II sites, are reported.
REFERENCE   4  (residues 1 to 731)
  AUTHORS   Qiao,H. and McMillan,J.R.
  TITLE     Gelsolin segment 5 inhibits HIV-induced T-cell apoptosis via
            Vpr-binding to VDAC
  JOURNAL   FEBS Lett. 581 (3), 535-540 (2007)
   PUBMED   17254575
  REMARK    GeneRIF: gelsolin G5 domain inhibits HIV-Vpr-induced T-cell
            apoptosis by blocking the interaction between Vpr and VDAC
REFERENCE   5  (residues 1 to 731)
  AUTHORS   Ewing,R.M., Chu,P., Elisma,F., Li,H., Taylor,P., Climie,S.,
            McBroom-Cerajewski,L., Robinson,M.D., O'Connor,L., Li,M.,
            Taylor,R., Dharsee,M., Ho,Y., Heilbut,A., Moore,L., Zhang,S.,
            Ornatsky,O., Bukhman,Y.V., Ethier,M., Sheng,Y., Vasilescu,J.,
            Abu-Farha,M., Lambert,J.P., Duewel,H.S., Stewart,I.I., Kuehl,B.,
            Hogue,K., Colwill,K., Gladwish,K., Muskat,B., Kinach,R.,
            Adams,S.L., Moran,M.F., Morin,G.B., Topaloglou,T. and Figeys,D.
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Mascot Search Results

Protein View

Match to: **gi|4504183** Score: **195** Expect: **6.1e-15**
glutathione transferase [Homo sapiens]

Nominal mass (M_r): **23569**; Calculated pI value: **5.43**
NCBI BLAST search of gi|4504183 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|121746 from Homo sapiens
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gi|4389051 from Homo sapiens
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gi|4389404 from Homo sapiens
gi|4389405 from Homo sapiens
gi|4389406 from Homo sapiens
gi|4389407 from Homo sapiens
gi|4389412 from Homo sapiens
gi|4389413 from Homo sapiens
gi|4389414 from Homo sapiens
gi|4389415 from Homo sapiens
gi|4699781 from Homo sapiens
gi|4699782 from Homo sapiens
gi|5822521 from Homo sapiens
gi|5822522 from Homo sapiens
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gi|598158 from Homo sapiens
gi|763405 from Homo sapiens
gi|32187525 from Homo sapiens
gi|47496669 from Homo sapiens
gi|54696760 from Homo sapiens
gi|54696762 from Homo sapiens
gi|61356575 from synthetic construct
gi|61356585 from synthetic construct
gi|119595055 from Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **54%**

Matched peptides shown in **Bold Red**

```
     1 MPPYTVVYFP VRGRCAALRM LLADQGQSWK EEVVTVETWQ EGSLKASCLY
    51 GQLPKFQDGD LTLYQSNTIL RHLGRTLGLY GKDQQEAALV DMVNDGVEDL
   101 RCKYISLIYT NYEAGKDDYV KALPGQLKPF ETLLSQNQGG KTFIVGDQIS
   151 FADYNLLDLL LIHEVLAPGC LDAFPLLSAY VGRLSARPKL KAFLASPEYV
   201 NLPINGNGKQ
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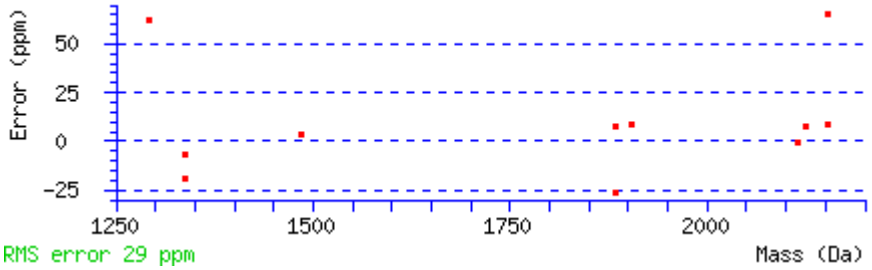
Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
1 - 12	1484.7664	1483.7591	1483.7534	4	0	-.MPPYTVVYFPVR.G Oxidation (M)
2 - 12	1337.7000	1336.6927	1336.7180	-19	0	M.PPYTVVYFPVR.G (Ions score 45
2 - 12	1337.7168	1336.7095	1336.7180	-6	0	M.PPYTVVYFPVR.G (No match)
20 - 30	1292.7105	1291.7032	1291.6231	62	0	R.MLLADQGQSWK.E Oxidation (M)
56 - 71	1883.9000	1882.8927	1882.9425	-26	0	K.FQDGDLTLYQSNTILR.H (Ions sco
56 - 71	1883.9650	1882.9577	1882.9425	8	0	K.FQDGDLTLYQSNTILR.H (No match
83 - 101	2116.9810	2115.9737	2115.9743	-0	0	K.DQQEAALVDMVNDGVEDLR.C (No ma
104 - 121	2155.0774	2154.0701	2154.0521	8	1	K.YISLIYTNYEAGKDDYVK.A (No mat
104 - 121	2155.2000	2154.1927	2154.0521	65	1	K.YISLIYTNYEAGKDDYVK.A (Ions s
122 - 141	2126.1765	2125.1692	2125.1531	8	0	K.ALPGQLKPFETLLSQNQGGK.T (No m
192 - 209	1904.0067	1902.9994	1902.9839	8	0	K.AFLASPEYVNLPINGNGK.Q (No mat



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LOCUS       NP_000843                 210 aa            linear   PRI 20-AUG-2007
DEFINITION  glutathione transferase [Homo sapiens].
ACCESSION   NP_000843
VERSION     NP_000843.1  GI:4504183
DBSOURCE    REFSEQ: accession NM_000852.2
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 210)
  AUTHORS   Vibhuti,A., Arif,E., Deepak,D., Singh,B. and Qadar Pasha,M.A.
  TITLE     Genetic polymorphisms of GSTP1 and mEPHX correlate with oxidative
            stress markers and lung function in COPD
  JOURNAL   Biochem. Biophys. Res. Commun. 359 (1), 136-142 (2007)
   PUBMED   17532303
  REMARK    GeneRIF: In conclusion, 105V/114V alleles of GSTP1 and 113H/139H
            alleles of mEPHX and the combination of genotypes with same alleles
            associated with imbalanced oxidative stress and lung function in
            patients.
REFERENCE   2  (residues 1 to 210)
  AUTHORS   Huang,G., Mills,L. and Worth,L.L.
  TITLE     Expression of human glutathione S-transferase P1 mediates the
            chemosensitivity of osteosarcoma cells
  JOURNAL   Mol. Cancer Ther. 6 (5), 1610-1619 (2007)
   PUBMED   17513610
  REMARK    GeneRIF: GSTP1 contributes to doxorubicin and cisplatin resistance
            in osteosarcoma, which may be mediated in part by the activation of
            extracellular signal-regulated kinase 1/2
REFERENCE   3  (residues 1 to 210)
  AUTHORS   Povey,A.C., Jury,F., Dippnall,W.M., Smith,A.E., Thomson,S.,
            Mackness,B., Mackness,M., Durrington,P. and Cherry,N.M.
  TITLE     GST CYP and PON1 polymorphisms in farmers attributing ill health to
            organophosphate-containing sheep dip
  JOURNAL   Biomarkers 12 (2), 188-202 (2007)
   PUBMED   17536768
  REMARK    GeneRIF: Risk of ill health attributed to
            organophosphate-containing sheep dip is associated with PON1
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Mascot Search Results

Protein View

Match to: **gi|4504965** Score: **137** Expect: **1.1e-07**
L-plastin [Homo sapiens]

Nominal mass (M_r): **70815**; Calculated pI value: **5.20**
NCBI BLAST search of gi|4504965 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|114651523 from Pan troglodytes
gi|114651525 from Pan troglodytes
gi|114651527 from Pan troglodytes
gi|114651529 from Pan troglodytes
gi|114651531 from Pan troglodytes
gi|114651533 from Pan troglodytes
gi|114651535 from Pan troglodytes
gi|114651537 from Pan troglodytes
gi|114651539 from Pan troglodytes
gi|114651541 from Pan troglodytes
gi|114651543 from Pan troglodytes
gi|114651545 from Pan troglodytes
gi|114651547 from Pan troglodytes
gi|114651549 from Pan troglodytes
gi|114651551 from Pan troglodytes
gi|1346733 from Homo sapiens
gi|189502 from Homo sapiens
gi|14043359 from Homo sapiens
gi|16307448 from Homo sapiens
gi|123988880 from synthetic construct
gi|123999207 from synthetic construct

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **123**
Number of mass values matched: **35**
Sequence Coverage: **57%**

Matched peptides shown in **Bold Red**

```
     1 MARGSVSDEE MMELREAFAK VDTDGNGYIS FNELNDLFKA ACLPLPGYRV
    51 REITENLMAT GDLDQDGRIS FDEFIKIFHG LKSTDVAKTF RKAINKKEGI
   101 CAIGGTSEQS SVGTQHSYSE EEKYAFVNWI NKALENDPDC RHVIPMNPNT
   151 NDLFNAVGDG IVLCKMINLS VPDTIDERTI NKKKLTPFTI QENLNLALNS
   201 ASAIGCHVVN IGAEDLKEGK PYLVLGLLWQ VIKIGLFADI ELSRNEALIA
   251 LLREGESLED LMKLSPEELL LRWANYHLEN AGCNKIGNFS TDIKDSKAYY
   301 HLLEQVAPKG DEEGVPAVVI DMSGLREKDD IQRAECMLQQ AERLGCRQFV
   351 TATDVVRGNP KLNLAFIANL FNRYPALHKP ENQDIDWGAL EGETREERTF
   401 RNWMNSLGVN PRVNHLYSDL SDALVIFQLY EKIKVPVDWN RVNKPPYPKL
   451 GGNMKKLENC NYAVELGKNQ AKFSLVGIGG QDLNEGNRTL TLALIWQLMR
   501 RYTLNILEEI GGGQKVNDDI IVNWVNETLR EAEKSSSISS FKDPKISTSL
   551 PVLDLIDAIQ PGSINYDLLK TENLNDDEKL NNAKYAISMA RKIGARVYAL
   601 PEDLVEVNPK MVMTVFACLM GKGMKRV
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
124 - 132	1154.8450	1153.8377	1153.5920	213	0	K.YAFVNWINK.A
133 - 141	1089.6979	1088.6906	1088.4557	216	0	K.ALENDPDCR.H
142 - 165	2638.9114	2637.9041	2637.3043	227	0	R.HVIPMNPNTNDLFNAVGDGIVLCK.M
166 - 178	1503.0802	1502.0729	1501.7446	219	0	K.MINLSVPDTIDER.T
218 - 233	1856.5044	1855.4971	1855.0971	216	0	K.EGKPYLVLGLLWQVIK.I
234 - 244	1233.9460	1232.9387	1232.6765	213	0	K.IGLFADIELSR.N
245 - 253	1012.8333	1011.8260	1011.6076	216	0	R.NEALIALLR.E
273 - 285	1577.0359	1576.0286	1575.6888	216	0	R.WANYHLENAGCNK.I
273 - 294	2552.7939	2551.7866	2551.1914	233	1	R.WANYHLENAGCNKIGNFSTDIK.D
286 - 294	994.7431	993.7358	993.5131	224	0	K.IGNFSTDIK.D
298 - 309	1432.0619	1431.0546	1430.7558	209	0	K.AYYHLLEQVAPK.G
310 - 326	1744.2310	1743.2237	1742.8509	214	0	K.GDEEGVPAVVIDMSGLR.E
327 - 333	903.6476	902.6403	902.4457	216	1	R.EKDDIQR.A
334 - 343	1235.8157	1234.8084	1234.5434	215	0	R.AECMLQQAER.L
348 - 357	1118.8245	1117.8172	1117.5768	215	0	R.QFVTATDVVR.G Gln->pyro-Glu (
348 - 357	1135.8553	1134.8480	1134.6033	216	0	R.QFVTATDVVR.G
362 - 373	1406.0924	1405.0851	1404.7877	212	0	K.LNLAFIANLFNR.Y
374 - 395	2539.7754	2538.7681	2538.2139	218	0	R.YPALHKPENQDIDWGALEGETR.E
402 - 412	1287.8983	1286.8910	1286.6190	211	0	R.NWMNSLGVNPR.V
402 - 412	1303.8969	1302.8896	1302.6139	212	0	R.NWMNSLGVNPR.V Oxidation (M)
413 - 432	2367.7341	2366.7268	2366.2158	216	0	R.VNHLYSDLSDALVIFQLYEK.I
433 - 441	1126.8794	1125.8721	1125.6295	216	1	K.IKVPVDWNR.V
435 - 441	885.6453	884.6380	884.4505	212	0	K.VPVDWNR.V
442 - 449	942.7171	941.7098	941.5334	187	0	R.VNKPPYPK.L
473 - 488	1676.1942	1675.1869	1674.8326	212	0	K.FSLVGIGGQDLNEGNR.T
489 - 500	1459.1587	1458.1514	1457.8428	212	0	R.TLTLALIWQLMR.R
489 - 500	1475.1609	1474.1536	1473.8377	214	0	R.TLTLALIWQLMR.R Oxidation (M)
489 - 501	1615.2804	1614.2731	1613.9439	204	1	R.TLTLALIWQLMRR.Y
501 - 515	1691.2394	1690.2321	1689.9050	194	1	R.RYTLNILEEIGGGQK.V
502 - 515	1535.1405	1534.1332	1533.8038	215	0	R.YTLNILEEIGGGQK.V
516 - 530	1800.3142	1799.3069	1798.9214	214	0	K.VNDDIIVNWVNETLR.E
546 - 570	2699.0918	2698.0845	2697.4840	223	0	K.ISTSLPVLDLIDAIQPGSINYDLLK.T
597 - 610	1586.1804	1585.1731	1584.8399	210	0	R.VYALPEDLVEVNPK.M
611 - 622	1387.9591	1386.9518	1386.6532	215	0	K.MVMTVFACLMGK.G
611 - 625	1719.3149	1718.3076	1718.8050	-289	1	K.MVMTVFACLMGKGMK.R Oxidation

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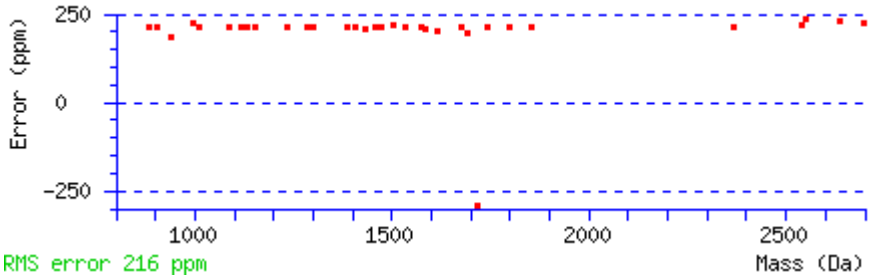

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LOCUS       NP_002289                627 aa            linear   PRI 27-JUN-2007
DEFINITION  L-plastin [Homo sapiens].
ACCESSION   NP_002289
VERSION     NP_002289.1  GI:4504965
DBSOURCE    REFSEQ: accession NM_002298.2
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 627)
  AUTHORS   Klemke,M., Rafael,M.T., Wabnitz,G.H., Weschenfelder,T.,
            Konstandin,M.H., Garbi,N., Autschbach,F., Hartschuh,W. and
            Samstag,Y.
  TITLE     Phosphorylation of ectopically expressed L-plastin enhances
```

Mascot Search Results

Protein View

Match to: **gi|10863927** Score: **224** Expect: **7.7e-18**
peptidylprolyl isomerase A [Homo sapiens]

Nominal mass (M_r): **18229**; Calculated pI value: **7.68**
NCBI BLAST search of gi|10863927 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|74136179 from Macaca mulatta
gi|113429091 from Homo sapiens
gi|113429184 from Homo sapiens
gi|114613182 from Pan troglodytes
gi|114613184 from Pan troglodytes
gi|114613186 from Pan troglodytes
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gi|114613190 from Pan troglodytes
gi|114613192 from Pan troglodytes
gi|114613194 from Pan troglodytes
gi|51702775 from Homo sapiens
gi|51702776 from Chlorocebus aethiops
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gi|51702778 from Papio anubis
gi|118572694 from Pongo pygmaeus
gi|118573115 from Gorilla gorilla gorilla
gi|118573116 from Hylobates lar
gi|118573117 from Nomascus leucogenys
gi|118573118 from Symphalangus syndactylus
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gi|31615576 from Homo sapiens
gi|31615579 from Human immunodeficiency virus 1
gi|31615580 from Human immunodeficiency virus 1
gi|55670663 from Homo sapiens
gi|55670664 from Homo sapiens
gi|55670666 from Homo sapiens
gi|62738674 from Homo sapiens
gi|62738675 from Homo sapiens
gi|99031691 from Homo sapiens
gi|99031692 from Homo sapiens
gi|30168 from Homo sapiens
gi|30309 from Homo sapiens
gi|2565299 from Papio hamadryas
gi|2565301 from Chlorocebus aethiops
gi|2565303 from Macaca mulatta
gi|12653801 from Homo sapiens
gi|13529080 from Homo sapiens
gi|15530260 from Homo sapiens
gi|32425484 from Homo sapiens
gi|49522214 from Homo sapiens
gi|51895760 from Homo sapiens
gi|77415502 from Homo sapiens
gi|82491985 from Pan paniscus
gi|82491987 from Pan troglodytes
gi|82491989 from Pongo pygmaeus
gi|82491991 from Gorilla gorilla
gi|82491993 from Hylobates lar
gi|82491995 from Nomascus leucogenys
gi|82491997 from Symphalangus syndactylus
gi|82491999 from Chlorocebus aethiops
gi|82492001 from Macaca mulatta
gi|90075960 from Macaca fascicularis
gi|123984411 from synthetic construct
gi|123998379 from synthetic construct

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **69%**

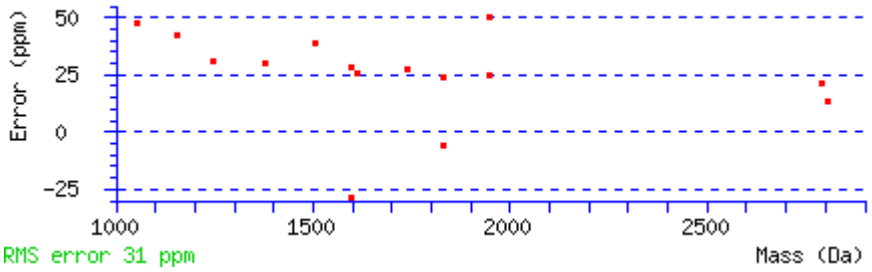
Matched peptides shown in **Bold Red**

```
   1 MVNPTVFFDI AVDGEPLGRV SFELFADKVP KTAENFRALS TGEKGFGYKG
  51 SCFHRIIPGF MCQGGDFTRH NGTGGKSIYG EKFEDENFIL KHTGPGILSM
 101 ANAGPNTNGS QFFICTAKTE WLDGKHVVFG KVKEGMNIVE AMERFGSRNG
 151 KTSKKITIAD CGQLE
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
2 - 19	1946.0494	1945.0422	1944.9946	24	0	M.VNPTVFFDIAVDGEPLGR.V (No mat
2 - 19	1946.1000	1945.0927	1944.9946	50	0	M.VNPTVFFDIAVDGEPLGR.V (Ions s
20 - 28	1055.5909	1054.5837	1054.5335	48	0	R.VSFELFADK.V (No match)
20 - 31	1379.7987	1378.7914	1378.7496	30	1	R.VSFELFADKVPK.T (No match)
56 - 69	1598.7000	1597.6927	1597.7381	-28	0	R.IIPGFMCQGGDFTR.H (Ions score
56 - 69	1598.7913	1597.7840	1597.7381	29	0	R.IIPGFMCQGGDFTR.H (No match)
56 - 69	1614.7820	1613.7747	1613.7331	26	0	R.IIPGFMCQGGDFTR.H Oxidation (
77 - 91	1831.9000	1830.8927	1830.9039	-6	1	K.SIYGEKFEDENFILK.H (Ions scor
77 - 91	1831.9557	1830.9484	1830.9039	24	1	K.SIYGEKFEDENFILK.H (No match)
83 - 91	1154.6211	1153.6138	1153.5655	42	0	K.FEDENFILK.H (No match)
92 - 118	2791.3879	2790.3807	2790.3218	21	0	K.HTGPGILSMANAGPNTNGSQFFICTAK.T
92 - 118	2807.3603	2806.3531	2806.3167	13	0	K.HTGPGILSMANAGPNTNGSQFFICTAK.T
132 - 144	1505.8036	1504.7963	1504.7377	39	1	K.VKEGMNIVEAMER.F (No match)
134 - 148	1741.8474	1740.8401	1740.7923	27	1	K.EGMNIVEAMERFGSR.N Oxidation
155 - 165	1247.6686	1246.6613	1246.6227	31	1	K.KITIADCGQLE.- (No match)



```
LOCUS       NP_066953                165 aa            linear   PRI 03-SEP-2007
DEFINITION  peptidylprolyl isomerase A [Homo sapiens].
ACCESSION   NP_066953 NP_001008741
VERSION     NP_066953.1  GI:10863927
DBSOURCE    REFSEQ: accession NM_021130.3
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 165)
  AUTHORS   Zhang,S., Joseph,G., Pollok,K., Berthoux,L., Sastry,L., Luban,J.
            and Cornetta,K.
  TITLE     G2 cell cycle arrest and cyclophilin A in lentiviral gene transfer
  JOURNAL   Mol. Ther. 14 (4), 546-554 (2006)
   PUBMED   16901758
  REMARK    GeneRIF: cyclophilin A and G2 cell cycle arrest appear to be two
            independent factors important for efficient lentiviral gene
            transfer
REFERENCE   2  (residues 1 to 165)
  AUTHORS   Kim,S.C., Sprung,R., Chen,Y., Xu,Y., Ball,H., Pei,J., Cheng,T.,
            Kho,Y., Xiao,H., Xiao,L., Grishin,N.V., White,M., Yang,X.J. and
            Zhao,Y.
  TITLE     Substrate and functional diversity of lysine acetylation revealed
            by a proteomics survey
```

Mascot Search Results

Protein View

Match to: **gi|4505753** Score: **183** Expect: **9.7e-14**
phosphoglycerate mutase 1 (brain) [Homo sapiens]

Nominal mass (M_r): **28900**; Calculated pI value: **6.67**
NCBI BLAST search of gi|4505753 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|89035644 from Homo sapiens
gi|109090155 from Macaca mulatta
gi|109090157 from Macaca mulatta
gi|109149555 from Macaca mulatta
gi|114632193 from Pan troglodytes
gi|130348 from Homo sapiens
gi|551174 from Homo sapiens
gi|9956014 from Homo sapiens
gi|14603144 from Homo sapiens
gi|15079726 from Homo sapiens
gi|44890768 from Homo sapiens
gi|49456279 from Homo sapiens
gi|49522678 from Homo sapiens
gi|56081766 from Homo sapiens
gi|57209137 from Homo sapiens
gi|67970637 from Macaca fascicularis
gi|119570323 from Homo sapiens
gi|119570324 from Homo sapiens
gi|119570329 from Homo sapiens
gi|119618138 from Homo sapiens
gi|119618141 from Homo sapiens
gi|123992778 from synthetic construct
gi|123999524 from synthetic construct

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **47%**

Matched peptides shown in **Bold Red**

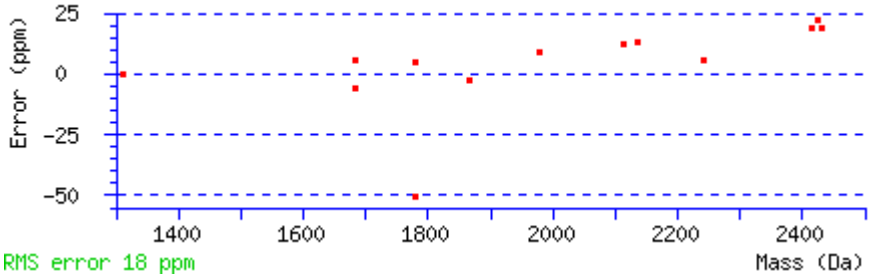
```
     1 MAAYKLVLIR HGESAWNLEN RFSGWYDADL SPAGHEEAKR GGQALRDAGY
    51 EFDICFTSVQ KRAIRTLWTV LDAIDQMWLP VVRTWRLNER HYGGLTGLNK
   101 AETAAKHGEA QVKIWRRSYD VPPPPMEPDH PFYSNISKDR RYADLTEDQL
   151 PSCESLKDTI ARALPFWNEE IVPQIKEGKR VLIAAHGNSL RGIVKHLEGL
   201 SEEAIMELNL PTGIPIVYEL DKNLKPIKPM QFLGDEETVR KAMEAVAAQG
   251 KAKK
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
11 - 21	1312.6022	1311.5949	1311.5956	-1	0	R.HGESAWNLENR.F (No match)
22 - 39	1979.8951	1978.8879	1978.8697	9	0	R.FSGWYDADLSPAGHEEAK.R (No mat
22 - 40	2136.0051	2134.9979	2134.9708	13	1	R.FSGWYDADLSPAGHEEAKR.G (No ma
47 - 61	1779.7000	1778.6927	1778.7822	-50	0	R.DAGYEFDICFTSVQK.R (Ions scor
47 - 61	1779.7975	1778.7902	1778.7822	5	0	R.DAGYEFDICFTSVQK.R (No match)
118 - 138	2417.1582	2416.1509	2416.1045	19	0	R.SYDVPPPPMEPDHPFYSNISK.D (No

118 - 138	2433.1516	2432.1443	2432.0995	18	0	R.SYDVPPPPMEPDHPFYSNISK.D Oxic
142 - 157	1868.8530	1867.8458	1867.8509	-3	0	R.YADLTEDQLPSCESLK.D (No match
142 - 162	2425.2083	2424.2010	2424.1478	22	1	R.YADLTEDQLPSCESLKDTIAR.A (No
163 - 176	1683.9000	1682.8927	1682.9032	-6	0	R.ALPFWNEEIVPQIK.E (Ions score
163 - 176	1683.9203	1682.9130	1682.9032	6	0	R.ALPFWNEEIVPQIK.E (No match)
223 - 240	2115.1531	2114.1458	2114.1194	12	0	K.NLKPIKPMQFLGDEETVR.K (No mat
223 - 241	2243.2341	2242.2269	2242.2143	6	1	K.NLKPIKPMQFLGDEETVRK.A (No ma



```
LOCUS       NP_002620                254 aa            linear   PRI 03-JUN-2007
DEFINITION  phosphoglycerate mutase 1 (brain) [Homo sapiens].
ACCESSION   NP_002620 XP_012219
VERSION     NP_002620.1  GI:4505753
DBSOURCE    REFSEQ: accession NM_002629.2
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 254)
  AUTHORS   Oh,S.J., Park,K.S., Ryan,H.F. Jr., Danon,M.J., Lu,J., Naini,A.B.
            and DiMauro,S.
  TITLE     Exercise-induced cramp, myoglobinuria, and tubular aggregates in
            phosphoglycerate mutase deficiency
  JOURNAL   Muscle Nerve 34 (5), 572-576 (2006)
   PUBMED   16881065
  REMARK    GeneRIF: We report two patients in whom phosphoglycerate mutase
            (PGAM) deficiency was associated with the triad of exercise-induced
            cramps, recurrent myoglobinuria, and tubular aggregates in the
            muscle biopsy.
REFERENCE   2  (residues 1 to 254)
  AUTHORS   Repiso,A., Ramirez Bajo,M.J., Corrons,J.L., Carreras,J. and
            Climent,F.
  TITLE     Phosphoglycerate mutase BB isoenzyme deficiency in a patient with
            non-spherocytic anemia: familial and metabolic studies
  JOURNAL   Haematologica 90 (2), 257-259 (2005)
   PUBMED   15710582
  REMARK    GeneRIF: Phosphoglycerate mutase may have a role in preventing
            non-spherocytic anemia [case report]
REFERENCE   3  (residues 1 to 254)
  AUTHORS   Rush,J., Moritz,A., Lee,K.A., Guo,A., Goss,V.L., Spek,E.J.,
            Zhang,H., Zha,X.M., Polakiewicz,R.D. and Comb,M.J.
  TITLE     Immunoaffinity profiling of tyrosine phosphorylation in cancer
            cells
  JOURNAL   Nat. Biotechnol. 23 (1), 94-101 (2005)
   PUBMED   15592455
REFERENCE   4  (residues 1 to 254)
  AUTHORS   Wang,Y., Cheng,Z., Liu,L., Wei,Z., Wan,M. and Gong,W.
  TITLE     Cloning, purification, crystallization and preliminary
            crystallographic analysis of human phosphoglycerate mutase
  JOURNAL   Acta Crystallogr. D Biol. Crystallogr. 60 (PT 10), 1893-1894 (2004)
   PUBMED   15388943
  REMARK    GeneRIF: Cloning, purification, crystallization and preliminary
            crystallographic analysis of human phosphoglycerate mutase
REFERENCE   5  (residues 1 to 254)
```

Mascot Search Results

Protein View

Match to: **gi|190533** Score: **151** Expect: **1.5e-10**
proteinase 3 [Homo sapiens]

Nominal mass (M_r): **26150**; Calculated pI value: **8.51**
NCBI BLAST search of gi|190533 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Glu->pyro-Glu (N-term E),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **48%**

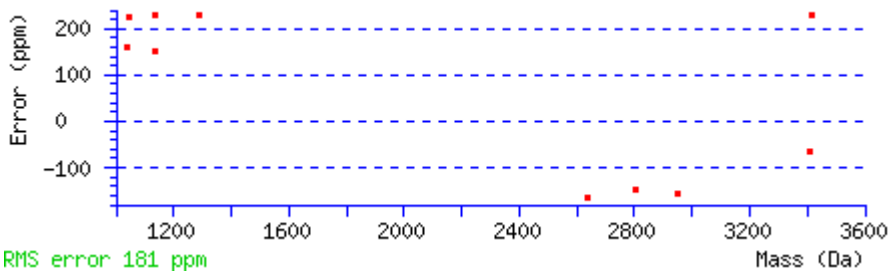
Matched peptides shown in **Bold Red**

```
     1 AARAAEIVGG HEAQPHSRPY MASLQMRGNP GSHFCGGTLI HPSFVLTAAH
    51 CLRDIPQRLV NVVLGAHNVR TQEPTQQHFS VAQVFLNNYD AENKLNDVLL
   101 IQLSSPANLS ASVATVQLPQ QDQPVPHGTQ CLAMGWGRVG AHDPPAQVLQ
   151 ELNVTVVTFF CRPHNICTFV PRRKAGICFG DSGGPLICDG IIQGIDSFVI
   201 WGCATRLFPD FFTRVALYVD WIRSTLRRVE AKGRP
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
1 - 27	2949.9934	2948.9861	2949.4450	-156	1	-.AARAAEIVGGHEAQPHSRPYMASLQMR.G
4 - 27	2635.8489	2634.8416	2635.2747	-164	0	R.AAEIVGGHEAQPHSRPYMASLQMR.G (
28 - 53	2805.9570	2804.9497	2805.3592	-146	0	R.GNPGSHFCGGTLIHPSFVLTAAHCLR.D
59 - 70	1291.0577	1290.0504	1289.7568	228	0	R.LVNVVLGAHNVR.T (No match)
175 - 206	3412.3865	3411.3792	3411.6051	-66	0	K.AGICFGDSGGPLICDGIIQGIDSFVIWG(
175 - 206	3413.3889	3412.3816	3411.6051	228	0	K.AGICFGDSGGPLICDGIIQGIDSFVIWG(
207 - 214	1042.7000	1041.6927	1041.5284	158	0	R.LFPDFFTR.V (Ions score 59)
207 - 214	1042.7714	1041.7641	1041.5284	226	0	R.LFPDFFTR.V (No match)
215 - 223	1134.8000	1133.7927	1133.6233	149	0	R.VALYVDWIR.S (Ions score 69)
215 - 223	1134.8892	1133.8819	1133.6233	228	0	R.VALYVDWIR.S (No match)



```
LOCUS       AAB59493                235 aa            linear   PRI 19-NOV-2002
DEFINITION  proteinase 3 [Homo sapiens].
ACCESSION   AAB59493
VERSION     AAB59493.1  GI:190533
DBSOURCE    locus HUMPRT3A01 accession M96838.1
            locus HUMPRT3A02 accession M96837.1
            locus HUMPRT3A03 accession M96839.1
KEYWORDS    .
SOURCE      Homo sapiens (human)
```

Mascot Search Results

Protein View

Match to: **gi|4506773** Score: **208** Expect: **3.1e-16**
S100 calcium-binding protein A9 [Homo sapiens]

Nominal mass (M_r): **13291**; Calculated pI value: **5.71**
NCBI BLAST search of gi|4506773 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|115444 from Homo sapiens
gi|7417327 from Homo sapiens
gi|34771 from Homo sapiens
gi|386958 from Homo sapiens
gi|516621 from Homo sapiens
gi|29126810 from Homo sapiens
gi|49457408 from Homo sapiens
gi|49457442 from Homo sapiens
gi|56205191 from Homo sapiens
gi|60822417 from synthetic construct
gi|60822435 from synthetic construct
gi|119573718 from Homo sapiens
gi|119573719 from Homo sapiens
gi|225793 from Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Methyl (C-term),Methyl (DE),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **67%**

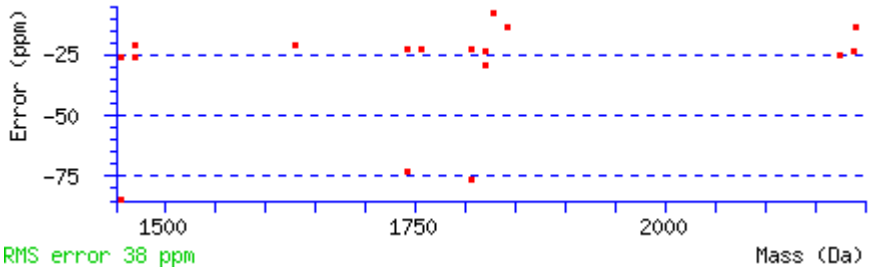
Matched peptides shown in **Bold Red**

```
     1 MTCKMSQLER NIETIINTFH QYSVKLGHPD TLNQGEFKEL VRKDLQNFLK
    51 KENKNEKVIE HIMEDLDTNA DKQLSFEEFI MLMARLTWAS HEKMHEGDEG
   101 PGHHHKPGLG EGTP
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
11 - 25	1806.8000	1805.7927	1805.9312	-77	0	R.NIETIINTFHQYSVK.L (Ions scor
11 - 25	1806.8972	1805.8899	1805.9312	-23	0	R.NIETIINTFHQYSVK.L (No match)
11 - 25	1820.9000	1819.8927	1819.9468	-30	0	R.NIETIINTFHQYSVK.L Methyl (DE
11 - 25	1820.9108	1819.9035	1819.9468	-24	0	R.NIETIINTFHQYSVK.L Methyl (C-
26 - 38	1455.6000	1454.5927	1454.7154	-84	0	K.LGHPDTLNQGEFK.E (Ions score
26 - 38	1455.6851	1454.6778	1454.7154	-26	0	K.LGHPDTLNQGEFK.E (No match)
26 - 38	1469.7000	1468.6927	1468.7310	-26	0	K.LGHPDTLNQGEFK.E Methyl (DE)
26 - 38	1469.7076	1468.7004	1468.7310	-21	0	K.LGHPDTLNQGEFK.E Methyl (C-te
58 - 72	1742.7000	1741.6927	1741.8192	-73	0	K.VIEHIMEDLDTNADK.Q (Ions scor
58 - 72	1742.7863	1741.7790	1741.8192	-23	0	K.VIEHIMEDLDTNADK.Q (No match)
58 - 72	1756.8027	1755.7955	1755.8349	-22	0	K.VIEHIMEDLDTNADK.Q Methyl (C-
58 - 72	1828.8854	1827.8781	1827.8924	-8	0	K.VIEHIMEDLDTNADK.Q Methyl (C-
58 - 72	1842.8905	1841.8832	1841.9081	-13	0	K.VIEHIMEDLDTNADK.Q Methyl (C-
73 - 85	1630.7621	1629.7548	1629.7895	-21	0	K.QLSFEEFIMLMAR.L Oxidation (M
94 - 114	2175.9084	2174.9012	2174.9552	-25	0	K.MHEGDEGPGHHHKPGLGEGTP.- (No
94 - 114	2189.9260	2188.9188	2188.9709	-24	0	K.MHEGDEGPGHHHKPGLGEGTP.- Meth
94 - 114	2191.9277	2190.9205	2190.9501	-14	0	K.MHEGDEGPGHHHKPGLGEGTP.- Oxid



```
LOCUS       NP_002956                114 aa            linear   PRI 20-AUG-2007
DEFINITION  S100 calcium-binding protein A9 [Homo sapiens].
ACCESSION   NP_002956
VERSION     NP_002956.1  GI:4506773
DBSOURCE    REFSEQ: accession NM_002965.3
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 114)
  AUTHORS   Benedyk,M., Sopalla,C., Nacken,W., Bode,G., Melkonyan,H., Banfi,B.
            and Kerkhoff,C.
  TITLE     HaCaT keratinocytes overexpressing the S100 proteins S100A8 and
            S100A9 show increased NADPH oxidase and NF-kappaB activities
  JOURNAL   J. Invest. Dermatol. 127 (8), 2001-2011 (2007)
   PUBMED   17429438
  REMARK    GeneRIF: HaCaT keratinocytes overexpressing the S100 proteins
            S100A8 and S100A9 show increased NADPH oxidase and NF-kappaB
            activities.
REFERENCE   2  (residues 1 to 114)
  AUTHORS   Anceriz,N., Vandal,K. and Tessier,P.A.
  TITLE     S100A9 mediates neutrophil adhesion to fibronectin through
            activation of beta2 integrins
  JOURNAL   Biochem. Biophys. Res. Commun. 354 (1), 84-89 (2007)
   PUBMED   17222807
  REMARK    GeneRIF: In contrast, neutrophil adhesion to fibronectin was
            completely inhibited by anti-beta2 integrins, suggesting that
            S100A9-induced specific activation of beta2 integrin is essential
            to neutrophil adhesion.
REFERENCE   3  (residues 1 to 114)
  AUTHORS   Klingelhofer,J., Senolt,L., Baslund,B., Nielsen,G.H., Skibshoj,I.,
            Pavelka,K., Neidhart,M., Gay,S., Ambartsumian,N., Hansen,B.S.,
            Petersen,J., Lukanidin,E. and Grigorian,M.
  TITLE     Up-regulation of metastasis-promoting S100A4 (Mts-1) in rheumatoid
            arthritis: putative involvement in the pathogenesis of rheumatoid
            arthritis
  JOURNAL   Arthritis Rheum. 56 (3), 779-789 (2007)
   PUBMED   17328050
  REMARK    GeneRIF: The pattern of S100A4 expression differed significantly
            from that of the proinflammatory proteins S100A9 and S100A12, which
            were restricted to phagocytes and granulocytes.
REFERENCE   4  (residues 1 to 114)
  AUTHORS   Shimada,S., Nakamura,M., Tanaka,Y., Tsutsumi,K., Katano,M.,
            Masuko,K., Yudoh,K., Koizuka,I. and Kato,T.
  TITLE     Crosslinking of the CD69 molecule enhances S100A9 production in
            activated neutrophils
  JOURNAL   Microbiol. Immunol. 51 (1), 87-98 (2007)
   PUBMED   17237603
  REMARK    GeneRIF: data suggest unidentified natural ligands for CD69 and/or
            CD69 autoantibodies possibly affect joint-composing cell types
            through increased production of S100A9 in neutrophils, providing
            insight into functions of CD69 on neutrophils in rheumatoid
```

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Baixar livros Multidisciplinar
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Baixar livros de Psicologia
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Baixar livros de Saúde Coletiva
Baixar livros de Serviço Social
Baixar livros de Sociologia
Baixar livros de Teologia
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Baixar livros de Turismo