# Measuring Intracellular hsp70 in Leukocytes by Flow Cytometry

**Linda L. Agnew**[1]

[1]Centre for Bioactive Discovery in Health and Ageing, School of Science and Technology, University of New England, Armidale, Australia

## ABSTRACT

Heat shock or stress proteins are constitutively expressed redox-sensitive proteins, the synthesis of which is induced in almost all organisms exposed to a range of stressors, including heat shock, oxidative stress, free radicals, UV radiation, and heavy metals. This unit details a method, with supporting protocols, for the measurement of their expression in peripheral blood leukocytes by flow cytometry. *Curr. Protoc. Toxicol.* 49:2.21.1-2.21.12. © 2011 by John Wiley & Sons, Inc.

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## INTRODUCTION

Heat shock proteins (hsp), or stress proteins as they are otherwise known, are a group of highly conserved proteins that represent between 2% and 15% of total cellular protein and are expressed by every living organism (Morimoto et al., 1994). As the name suggests, they are expressed when cells are exposed to stressful stimuli such as free radical attack, smoking, UV light, heavy metals, ozone, or fever; however they are also expressed constitutively, albeit at much lower levels, in the unstressed cell (Welch, 1992). These proteins allow cells to adapt to gradual changes in their environment. The main functions of hsps are to regulate apoptosis and to act as intracellular molecular chaperones that facilitate protein folding, biogenesis, and assembly (Morimoto et al., 1994). An important physiological function for the hsps is their role in the assembly and transport of newly synthesized proteins within cells, as well as in the removal of denatured proteins. The hsps are therefore important in preventing damage and in cellular repair processes after injury. There is well documented evidence that increased production of hsps protects cells against subsequent lethal stress induced, e.g., by oxidative stress, cytotoxins, heat stress, and cellular damage after ischemia or sepsis-induced injury (Jolly and Morimoto, 2000). The transport of proteins across membranes (van der Vies et al., 1992; Matthew and Morimoto, 1998) and their role in the ubiquitin-dependent protein-degradation pathway (Callis, 1995) are also important functions of hsps. Another key function of hsps is the regulation of apoptosis and cell death through both chaperone-dependent and -independent pathways (Takayama et al., 2003). The involvement of hsps in a number of human disease states has emphasized the important role of these highly conserved proteins in the modulation of the immune response.

This unit describes a method for detecting intracellular stress protein expression in leukocytes by flow cytometry using a whole-blood method (see Basic Protocol 1; also see Support Protocol 1 for blood collection). An adaptation of this method to enable high-throughput analysis using a 96-well plate format is also described (Alternate Protocol). Flow cytometry is a method that can be used to measure specific pre-existing hsps as well as those synthesized during the designated stress treatment; therefore, a method for inducing hsps by heat shock is also described (Support Protocol 2).

Flow cytometric methods hold several advantages over the more traditional methods of determining levels of heat shock protein expression, such as western blotting


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(immunoblotting), 1-D SDS-PAGE, and ELISA. These advantages include the specific information provided by this technique as to which cells are expressing the protein(s) of interest, and the significantly smaller volume of blood required to conduct the flow cytometric analysis. The information gained from flow cytometric analysis of cell populations at a single-cell level is invaluable to the monitoring of their expression in a range of disease states.

This unit uses whole-blood-derived leukocytes to measure basal and heat shock–induced hsp70; however, it should be noted that the protocols may be adapted to measure other hsps in a range of cell types and under a range of different stress-inducing conditions.

*NOTE:* All experiments must be approved by the appropriate institutional and or national review boards or ethics committees and, in addition, human subjects must provide written informed consent.

***BASIC PROTOCOL 1***

## CELL PREPARATION AND STAINING

This protocol describes the utilization of whole-blood samples for the determination of intracellular hsp70 levels in leukocyte subpopulations. The protocol includes the staining of extracellular markers with fluorochrome-labeled antibodies, hemolysis of red blood cells, permeabilization of leukocytes, staining with fluorochrome-labeled anti-hsp70, and fixation of cells.

*CAUTION:* Avoid ingestion, inhalation, or skin contact with human or animal blood products to avoid exposure to blood-borne pathogens. Ensure that protective equipment such as lab coat, gloves, and glasses are worn. All human/animal waste must be disposed of according to Occupational Health Safety and Environmental Protection guidelines. Researchers should follow their institution's specific Standard Operating Procedures for the disposal of solids, liquids, sharps, and formaldehyde waste.

### *Materials*

Fluorochrome-labeled leukocyte extracellular marker antibodies (Becton Dickinson Biosciences), e.g.:
- anti-CD3 conjugated to PerCP (cat. no. 347344)
- anti-CD3 conjugated to PerCP-Cy5.5 (cat. no. 340949)
- anti-CD4 conjugated to PE-Cy7 (cat. no. 348789)
- anti-CD8 conjugated to APC-Cy7 (cat. no. 348793)
- anti-CD14 conjugated to PE (cat. no. 347497)
- anti-CD14 conjugated to APC (cat. no. 340436)
- anti- CD16 conjugated to PE (cat. no. 555407)
- anti-CD19 conjugated to PE (cat. no. 555413)
- anti-CD36 conjugated to PE (cat. no. 555455)
- anti-CD38 conjugated to APC (cat. no. 340439)
- anti-CD45RA conjugated to APC (cat. no. 550855)
- anti-CD45RO conjugated to PE (cat. no. 555493)
- anti-CD56 conjugated to APC (cat. no. 555518)
- anti-HLA-DR conjugated to PerCP (cat. no. 347364)

FACS wash buffer (see recipe)

Whole blood sample(s), heparinized and heat shocked (see Support Protocols 1 and 2)

Whole blood sample, heparinized but not heat shocked (see Support Protocol 1), as control

1× FACS lysing solution (see recipe)

1× FACS permeabilizing solution (see recipe)

Fluorochrome-labeled isotype-control antibody: an antibody raised in the same species, of the same isotype and with the same fluorochrome as the intracellular hsp antibody, should be used to detect nonspecific binding; if using SPA-810FI then an anti-mouse IgG1-FITC antibody (such as Sigma-Aldrich F6397) should be used

Fluorochrome-labeled intracellular hsp70 antibody: the antibody should specifically recognize the inducible Hsp70 (HspA1A/HspA1B) (e.g., anti-hsp70-FITC, SPA-810FI, or anti-hsp70- R phycoerythrin (PE) SPA-810PE; Assay Designs, *http://www.enzolifesciences.com/assay-designs/*)

1% paraformaldehyde (PFA; see recipe)

Polystyrene 12 × 75–mm FACS tubes

Centrifuge

***Staining with extracellular marker antibodies and red cell lysis***

1. Aliquot extracellular marker antibodies, appropriately diluted in FACS wash buffer, to appropriately labeled FACS tubes.

   *Follow manufacturer's instructions regarding appropriate antibody concentrations. For ease of mixing, the required volume of antibody should first be added to the FACS wash buffer to a final volume of 50 µl per tube. This is particularly useful where multiple antibodies are to be used in each tube, as it is the most efficient way of dispensing the antibodies.*

   *There are a wide range of fluorochrome-conjugated CD marker antibodies available from many suppliers. Researchers are advised to select the appropriate combinations of antibodies that will detect the cell types of interest and whose fluorochromes are detectable by the flow cytometer to be used.*

   *Each group of extracellular markers should have a tube for control, another for heat shock, and another for the isotype control for each patient/animal sample. Researchers should also always include tubes containing cells with no antibody as a background control.*

   *It is not necessary to include isotype control tubes for both heat-shocked and non-heat-shocked blood as the results do not differ and the isotype control is merely used to set gating parameters for the hsp-stained samples from each individual. A single isotype control tube for all treatment conditions is sufficient.*

2. Add 100 µl of each respective whole blood sample to each corresponding antibody tube and vortex gently.

   *Blood should be gently mixed by inverting the tube several times or by pipetting up and down before taking the aliquot.*

3. Incubate at room temperature in the dark, for 15 min.

   *All incubation steps should be undertaken in the dark to prevent deterioration of fluorescence.*

4. Add 2 ml of 1 × FACS lysing solution, then vortex.

   *Incomplete lysis of erythrocytes will cause erroneous results.*

5. Incubate at room temperature for 10 min in the dark.

6. Centrifuge 7 min at 378 × *g*, room temperature.

7. Carefully aspirate or decant supernatant.

***Intracellular antibody staining, cell permeabilization, and fixing***

8. Add 500 µl of 1 × FACS permeabilizing solution and vortex gently.

   *Detection of intracellular antigens requires a cell permeabilization step prior to staining.*

9. Incubate at room temperature for 10 min in the dark.

10. Add 2 ml FACS wash buffer.
11. Centrifuge 7 min at 486 × $g$, room temperature.
12. Carefully aspirate or decant supernatant.
13. Add fluorochrome-conjugated anti-hsp70 or fluorochrome-conjugated isotype control antibody (diluted in FACS wash buffer) to appropriate tubes, then vortex gently.

    *Follow manufacturer's instructions regarding appropriate antibody concentration. For ease of mixing the required volume of antibody should be first added to the FACS wash buffer to a final volume of 50 µl per tube.*
14. Incubate 30 min at room temperature in the dark.
15. Add 2 ml FACS wash buffer and vortex gently.
16. Centrifuge 7 min at 486 × $g$, room temperature.
17. Carefully aspirate or decant supernatant.
18. Add 200 µl 1% paraformaldehyde in PBS and vortex gently.
19. Store at 4°C in the dark until flow cytometric analysis (see Basic Protocol 2).

    *Analysis should be performed on a multicolor flow cytometer with appropriate analysis software within 24 hr of staining, as described in Basic Protocol 2.*

***ALTERNATE PROTOCOL***

## CELL STAINING FOR FACS IN 96-WELL PLATE FORMAT

This protocol describes an alternate method to Basic Protocol 1 that allows for efficient processing of large numbers of samples.

***Additional Materials*** *(also see Basic Protocol 1)*

96 well V-bottom microplates
Adhesive plate sealers
Centrifuge with microtiter plate adaptors

***Stain with extracellular marker antibodies and lyse red blood cells***

1. Aliquot extracellular marker antibodies, appropriately diluted in FACS wash buffer, to appropriate wells of a 96-well V-bottom microplate.

   *Follow manufacturer's instructions regarding appropriate antibody concentrations. For ease of mixing, the required volume of antibody should first be added to the FACS wash buffer to a final volume of 50 µl per well. This is particularly useful where multiple antibodies are to be used in each well, as it is the most efficient way of dispensing the antibodies.*

   *Each group of extracellular markers should have a tube for control, another for heat shock, and another for the isotype control for each patient/animal sample. Researchers should also always include tubes containing cells with no antibody as a background control.*
2. Add 100 µl of each respective whole blood sample to appropriate wells. Gently mix by pipetting up and down.

   *Blood should be gently mixed by inverting the tube several times or by pipetting up and down before taking the aliquot.*
3. Incubate at room temperature, in the dark, for 15 min.

   *All incubation steps should be undertaken in the dark to prevent deterioration of fluorescence.*
4. Add 90 µl FACS wash buffer to each well.
5. Cover with adhesive plate sealer.

6. Centrifuge 3 min at 400 × *g*, room temperature, in a centrifuge with a microtiter plate adaptor.
7. Remove plate sealer and carefully aspirate or flick plate to remove supernatants.
8. Repeat steps 4 to 7.
9. Add 200 µl/well of 1× FACS lysing solution, then mix well by pipetting up and down.

   *To obtain complete lysis of erythrocytes, adequate pipet mixing is essential.*
10. Cover with adhesive plate sealer.
11. Incubate at room temperature in the dark for 10 min.
12. Centrifuge 3 min at 400 × *g*, room temperature.
13. Remove plate sealer and carefully aspirate or flick plate to remove supernatant.

***Intracellular antibody staining, cell permeabilization, and fixing***

14. Add 100 µl/well of 1× FACS permeabilizing solution, then mix gently by pipetting up and down.

    *Detection of intracellular antigens requires a cell permeabilization step prior to staining.*
15. Incubate at room temperature for 10 min in the dark.
16. Add 90 µl/well of FACS wash buffer, then mix by pipetting up and down.
17. Cover with adhesive plate sealer.
18. Centrifuge 3 min at 400 × *g*, room temperature.
19. Remove plate sealer and carefully aspirate or flick plate to remove supernatant.
20. Add fluorochrome-conjugated anti hsp70 or fluorochrome-conjugated isotype control antibody, appropriately diluted in FACS wash buffer, to the appropriate wells.

    *Follow manufacturer's instructions regarding appropriate antibody concentration. For ease of mixing, the required volume of antibody should first be added to FACS wash buffer to a final volume of 50 µl per well.*
21. Incubate 30 min at room temperature in the dark.
22. Add 90 µl FACS wash buffer, then mix by pipetting up and down.
23. Cover with adhesive plate sealer.
24. Centrifuge 3 min at 400 × *g*, room temperature.
25. Remove plate sealer and carefully aspirate or flick plate to remove supernatant.
26. Repeat steps 22 to 25 two more times.
27. Add 200 µl 1% paraformaldehyde in PBS, then mix by pipetting up and down.
28. Cover with adhesive plate sealer.
29. Store at 4°C in the dark until ready to perform flow cytometric analysis (Basic Protocol 2).

    *Analysis should be done on a multicolor flow cytometer with appropriate analysis software within 24 hr of staining. If the research facility's flow cytometer does not have a high-throughput screening (HTS) plate reader accessory, then researchers should transfer samples to appropriately labeled polystyrene 12 × 75 mm FACS tubes for flow cytometric analysis.*

*BASIC PROTOCOL 2*

## FLOW CYTOMETRY

Researchers are advised to follow the manufacturer's operating instructions for their particular multicolor flow cytometer. Both forward scatter (FSC) and side scatter (SSC) data should be collected in conjunction with fluorescence emission data. Lymphoid cells should be selected by gating on forward scatter (FSC) versus side scatter (SSC). Gating should be applied to differentiate between positive and negative cell populations based on staining with leukocyte extracellular marker mAbs. A sequential gating strategy should then be employed to identify the hsp70-producing cells within the leukocyte subpopulations.

Data from a minimum of 10,000 cells should be acquired for each sample to ensure that a statistically relevant number of events are collected. Histograms of fluorescence for hsp70-specific labeling should be constructed, and percentages of positive and negative cells determined using isotype control gating. The intensity of the labeling is determined as mean fluorescence intensity (MFI). The ratio of hsp70 expression after heat shock as compared to control levels can be expressed a fold-increase. Results should be analyzed using an appropriate software package.

Detailed protocols for the abovementioned flow cytometric procedures may be found in Robinson et al. (2011).

*SUPPORT PROTOCOL 1*

## COLLECTION OF WHOLE BLOOD

Collection of blood must be performed carefully to ensure the integrity of the sample, and, therefore, the data. The protocol below is designed for collection of blood from larger specimens, but the basic principle—inclusion of heparin to prevent coagulation—is globally applicable.

### *Materials*

Animal of interest
10 ml sodium heparin vacutainer blood collection tubes
Phlebotomy equipment (e.g., needles and syringes) for venous blood collection

1. Collect approximately 6 ml venous blood into sodium heparin blood collection tubes using standard venipuncture techniques.

   CAUTION: *Avoid ingestion, inhalation, or skin contact with human or animal blood products to avoid exposure to blood-borne pathogens. Blood should be stored at room temperature and NOT refrigerated, as some hsps are induced by cold shock. Blood should be used as soon after collection as possible.*

*SUPPORT PROTOCOL 2*

## HEAT SHOCK

Heat shock treatment of samples is one of the best methods of inducing hsp synthesis. Alternate methods, including oxidative stress, are described in Agnew and Watson (2006).

### *Materials*

Heparinized whole blood (from Support Protocol 1)
1.5-ml microcentrifuge tubes
Circulating water bath set at 37°C
Circulating water bath set at 42.5°C

1. Transfer whole blood into two 1.5-ml microcentrifuge tubes (1 ml in each).

   *Label one tube control and the other heat shock.*

2. Place both tubes in a 37°C water bath for at least 15 min to rest.

3. Remove the heat shock tube and place in a 42.5°C water bath for 1 hr. Leave the control tube in the 37°C water bath for 1 hr.

   *Do not heat shock human blood above 43°C, as protein synthesis will be inhibited. This technique can be applied to measure leukocyte hsp70 in other species. Researchers should determine species-specific optimal heat shock temperatures (Agnew and Colditz, 2008)*

4. Remove the heat shock sample from the 42.5°C water bath and place in the 37°C water bath with the control sample. Incubate both tubes for a further 3 hr as a recovery period.

   *The optimum recovery period at 37°C for hsp70 in human blood is about 3 hr following a heat shock. Note that the recovery period is dependent on the nature of the hsp, species, and cell type under investigation. Researchers are therefore advised to establish the appropriate species and cell-specific conditions for their assay.*

## REAGENTS AND SOLUTIONS

*Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see* ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

### *FACS lysing solution, 1×*

Dilute 10× FACS lysing solution (Becton Dickinson, product no. 349202) 1:10 with distilled water. Keep at room temperature.

### *FACS permeabilizing solution, 1×*

Dilute 10× FACS permeabilizing solution 2 (Becton Dickinson, product no. 340973) 1:10 with distilled water. Keep at room temperature.

### *FACS wash buffer*

To phosphate-buffered saline (PBS; *APPENDIX 2A*) add 0.5% (w/v) bovine serum albumin and 0.1% (w/v) $NaN_3$. Keep at 4°C for up to 1 month.

### *Paraformaldehyde (PFA) solution, 1%*

For 10 ml of 1% PFA solution, place 0.1 g paraformaldehyde powder (Sigma-Aldrich) in a small glass tube. Add 0.5 ml distilled water. Add 1 drop of 0.5 to 1.0 M sodium hydroxide. Heat to 80°C for 2 to 3 min, until paraformaldehyde has dissolved. Do not overheat. Add 9.5 ml phosphate-buffered saline (PBS; *APPENDIX 2A*). Adjust pH to 7.4 with HCl if necessary. Cool completely before use and store at room temperature.

CAUTION: *Paraformaldehyde is flammable, has irritant vapor, and is carcinogenic. Preparation should be carried out in a fume hood while wearing appropriate protective gear.*

## COMMENTARY

### Background Information

The cellular stress response was first observed in *Drosophila busckii* salivary glands that had been exposed to an increased temperature (Ritossa, 1962). The increase in transcriptional activity of a set of genes, at a particular locus, was evidenced by a novel pattern of chromosomal puffing. The proteins coded for by these genes were later named the heat shock proteins, due to their inducibility by increases in temperature (Tissières et al., 1974).

The stress proteins belong to a multi-gene family and range in size from 8 to 150 kDa. Hsps are conveniently classified according to their molecular weight—for example, the 70-kDa protein is named hsp70, and each hsp species has a unique mechanism of action (Craig et al., 1993, Whitley et al., 1999).

The hsp70 family is the most conserved and widely researched group of proteins within the stress protein superfamily (Hunt and Morimoto, 1985). The hsp70 family comprises the constitutively expressed 73-kDa heat shock cognate protein (hsc70), the 72-kDa inducible heat shock protein (hsp70), both of which are cytosolic proteins, the

endoplasmic reticulum glucose regulated protein (grp78), and the mitochondrial glucose regulated protein (grp75). Hsp70 homologs contain peptide-binding and ATPase domains that are able to stabilize protein structures in assembly-competent and unfolded states for extended periods of time (Craig et al., 1994; James et al., 1997). The C-terminal domain binds unfolded proteins and peptides, and the N-terminal ATPase domain controls the actions of the peptide-binding domain (reviewed in Bukau and Horwich, 1998).

Hsp70 has diverse roles that include involvement in the ubiquitin/proteosome pathway, chaperoning proteins into degradation pathways, binding of newly synthesized amino acid chains onto ribosomes, and maintenance of translocation-competent folding of mitochondrial and ER proteins in the cell cytosol (reviewed in Pilon and Schekman, 1999).

In humans, at least eleven genes are known to encode the hsp70 family members (Tavaria et al., 1996). The hsp70 genes are located on chromosome 6, and are found within the major histocompatibility complex (MHC). These genes are *hsp70-1* [*hspA1A*] and *hsp70-2* [*hspA1B*], which code for the inducible hsp70. Another gene, *hsp70-hom* [*hspA1L*], which encodes a testis-specific-hsp70, is also located on chromosome 6 (Milner and Campbell, 1990; Fujimoto et al., 1992). There are also two hsp70 genes located on chromosome 1. These genes both encode the inducible form of hsp70 and are known as *hsp70B′* [*hsp70A6*] (Leung et al., 1990) and *hsp70B* [*hsp70A7*] (Voellmy et al., 1985; Leung et al., 1992).

The involvement of hsps in a number of human disease states has emphasized the important role of these highly conserved proteins in the modulation of the immune response. It appears that these proteins are not only intracellular molecules that have essential housekeeping and cytoprotective roles, but they also function as intercellular signaling molecules (Pockley, 2002). The immunoregulatory roles of hsps are yet to be fully elucidated; however, their ability to stimulate the innate immune system may, in turn, prime the adaptive immune system (Lehner et al., 2000). Current understanding of the roles of hsps in the immune response can be characterized by three main mechanisms. The first involves the ability of cytosolic- and ER-located hsps to noncovalently bind immunogenic peptides such as MHC class I epitopes and their elongated precursors (Nieland et al., 1996; Ishii et al., 1999). Secondly, hsp-peptide complexes are able to be cross-presented to $CD8^+$ T lymphocytes via the MHC class I pathway (Udono and Srivastava, 1993; Breloer et al., 1999; Singh-Jasuja et al., 2000). It has been proposed that hsp-chaperoned antigenic peptides are endocytosed by antigen-presenting cells (APC) through the common heat shock protein receptor CD91 (Binder et al., 2000; Basu et al., 2001). Thirdly, hsps have the ability to promote dendritic cell maturation and the release of pro-inflammatory cytokines (Asea et al., 2000).

Flow cytometry is a method of measuring particular physical and chemical characteristics of cells as they travel in suspension, one by one, past a sensing point. Forward and side scatter of light by cells provides information on cell size and cytoplasmic granularity. The staining of cells with specific antibodies tagged with different fluorochromes facilitates detection of cell surface antigens and intracellular molecules. Flow cytometry is a rapid, quantitative method for determining intracellular hsp70 expression in individual cells from a heterogeneous population such as peripheral blood mononuclear cells (PBMC; Bachelet et al., 1998). Leukocytes express distinct assortments of cell surface antigens, many of which reflect either different stages of their lineage-specific differentiation or different states of activation or inactivation. The Clusters of Differentiation (CD) nomenclature assigns a number that includes any antibody having an identical and unique reactivity pattern with different leukocyte populations. To date, molecules CD1 to CD350 have been characterized (Zola et al., 2007). CD antigens are differentially expressed on leukocytes, and distinct cell subpopulations can be identified according to their patterns of CD antigen expression (Goldsby et al., 1997). The ability to stain cells for multiple extracellular markers in a single tube has a range of benefits for the researcher. This method uses less blood, eliminates potential errors, and allows for the comparison of dual-positive cell types as compared to multiple tubes and a flow cytometer with fewer lasers.

Multicolor flow cytometric analysis enables examination of complex cellular interactions in mixed cell populations. The sensitivity of this technique allows simultaneous analysis of cell surface markers and intracellular molecules at the single-cell level. Since cell surface molecules are involved in mediating immune responses, flow cytometry may provide important information regarding a number of related parameters including cell lineage, activation status, adhesion, migration,

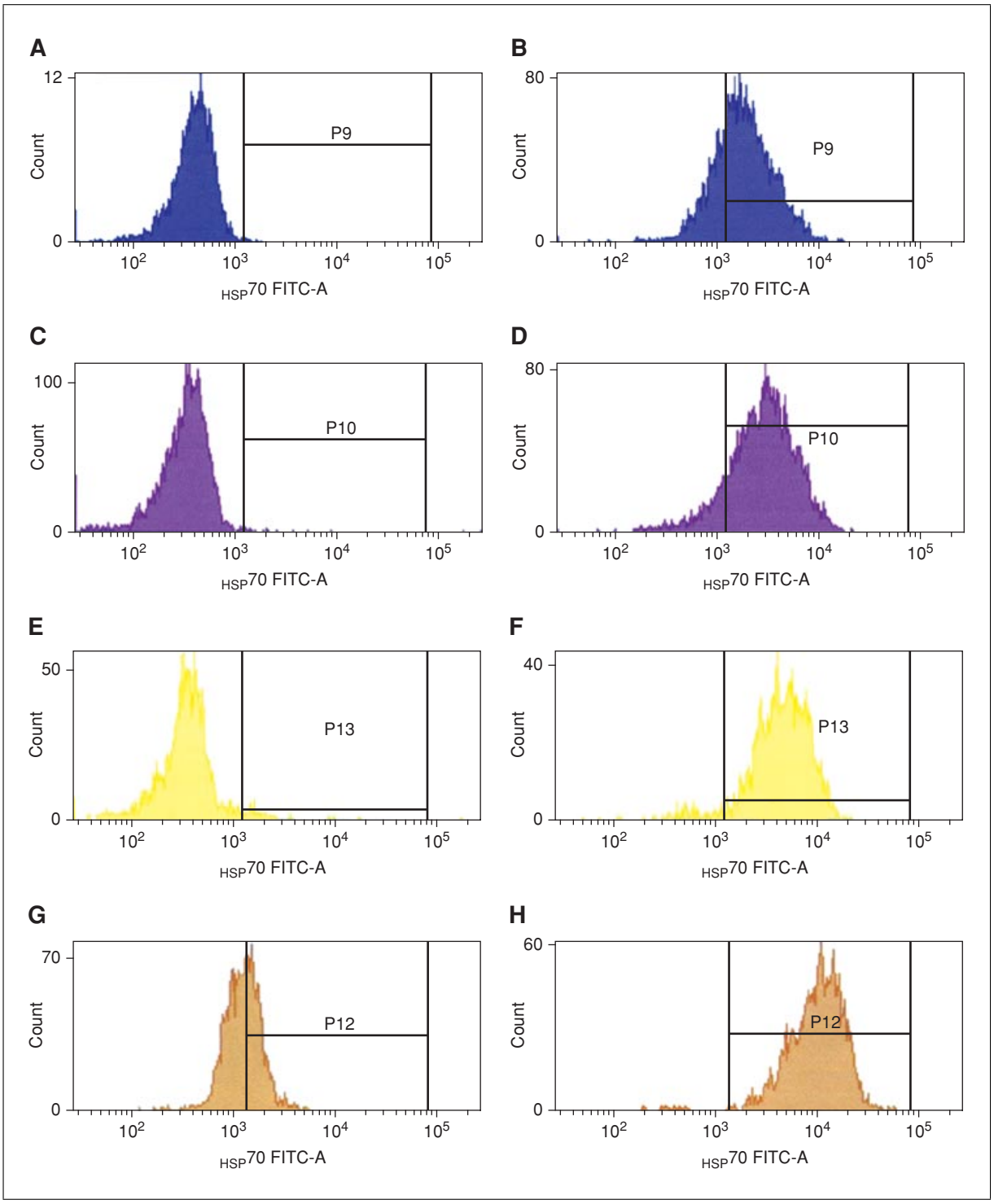


**Figure 2.21.1** Histograms illustrating hsp70 expression in a range of leukocyte subpopulations. Gating has been applied using an isotype control. (**A**) $CD3^+/CD4^+$ T (helper) lymphocytes under control conditions (37°C). (**B**) $CD3^+/CD4^+$ T (helper) lymphocytes after heat shock (42.5°C for 1 hr). (**C**) $CD3^+/CD8^+$ T (cytotoxic) lymphocytes under control conditions (37°C). (**D**) $CD3^+/CD8^+$ T (cytotoxic) lymphocytes after heat shock (42.5°C for 1 hr). (**E**) $CD16^+/CD56^+$ natural killer cells under control conditions (37°C). (**F**) $CD16^+/CD56^+$ natural killer cells after heat shock (42.5°C for 1 hr). (**G**) $CD14^+$ monocytes under control conditions (37°C). (**H**) $CD14^+$ monocytes after heat shock (42.5°C for 1 hr).

ability to respond to stimuli, and interaction with other cells. Quantitative comparisons of both the constitutive and inducible forms of hsp70 as measured by western immunoblot and flow cytometry have shown excellent agreement, demonstrating the accuracy of flow cytometric analysis of hsp expression (He and Fox, 1996). Flow cytometric methods have demonstrated that hsp70 is constitutively expressed in human leukocytes, but that the level of expression varies considerably between different cell types (Oehler et al., 2001). This method holds several advantages over the more traditional methods of determining levels of hsp expression, such as western blotting (immunoblotting), 1-D SDS-PAGE, and ELISA. These advantages include the specific information provided by this technique as to which cells are expressing the protein of interest and the significantly smaller volume of blood required to conduct the flow cytometric analysis.

## Critical Parameters and Troubleshooting

***Cell staining and flow cytometry***

Appropriate controls to detect background fluorescence and nonspecific binding of antibodies should be used. Tubes containing nonlabeled cells are used to detect background signals, and isotype controls are used to detect nonspecific binding.

Compensation for multi-fluorochrome spectral overlap should be made using single-fluorochrome-labeled cells (e.g., if undertaking dual staining with FITC and PE, then FITC-only and PE-only stained cells or compensation beads should be used). For more detail regarding the fundamental principles underpinning standardization and calibration in cytometry, see Mittag and Tarnok (2009) and Robinson et al. (2011).

Where fluorochrome-labeled extracellular marker antibodies are not available, researchers are advised that Basic Protocols 1 and 2 can be adapted to include additional steps for the use of unlabeled primary antibodies followed by a second-step reaction with species- and isotype-compatible fluorochrome-labeled secondary antibodies. If using a second-step reaction, researchers are advised that additional controls are required. These include tubes containing the secondary antibody only.

***Stress treatment***

The kinetics of induction of hsps in PBMC has been found to be different for a number of hsps. Hsp70 attains maximum induction levels at the 3-hr recovery time, whereas hsp40 shows maximum induction at the 1-hr recovery time (Rao et al., 2003). Hsps 110, 90, and 60 also show maximal levels of induction at the 3-hr recovery time (D.V. Rao, pers. comm.).

It should be noted that the kinetics of induction of the heat shock response may vary widely with different cell types. Appropriate conditions for a range of cell lines has been reported (Wieten et al., 2010). Researchers are therefore advised to establish the appropriate species and cell-specific conditions for their assay.

## Anticipated Results

Hsp70 expression is variable in leukocyte subtypes; the protein is preferentially expressed by monocytes (Bachelet et al., 1998). Leukocytes examined by flow cytometry display differential patterns of hsp70 expression at both basal and heat shock levels. The common leukocyte cell types (helper T lymphocytes, cytotoxic T lymphocytes, B lymphocytes, natural killer cells, and monocytes) display a significant up-regulation in hsp70 after a mild, nonlethal heat shock (Fig. 2.21.1).

Using an isotype control antibody reduces the risk of false-positive results, an issue of particular importance when examining cell subsets expressing low levels of hsp70.

## Time Considerations

Venipuncture takes approximately 15 min. Stress treatment of whole blood takes approximately 4.5 hr. The staining of extracellular markers with fluorochrome-labeled antibodies, lysis of red blood cells, permeabilization of leukocytes, staining with fluorochrome-labeled anti-hsp70, and fixation of cells takes around 2 hr. The time required for flow cytometry will depend on the instrument used, instrument setup times, and the number of samples to be run.

These times are approximate and will vary depending on the number of samples being assayed at the one time. Generally, if using Basic Protocol 1, one person can comfortably handle six samples at the one time.

## Literature Cited

Agnew, L.L. and Colditz, I.G. 2008. A method for measuring cellular stress in sheep and cattle. *Vet. Immunol. Immunopathol.* 123:197-204.

Agnew, L.L. and Watson, K. 2006. Detection of stress proteins as biomarkers of oxidative stress. *Curr. Protoc. Toxicol.* 28:17.8.1-17.8.25.

Asea, A., Kraeft, S.K., Kurt-Jones, E.A., Stevenson, M.A., Chen, L.B., Finberg, R.W., Koo, G.C., and Calderwood, S.K. 2000. Hsp 70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat. Med.* 6:435-442.

Bachelet, M., Mariethoz, E., Banzet, N., Souil, E., Pinot, F., Polla, C.Z., Durand, P., Bouchaert, I., and Polla, B.S. 1998. Flow cytometry is a rapid and reliable method for evaluating heat shock protein 70 expression in human monocytes. *Cell Stress Chaperones* 3:168-176.

Basu, S., Binder, R.J., Ramalingam, T., and Srivastava, P.K. 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* 14:303-313.

Binder, R.J., Han, D.K., and Srivastava, P.K. 2000. CD91: A receptor for heat shock protein gp96. *Nat. Immunol.* 1:151-155.

Breloer, M., Fleischer, B., and Bonin, A.V. 1999. In vivo and in vitro activation of T cells after administration of Ag-negative heat shock proteins. *J. Immunol.* 162:3141-3147.

Bukau, B. and Horwich, A.L. 1998. The hsp90 and hsp70 chaperone machines. *Cell* 92:351-366.

Callis, J. 1995. Regulation of protein degradation. *Plant Cell* 7:845-857.

Craig, E.A., Gambill, B.D., and Nelson, R.J. 1993. Heat shock proteins: Molecular chaperones of protein biogenesis. *Microbiol. Rev.* 57:402-414.

Craig, E.A., Weissmann, J.A., and Horwich, A.L. 1994. Heat shock proteins and molecular chaperones: Mediators of protein conformation and turnover in the cell. *Cell* 78:365-372.

Fujimoto, H., Ito, Y., Ando, A., Matsumoto, M., Fujio, K., Miura, K., Shirai, M., and Inoko, H. 1992. A human hsp70 homologue gene located in HLA class III region is expressed in the testicular germ cells. *In* Proceedings of the Eleventh International Histocompatibility Workshop and Conference. HLA 1991. Vol II. (K. Tsugi, M. Aizawa, and T. Sasazuki, eds.) pp. 154-157. Oxford University Press, Oxford, U.K.

Goldsby, R.A., Kindt, T.J., and Osbourne, B.A. 1997. Kuby Immunology, 3rd edition. W.H. Freeman & Company, New York.

He, L. and Fox, M.H. 1996. Comparison of flow cytometry and Western blotting to measure hsp70. *Cytometry* 25:280-286.

Hunt, C. and Morimoto, R.I. 1985. Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. *Proc. Natl. Acad. Sci. U.S.A.* 82:6455-6459.

Ishii, T., Udono, H., Yamano, T., Ohta, H., Uenaka, A., Ono, T., Hizuta, A., Tanaka, N., Srivastava, P.K., and Nakayama, E. 1999. Isolation of MHC class I-restricted tumor antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. *J. Immunol.* 162:1202-1309.

James, P., Pfund, C., and Craig, E.A. 1997. Functional specificity among hsp70 molecular chaperones. *Science* 275:387-391.

Jolly, C. and Morimoto, R.I. 2000. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J. Natl. Cancer Inst.* 92:1564-1572.

Lehner, T., Bergmeier, L.A., Wang, Y., Tao, L., Sing, M., Spallek, R., and van der Zee, R. 2000. Heat shock proteins generate β-chemokines which function as innate adjuvants enhancing adaptive immunity. *Eur. J. Immunol.* 30:594-603.

Leung, T.K., Rajendran, M.Y., Monfries, C., Hall, C., and Lim, L. 1990. The human heat shock protein family: Expression of a novel heat-inducible HSP70 (HSP70B') and isolation of its DNA and genomic DNA. *Biochem. J.* 267:125-132.

Leung, T.K., Hall, C., Rajendran, M.Y., Spurr, N.K., and Lim, L. 1992. The human heat shock genes HSPA6 and HSPA7 are both expressed and localize to chromosome 1. *Genomics* 12:74-79.

Matthew, A. and Morimoto, R.I. 1998. Role of the heat-shock response in the life and death of proteins. *Ann. N.Y. Acad. Sci.* 851:99-111.

Milner, C.M. and Campbell, R.D. 1990. Structure and expression of the three MHC-linked hsp70 genes. *Immunogenetics* 36:242-251.

Mittag, A. and Tarnok, A. 2009. Basics of standardization and calibration in cytometry: A review. *J. Biophotonics.* 2:470-481.

Morimoto, R.I., Tissieres, A., and Georgopoulos, C. 1994. The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Nieland, T.J., Tan, M.C., Monne-van Muijen, M., Koning, F., Kruisbeek, A.M., and van Bleek, G.M. 1996. Isolation of an immunodominant viral peptide that is endogenously bound to the stress protein GP96/GRP94. *Proc. Natl. Acad. Sci. U.S.A.* 93:6135-6139.

Oehler, R., Pusch, E., Zellner, M., Dungel, P., Hergovics, N., Homoncik, M., Eliasen, M.M., Brabec, M., and Roth, E. 2001. Cell type-specific variations in the induction of hsp70 in human leukocytes by fever-like whole body hyperthermia. *Cell Stress Chaperones* 6:306-315.

Pilon, M. and Schekman, R. 1999. Protein translocation: How hsp70 pulls it off. *Cell* 97:679-682.

Pockley, A.G. 2002. Heat shock proteins as regulators of the immune response. *Lancet* 362:469-476.

Rao, D.V., Boyle, G.M., Parsons, P.G., Watson, K., and Jones, G.L. 2003. Influence of ageing, heat shock treatment and in vivo total antioxidant status on gene-expression profile and protein synthesis in human peripheral lymphocytes. *Mech. Ageing Dev.* 124:55-69.

Ritossa, F. 1962. A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia* 18:571-573.

Robinson, J.P., Darzynkiewicz, Z., Dean, P.N., Dressler, L.G., Rabinovitch, P.S., Stewart, C.C., Tanke, H.J., and Wheeless, L.L. 2011. Current Protocols in Cytometry. John Wiley & Sons, Hoboken, N.J.

Singh-Jasuja, H., Toes, R.E.M., Spee, P., Munz, C., Hilf, N., Schoenburger, S.P., Ricciardi-Castagnoli, P., Neefjees, J., Rammensee, H-G., Arnold-Schild, D., and Schild, H. 2000. Cross-presentation of glycoprotein 96-associated antigens on Major Histocompatibility Complex class I molecules requires receptor-mediated endocytosis. *J. Exp. Med.* 191:1965-1974.

Takayama, S., Reed, J.C., and Homma, S. 2003. Heat shock proteins as regulators of apoptosis. *Oncogene* 22:9041-9047.

Tavaria, M., Gabriele, T., Kola, I., and Anderson, R.L. 1996. A hitchhiker's guide to the human hsp70 family. *Cell Stress Chaperones* 1:23-28.

Tissières, A., Mitchell, H.K., and Tracy, U.M. 1974. Protein synthesis in salivary glands of *Drosophila melanogaster*: Relation to chromosome puffs. *J. Mol. Biol.* 85:389-398.

Udono, H. and Srivastava, P.K. 1993. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J. Exp. Med.* 178:1391-1396.

van der Vies, S.M., Viitanen, P.V., Gatenby, A.A., Lorimer, G.H., and Jaenicke, R. 1992. Conformational states of ribulosebisphosphate carboxylase and their interaction with chaperonin 60. *Biochemistry* 31:3635-3644.

Voellmy, R., Ahmed, A., Schiller, P., Bromley, P., and Rungger, D. 1985. Isolation and functional analysis of human 70,000-dalton heat shock protein gene segment. *Proc. Natl. Acad. Sci. U.S.A.* 82:4949-4953.

Welch, W.J. 1992. Mammalian stress response: Cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol. Rev.* 72:1063-1081.

Whitley, D., Goldberg, S.P., and Jordan, W.D. 1999. Heat shock proteins: A review of the molecular chaperones. *J. Vasc. Surg.* 29:748-751.

Wieten, L., van der Zee, R., Goedemans, R., Sijtsma, J., Serafini, M., Lubsen, N.H., van Eden, W., and Broere, F. 2010. Hsp70 expression and induction as a readout for detection of immune modulatory components in food. *Cell Stress Chaperones* 15:25-37.

Zola, H., Swart, B., Banham, A., Barry, S., Beare, A., Bensussan, A., Boumsell, L., Buckley, C., Bühring, H.J., Clark, G., Engel, P., Fox, D., Jin, B.Q., Macardle, P.J., Malavasi, F., Mason, D., Stockinger, H., and Yang, X. 2007. CD molecules 2006-human cell differentiation molecules *J. Immunol. Methods* 319:1-5.