

Isolation of Cytotoxic T Cell and NK Granules and Purification of Their Effector Proteins

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ABSTRACT

Killer lymphocytes induce apoptosis by the release of cytotoxic mediators from specialized secretory lysosomes, called cytotoxic granules, into the immunological synapse formed with a cell targeted for elimination. Methods are presented here for isolating CTL and NK cell cytotoxic granules using cell disruption by nitrogen cavitation followed by continuous Percoll density gradient fractionation. Protocols are also given for purifying the key cytolytic molecules (perforin, granzyme A, granzyme B, and granulysin) from isolated cytotoxic granules by fast protein liquid chromatography. *Curr. Protoc. Cell Biol.* 47:3.37.1-3.37.29. © 2010 by John Wiley & Sons, Inc.

Keywords: cytotoxic granules • CTL • NK cells • perforin • granzyme • granulysin

INTRODUCTION

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells play key roles in the immune response against virus-infected cells and tumors. These killer cells eliminate infected or transformed cells principally by releasing the contents of cytotoxic granules that contain programmed cell death-inducing serine proteases, called granzymes (for granule enzyme), and membranolytic pore-forming molecules, perforin (PFN) and granulysin (GNLY; Russell and Ley, 2002; Voskoboinik et al., 2006; Pipkin and Lieberman, 2007; Chowdhury and Lieberman, 2008). Recognition and binding of target cells by CTL and NK cells leads to highly polarized exocytosis of cytotoxic granules into the immunological synapse formed with the target cell. Granzyme entry into the target cell requires perforin.

Cytotoxic granules are complex secretory lysosomes that contain both effector molecules responsible for target cell elimination and resident lysosomal proteins (Table 3.37.1). These include PFN, calreticulin, and granzymes, which are complexed with a chondroitin sulfate-rich proteoglycan termed serglycin (for its alternating serine-glycine backbone; Lieberman, 2003). The dense core of activated human CTL and NK cells cytotoxic granules also contain GNLY, a member of the saposin-like protein (SAPLIP) family that exhibits a broad spectrum of antimicrobial activity, killing bacteria, fungi, and parasites (Stenger et al., 1998). Other than calreticulin, the expression of these proteins is largely restricted to activated CTL and NK cells. However, some of the granzymes, especially granzyme B, are also expressed together with mast cell proteases, but without perforin, in activated myeloid cells and some other cell types (Rissoan et al., 2002; Strik et al., 2007).

Protocols have been developed for cytotoxic granule isolation in order to study the composition of these organelles, as well as to purify native PFN and granzymes for in vitro studies of their respective roles in inducing target cell apoptosis. There is also increasing evidence for noncytotoxic functions of the granzymes in inflammation. Methods to

Table 3.37.1 CTL and NK Cell Cytotoxic Granule Contents

Protein	Function
<i>Dense core</i>	
Perforin	Pore formation; internalization of granzymes
Granzymes	Serine proteases
Granulysin	Microbicidal activity
Calreticulin	Calcium storage and perforin inhibitor
Cathepsin C	Granzyme processing
Serglycin	Proteoglycan matrix
<i>Periphery (resident lysosomal proteins)</i>	
Lamp-1	Lysosomal membrane proteins
Lamp-2	Lysosomal membrane proteins
CD63	Lysosomal membrane proteins
H ⁺ ATPase	Lysosomal acidification
Cathepsin B	Protection of killer cell from perforin
Cathepsin D	Lysosomal hydrolases
A-glucosidase	Lysosomal hydrolases
B-glucuronidase	Lysosomal hydrolases

express and purify recombinant versions of these proteins are also available (Sun et al., 1999; Martinvalet et al., 2008b; Sutton et al., 2008), but the procedure for purifying recombinant perforin is very challenging and not reproducible in our hands.

Basic Protocol 1 describes the isolation of cytotoxic granules from human lymphokine-activated killer (LAK) cells obtained from interleukin-2 and phytohemagglutinin (IL2/PHA)-activated peripheral blood mononuclear cells (PBMC) or human NK cell lines (i.e., YT-Indy) using a modification of the Borregaard method (Borregaard et al., 1983). This method (and all of the procedures described here) can also be used to isolate rodent granules and purify their cytolytic molecules. The Borregaard method is based on nitrogen cavitation of activated effector cells designed to disrupt the plasma membrane without lysing cytotoxic granules. The cytotoxic granules are then separated from other cell components by Percoll density gradient fractionation. Cytotoxic granules may also be isolated by the same method from RNK-16 cells, a rat NK-like leukemia cell line, grown as ascites for higher yields (see Alternate Protocol 1). Once the cytotoxic granules have been isolated, they can be lysed and either used directly to treat target cells to induce apoptosis (Support Protocols 9 and 10), or their cytolytic effector molecules can be purified by fast protein liquid chromatography (FPLC; Basic Protocol 2 and Alternate Protocol 2). We describe the purification of PFN (Basic Protocol 2), and the major granzymes—GzmA, GzmB, and GNLY (Alternate Protocol 2). PFN and granzyme expression can be detected immunologically (Support Protocols 1 and 2). After purification, PFN activity may be measured by different methods, including hemoglobin release assay (Support Protocol 3) or propidium iodide uptake (Support Protocol 4). GzmA and GzmB proteolytic activity is tested by peptide substrate (BLT and AAD) hydrolysis (Support Protocols 5 and 6), and GNLY activity by a colony-forming unit assay (Support Protocol 7) and turbidimetry (Support Protocol 8). We also describe two common assays (chromium release assay and annexin-V assay) to assess PFN and granzyme-induced cell death and apoptosis obtained by these methods (Support Protocols 9 and 10).

BORREGAARD METHOD OF CTL AND NK CELL CYTOTOXIC GRANULE ISOLATION

BASIC PROTOCOL 1

This is a 1-day protocol. CTL or NK cells are disrupted by nitrogen cavitation in a physiological buffer to release cytotoxic granules from whole cells. Isolated granules obtained from human LAK cells contain PFN and both major granzymes, GzmA and GzmB, as well as GNLY. YT-Indy NK cells contain PFN, GzmB, and GNLY, but not GzmA or K, and they are especially recommended for high-yield hGzmB purification. Alternatively, cytotoxic granules can be isolated from rat RNK-16 NK-like leukemia cells, serially passaged in vivo as ascites in Fischer F344 rats for higher yield purification (Alternate Protocol 1).

Granules may be purified from various sources, three of which are described in this protocol. The advantage of using human cells is that all effector molecules will be of the same species and from the same source. Additionally, primary human cells are rapidly dividing, and the transformed human NK lines highly over-express granzymes. The disadvantages of these techniques are the cost of IL-2 when expanding primary human cells and that the transformed human NK lines expand slowly and require a large amount of culture medium.

Materials

Human blood *or* buffy coats for LAK cell production *or* YT-Indy NK cells (ATCC)
Ficoll-Paque PLUS (GE Healthcare)
K10 medium (see recipe)
K10 + IL-2: K10 medium plus 1000 IU/ml human IL-2 (Chiron)
PHA-P (Sigma)
Hanks' balanced salt solution (HBSS) without calcium, magnesium, and phenol red
Relaxation buffer (see recipe)
Continuous 40% (w/v) adjusted Percoll gradient (see recipe)
Solubilization buffer (see recipe)

50-ml centrifuge tubes
Centrifuge
1000- μ l pipettor *or* serological pipet
Cavitation/disruption bomb (Parr Instrument Company)
Magnetic stirrer
Refrigerated centrifuge with Sorvall SS-34 rotor (or equivalent), and corresponding 26-ml rigid polycarbonate tube (Beckman Instruments)
20-G spinal needle (Popper & Sons)
Beckman ultracentrifuge with Sw28Ti rotor (or equivalent) and thick-wall polycarbonate tubes (cat. no. 355631)

Additional reagents and equipment for counting the cells (*UNIT 1.1*), flow cytometry (Support Protocol 2), and fluorescence microscopy (Support Protocol 1)

NOTE: All procedures, buffers, tubes, and equipment must be maintained at 4°C during the purification unless otherwise noted.

NOTE: Make sure to handle human blood products with universal precautions following the rules of your institution.

Isolate and culture cells

1. For purification from NK cell lines, skip to step 8. For purification from PBMC or human buffy coats, layer 30 ml of whole blood or buffy coat onto 20 ml prewarmed Ficoll in a 50-ml centrifuge tube. Layer the blood on top of the Ficoll by slowly

running the blood down the side of the tube, making sure not to disrupt the phase layers during layering.

2. Centrifuge the tubes 30 min at 950 g, room temperature, and turn off the brake of the centrifuge.

Active braking will disturb the lymphocyte layer. Do not centrifuge at 4°C, as this will alter cell/Ficoll density.

3. Remove tubes carefully from the centrifuge.

The top layer will be plasma, the middle white layer (interface) will be PBMC, the clear layer will be Ficoll, and the bottom layer will be red blood cells.

4. Carefully remove the lymphocyte layer from the tubes with a 1000- μ l pipettor or serological pipet, making sure to remove the least amount of Ficoll possible.

5. Wash the PBMC three times, each time for 5 min in 50-ml 37°C K10 medium to remove Ficoll and resuspend in 10 to 50 ml K10. Count an aliquot of the cells (UNIT 1.1).

6. Culture cells at 10^6 cells/ml for 3 days in K10+IL-2 supplemented with 4 μ g/ml PHA-P in 75-cm² cell culture flasks at 37°C, 5% CO₂.

Alternatively, one may select for CD3⁺CD8⁺ cells to enrich for cytotoxic T cells or for CD3⁻CD56⁺ cells to enrich for NK cells. Reduced doses of IL-2 may be used, but high doses of IL-2 improve yields by up-regulating expression of the effector proteins.

7. On the fourth day, resuspend the cells in fresh K10+IL-2 (without PHA-P) and reseed at 10^6 cells/ml and expand culture, splitting every several days to maintain a density of 1×10^6 cells/ml, until the desired cell number is achieved.

At least 5×10^9 total cells are suggested for granule purification.

These LAK cells should grow very rapidly; expanding from $0.1-1 \times 10^8$ cells (the expected yield of PBMCs is at least 10^6 cells/ml of blood) should take less than 2 weeks.

Cells should be harvested 1 to 3 weeks after PHA stimulation for optimal expression of cytotoxic molecules.

8. For cultures beginning with NK cell lines, maintain YT-Indy cells in K10 medium at densities of $0.1-1 \times 10^6$ cells/ml. Split the cells by diluting 1:2 to 1:3, adding new medium to older medium.

Do not completely change medium as the cells rely on secreted IL-2 to proliferate.

9. Expand the cells to $3-5 \times 10^9$ total cells, which is adequate for granule purification.

The cells do not divide very rapidly, so expect the expansion to take ~3 weeks.

10. After cell expansion, test expression of PFN and/or granzymes contained in cytotoxic granules by flow cytometry (Support Protocol 2) or fluorescence microscopy (Support Protocol 1) before isolation of cytotoxic granules or effector molecule purification (Fig. 3.37.1).

Disrupt cells by nitrogen cavitation

11. Prechill the cavitation/disruption bomb at 4°C overnight before procedure.

12. Collect at least $3-5 \times 10^9$ cells, wash the cells twice with cold HBSS (keep at 4°C at all times), and then resuspend at 10^8 cells/ml in relaxation buffer.

13. Place cells in the cavitation bomb on top of a magnetic stirrer.

If the cell suspension is ≤ 50 ml, place the cells in a 50-ml centrifuge tube and then place the tube in the bomb with ice surrounding the tube of cells.

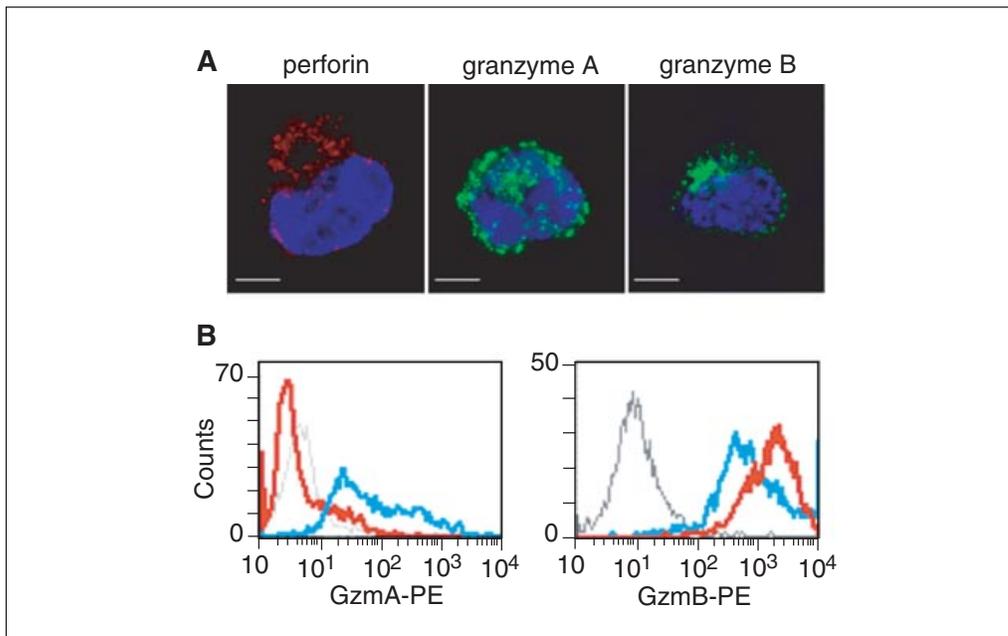


Figure 3.37.1 Analysis of cytotoxic granule content. **(A)** Cytotoxic granules of day 10 LAK cells were stained with anti-PFN, anti-GzmA, or anti-GzmB monoclonal antibodies before fluorescence microscopy analysis. Scale bars: 5 μm . **(B)** Jurkat (gray), YT-Indy (red), or day 10 LAK cells (blue) cells were stained with GzmA (left panel) or GzmB (right panel) monoclonal antibodies and expression was determined by FACS analysis. Jurkat cells do not express GzmA or GzmB while YT-Indy cells only express GzmB. LAK cells express both GzmA and GzmB. For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb0337>.

14. Slowly pressurize to 450 psi with N_2 and stir at 4°C for 20 min. Then, gradually release pressure and harvest the contents dropwise into a new tube on ice. If pressure drops below 250 psi, release remaining pressure quickly through the intake valve and collect remaining sample.

The yield of granules is markedly influenced by the cavitation process. Excessive cavitation will rupture the cytotoxic granules. The stir plate must be adjusted to a speed that minimizes foaming.

Concentrate granules and remove nuclear and cytoplasmic contamination

15. To remove nuclei by differential centrifugation, centrifuge the sample 10 min at $400 \times g$, 4°C , and retain the supernatant.
16. Wash the pellet twice in equal volumes of relaxation buffer, centrifuge 5 min at $400 \times g$, 4°C , and add supernatants to the original supernatant.
17. Centrifuge the pooled supernatants 5 min at $400 \times g$, 4°C .

The final post-nuclear supernatant (PNS) should be free of nuclei and whole cells.

18. Centrifuge the PNS 10 min at $15,000 \times g$, 4°C , to pellet granules and heavy organelles and resuspend the pellet in 20 ml relaxation buffer with a 20-G spinal needle.

Fractionate and collect cytotoxic granules

19. Perform a 20-ml continuous gradient of 40% adjusted Percoll (4°C) in four rigid polycarbonate tubes by centrifuging 10 min at $48,000 \times g$, 4°C , in an SS-34 rotor.

“Adjusted” means that the Percoll concentration is adjusted with relaxation buffer to a final concentration of 40%.

20. Carefully, layer 5 ml of PNS/tube onto the 20 ml of preformed gradient of 40% adjusted Percoll. Centrifuge for 35 min at $48,000 \times g$, 4°C .

21. Extract cytotoxic granules with a 20-G spinal needle from the bottom of the gradient by harvesting the bottom 5 to 7 ml from each tube.

Granules should form a visible refractile layer.

22. Pool the granule fractions and then remove Percoll by pelleting it by centrifugation overnight at $64,000 \times g$ in 28-ml thick-wall polycarbonate tubes, 4°C , in a Sw28Ti rotor.

Granules form a thin layer above the Percoll pellet.

Pellet granules

23. Remove the supernatant and resuspend the granules in solubilization buffer with a 20-G spinal needle (2 ml per 10^9 original cells) and store at -80°C .

After resuspension, the granules can be stored indefinitely at -80°C before further protein purification.

Disruption of the granule membranes and solubilization of granule proteins is optimal after at least one freeze/thaw cycle after storage overnight at -80°C followed by two freeze/thaw cycles in liquid N_2 .

The solubilized granules can also be used directly to treat cells for apoptosis assays provided the total salt is diluted to 150 mM NaCl with HE buffer (Support Protocol 9).

ALTERNATE PROTOCOL 1

PURIFICATION OF CYTOTOXIC GRANULES FROM RAT RNK-16 CELLS

The advantage of this method is that RNK-16 granule isolation described below is best suited for high-yield purification. The disadvantage of this technique is the need to use rats to serially passage RNK-16 NK-like leukemia cells as ascites *in vivo* since these cells do not grow in tissue culture. Typically, ascites from each rat yields $1-5 \times 10^9$ cells.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Materials

- F344 rats (200 to 225 g; Harlan Laboratories)
- Pristane (2,5,10,14-tetramethyl-pentadecane; Sigma)
- RNK-16 cells
- Hanks' balanced salt solution (HBSS) without calcium, magnesium, and phenol red
- HHH buffer (see recipe)
- Ice
- Red blood cell lysis buffer (Sigma)
- 3-ml syringe and 23-G needle
- Dissecting pad
- Scissors
- 10-ml pipet
- Additional reagents and equipment for euthanizing the rat (Donovan and Brown, 2006)

Prepare the peritoneal cavity

1. Inject F344 rats intraperitoneally with 1.5 ml of sterile Pristane using a 3-ml syringe with a 23-G needle 4 to 7 days before RNK-16 cell injection to prepare the peritoneal cavity to host the cells.

Grow RNK-16 cells in rats

2. Wash $5\text{--}10 \times 10^7$ RNK-16 cells/rat twice, each time in 50 ml HBSS and resuspend in 2 ml of HBSS per rat for intraperitoneal (i.p.) injection.
3. Inject about $5\text{--}10 \times 10^7$ RNK-16 cells into each rat i.p. using a 3-ml syringe and 23-G needle.
4. Beginning 11 days post i.p. injection of RNK-16 cells, monitor rats for ascites production. Monitor rats every day for ascites production by abdominal palpation and progressive ascites fluid distention of the abdomen.

Most of the time, rats are sacrificed 12 to 15 days post i.p. injection and RNK-16 cells are harvested immediately.

Harvest RNK-16 cells

5. Sacrifice the rat using CO₂ asphyxiation (Donovan and Brown, 2006) and perform bilateral thoracotomy (a small incision into the pleural space of the chest) 5 min after CO₂ exposure for euthanasia before harvesting the cells.
6. To harvest cells, pin the rat on its back to a dissecting pad and using scissors make a midline vertical slit in the abdominal skin to expose the peritoneal membrane without damaging it.
7. Then make a small incision with scissors into the left peritoneal membrane and slowly inject 10 ml of cold HHH buffer with a 10-ml pipet into the peritoneal cavity.
8. Mix carefully with the pipet to mobilize the ascitic fluid from the cavity, withdraw the peritoneal wash fluid containing RNK16 cells, and place on ice until the end of the procedure.
9. Repeat washing and harvesting cells until washes are clear.

Expect to use 100 to 150 ml HHH buffer to extract all RNK-16 cells in one rat.

10. Centrifuge 5 min at $200 \times g$, 4°C, and remove the supernatant (carefully take the top layer first, which is pristane, and then remove the HHH buffer).

The pristane is a distinguishable white layer above the HHH buffer.

If RNK-16 cells are contaminated with blood, incubate the pellet with red blood cell lysis buffer for 10 min at 4°C.

11. Wash the cells three times, each time with at least 100 ml cold HBSS (keep at 4°C at all times) and then resuspend the cells at 10^8 /ml in relaxation buffer.

Aliquots of RNK-16 cells that appear viable by trypan blue staining from rats that produce a high yield of cells without blood contamination are used for re-injection or immediately frozen for future use.

Isolate granules

12. Proceed immediately with cell disruption by nitrogen cavitation and Percoll gradient fractionation of cytotoxic granules (Basic Protocol 1, steps 11 through 23).

PURIFICATION OF PERFORIN FROM CYTOTOXIC GRANULES

Rodent and human granzymes and PFN are cross-reactive at inducing cell death across species, although with some differences in substrate activity (Casciola-Rosen et al., 2007; Cullen et al., 2007). PFN can be purified from cytotoxic granules isolated from human LAK cells, YT-Indy NK cells, or rat RNK-16 cells. Because YT-Indy NK cells lack GzmA and GzmK expression and have high GzmB yields, those cells are preferred for GzmB purification, but GzmB can also be purified from rat RNK-16 cells. Because

BASIC PROTOCOL 2

Subcellular Fractionation and Isolation of Organelles

3.37.7

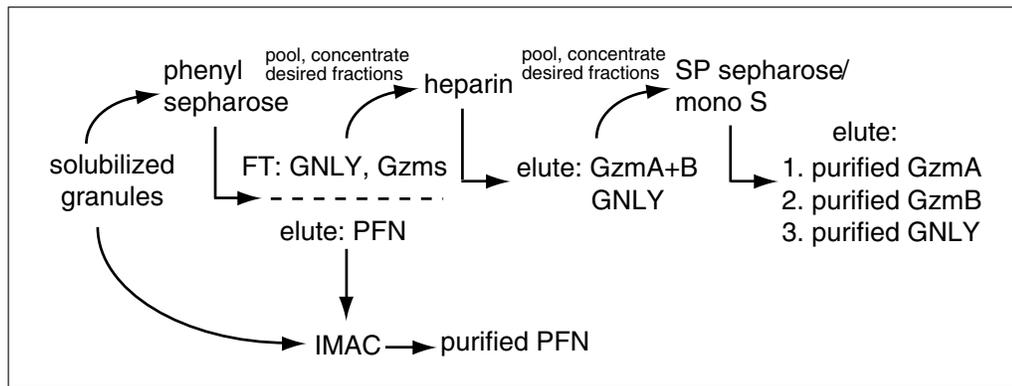


Figure 3.37.2 Procedure for purification of cytotoxic granule effector proteins by fast protein liquid chromatography (see Basic Protocol 2 and Alternate Protocol 2 for details). Abbreviations: FT, flow through; IMAC, immobilized metal affinity chromatography.

GNLY is not expressed in rodents, YT-Indy or human LAK cells preferentially are used for GNLY purification. A schematic of the different purification procedures is described in Figure 3.37.2.

For perforin isolation, an IMAC (immobilized metal affinity chromatography) purification method has been optimized by Winkler et al. (1996) to separate PFN from other granule components.

Materials

- Cytotoxic granules (Basic Protocol 1), frozen
- Ice
- Equilibration buffer A1 (see recipe)
- IMAC resin charged with Cobalt (TALON Superflow Metal Affinity Resin; Clontech)
- 0.5 M EGTA in H₂O
- Elution buffer B1 (see recipe)
- BCA assay (Pierce)
- Rabbit anti-PFN (Cell Signaling, cat. no. 3693)
- 0.45- μ m low-protein-binding syringe filter (Millipore)
- 10-ml desalting columns (Econo-Pac 10DG disposable chromatography columns; Biorad)
- 50-ml Superloop (Amersham)
- Fast Protein Liquid Chromatography (FPLC) system (Biorad) and columns (Pharmacia Biotechnology)
- Ultrafiltration concentrator (Amicon Ultra centrifugal filter device with low binding; Millipore)
- Nitrocellulose membrane
- Additional reagents and equipment for hemoglobin release assay (Support Protocol 2), BCA assay (APPENDIX 3H), SDS-PAGE (UNIT 6.1), immunoblotting (UNIT 6.2), and silver staining (UNIT 6.6)

NOTE: All buffers and suspensions are kept on ice.

Prepare granules

1. Place the frozen tube of solubilized granules (Basic Protocol 1, step 23) on ice for 2 hr, mixing the suspension by inverting the tube every 15 to 20 min.
2. Centrifuge the suspension 10 min at 15,000 \times g, 4°C.

- Carefully decant the supernatant and filter through a 0.45- μm low-protein-binding syringe filter to remove membrane fragments

If the filter is blocked, carefully change the filter.

Desalt the suspension

- During step 1, equilibrate desalting columns with 25 ml of cold equilibration buffer A1. Prepare one column for every 2.5 ml of supernatant.
- Run 2.5 ml of supernatant through each desalting column, discard the flow-through, and then add 3.5 ml of equilibration buffer A1. Retain the flow-through and combine all samples for FPLC purification.
- Filter the sample again through a 0.45- μm low-protein binding syringe filter.

It is very important to note that at this point perforin is no longer in a chelated buffer and can be inactivated by multimerizing in the presence of trace amounts of Ca^{2+} . Therefore, the FPLC step must be performed as quickly as possible.

Perform FPLC

- During step 5, prepare a 5-ml column of IMAC TALON Superflow resin and equilibrate it with 10 column volumes (CV) of equilibration buffer A1.
- Load filtered sample into a superloop and attach to an FPLC workstation.

Add 50 or 20 μl of 0.1 M EGTA into each fraction collector tube before FPLC run (if collecting 5- or 2-ml fractions to yield 1 mM final concentration of EGTA) to prevent perforin inactivation.

- Run the following FPLC program with equilibration buffer A1 and elution buffer B1:
 - Wash the column with 100% buffer A1 at 1.5 ml/min for 1 CV.
 - Inject sample at 1 ml/min.
 - Wash the column with a linear gradient of 1% to 2% buffer B1 at 1 ml/min for 5 CVs.
 - Then elute with a linear gradient of 2% to 40% buffer B1 at 1.5 ml/min for 7 CVs and 40% to 100% buffer B1 at 1.5 ml/min for 5 CVs.

Elution fractions are continuously monitored for absorbance at 280 nm. With this method one peak, absorbing at 280 nm, containing perforin is observed (Fig. 3.37.3A).

Identify PFN-containing fractions

- Assay 10 to 20 μl of each fraction for hemolytic activity by a hemoglobin release assay (see Support Protocol 2; Fig. 3.37.3B).
- Then, collect and pool all hemolytic positive fractions (containing perforin).
- Concentrate with an ultracentrifugation concentrator to a final volume of 200 to 500 μl (10 mg/ml) to prevent loss of PFN due to binding to concentrator tube membrane.
- Determine protein concentration by BCA assay (APPENDIX 3H) using BSA for calibration.
- Divide purified active perforin and store indefinitely at -80°C .

Since PFN is not stable with freeze thawing and loses activity at 4°C within a week, it is important to store it in small aliquots in an amount that will be used for just a few experiments. We generally divide our samples into 5- μl aliquots.

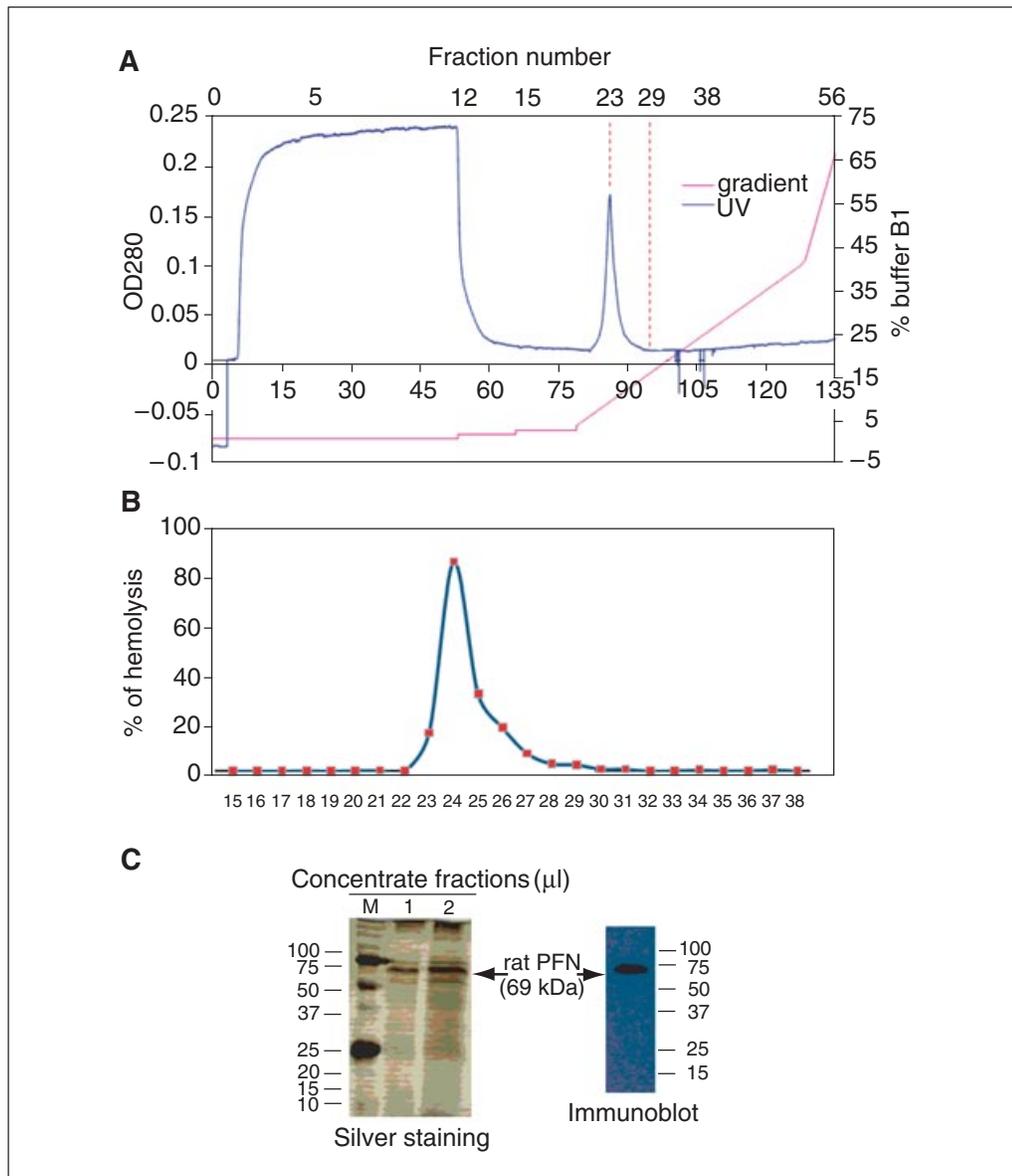


Figure 3.37.3 Purification of PFN from cytotoxic granules by fast protein liquid chromatography. **(A)** The profile of eluted protein from the FPLC is shown. Perforin activity is eluted over an imidazole gradient (pink line) and coincides with a single absorption peak at 280 nm (blue line) as shown by **(B)** testing the hemolytic activity of collected fractions by hemoglobin release assay. **(C)** After concentration of hemolytic positive fractions, 1 and 2 μ l were used to assess the purity of the purified PFN by electrophoresis on a 12% polyacrylamide gel. Rat PFN (69 kDa) is visualized by silver staining (left) and an immunoblot (right). Abbreviations: M, protein markers. For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb0337>.

Analyze purity

- Analyze purified perforin by SDS-PAGE (UNIT 6.1) and immunoblotting (UNIT 6.2) to assess purity. Stain the protein gel by silver staining (UNIT 6.6). Transfer to nitrocellulose membrane and detect with rabbit anti-PFN (Fig. 3.37.3C).

The staining is performed according to the manufacturer's protocol (dilution 1:1000, incubation overnight at 4°C).

Immunoblots can also be probed for granzymes and GNLV to verify that there is no cross-contamination.

It is important to note that cell sensitivity to PFN follows a very steep dose response curve that differs from cell to cell and varies even for the same cell under different culture

conditions (such as variations in cell density; Keefe et al., 2005). At high concentrations (termed lytic) perforin on its own triggers necrosis by causing irreparable membrane damage. At lower concentrations (termed sublytic) perforin on its own triggers necrosis in only a minority of cells (generally 5% to 20%, assessed by propidium iodide incorporation; Support Protocol 4), but is able to deliver granzymes into cells to induce apoptotic death. At still lower concentrations (when PI incorporation is <5%), perforin is not active at delivering granzymes. Sublytic concentrations are used to load granzymes into target cells to mimic CTL or NK cell-mediated cell death (which is apoptotic rather than necrotic). Because of this steep dose dependence (often a factor of 2 is all that distinguishes an inactive concentration from a lytic concentration), our practice is to titrate the perforin concentration to be used for each experiment on the day of the experiment or the day before (see Support Protocol 4).

PURIFICATION OF GRANZYME AND GRANULYSIN FROM CYTOTOXIC GRANULES

ALTERNATE PROTOCOL 2

A method adapted from Shi et al. (2000) is detailed here for purifying human GzmB and GNLY from the cytotoxic granules of YT-Indy cells, an autonomously growing human NK cell line expressing high levels of GzmB, perforin, and GNLY. YT-Indy cells do not express GzmA or GzmK (Froelich et al., 1996), which facilitates the purification of GzmB. However, RNK cell granules can also be used as starting material. RNK cells do not express GNLY, but express GzmA, GzmK, and GzmB. The granzymes are all highly basic (they are among the most basic proteins in mammalian cells with pI >9), making them easy to separate from other granule components. However, because of the similar chemical properties of these homologous enzymes, the purified granzymes need to be carefully screened by immunoblot and activity assay for cross-contamination. Since GzmA and GzmB have distinct substrate activity (GzmA is a tryptase, while GzmB cuts after aspartic acid residues), either antibodies or protease assays can be used to detect contamination. It is possible to separate GzmA and GzmB from LAK or RNK cell granules using a Mono-S column for the final step purification as previously described (Hanna et al., 1993). However, this must be done with care. An alternative is expression and purification of recombinant GzmA or GzmB as described elsewhere (Martinvalet et al., 2008b).

Materials

- Granules from YT-Indy cells (Basic Protocol 1)
- Phenyl Sepharose chromatography elution buffers A2 and B2 (see recipes)
- Liquid nitrogen
- Heparin chromatography buffers A3 and B3 (see recipes)
- SP Sepharose (or MonoS) chromatography elution buffers A4 and B4 (see recipes)
- BCA protein assay kit (Pierce)
- Fast Protein Liquid Chromatography (FPLC) and columns (GE Healthcare)
- Centrifuge
- 0.45- μ m low-protein-binding syringe filter (Millipore)
- Phenyl Sepharose High Performance, SP Sepharose High Performance, 5 ml of each densely packed in a column (GE Healthcare)
- Ultracentrifugation concentrator
- HiTrap heparin column, 5 ml (GE Healthcare)
- MonoS column (GE Healthcare)
- Additional reagents and equipment for SDS-PAGE (UNIT 6.1), staining with Coomassie brilliant blue (UNIT 6.6), and immunoblotting (UNIT 6.2)

NOTE: For the purification of rat granzymes the use of a MonoS column (GE Healthcare) instead of SP Sepharose is highly recommended.

Subcellular Fractionation and Isolation of Organelles

3.37.11

Prepare for purification

1. Attach all columns to the FPLC system in a cold room.
2. Solubilize cytotoxic granules from YT-Indy cells in 10 ml buffer A2.
3. Subject the samples to three freeze/thaw cycles in liquid N₂.
4. Centrifuge 15 min at 15,000 × g, 4°C.
5. Collect the supernatant, filter it through a 0.45-μm low-protein-binding filter, and store on ice until use.

Remove PFN

6. Equilibrate the Phenyl Sepharose column with 10 CV of buffer A2.

A Phenyl Sepharose column is used to separate PFN, which binds to the column, from granzymes and GNLY, which do not bind.

7. Load the filtered supernatant manually at ~1 ml/min.
8. After the sample loading, wash the column with 10 ml buffer A2 until the UV detector baseline is reached (usually 2 CVs). Collect flowthrough and initial wash fractions, which contain granzymes and GNLY.

Collect 2-ml fractions for a total of 20 ml (10 fractions).

9. Pool and concentrate these fractions to a volume <1 ml using an ultracentrifugation concentrator with a molecular cutoff <10 kDa before loading on the heparin column.
10. Elute perforin.

PFN can be eluted from the Phenyl Sepharose column using the following program (flow: 1 ml/min): wash 10 min with buffer A2, elute with a 0–2 M NaCl (0% to 100% buffer B2) linear gradient for 20 min. Peak fractions contain perforin and are pooled, concentrated to a final volume of ~2.5 ml, and stored at –80°C until IMAC purification (Basic Protocol 2).

Pass the sample through the heparin column

11. Equilibrate the HiTrap heparin column with 10 CVs of buffer A3.
12. Dilute the concentrated flowthrough and wash fractions from the Phenyl Sepharose column 20-fold in buffer A3 prior to loading manually on the heparin column at 1 ml/min.
13. Wash the column until the UV absorbance baseline is reached.
14. Elute granzymes and GNLY from the heparin column using the following program at a flow rate of 1 ml/min:
 - a. Wash with 2 CVs of buffer A3.
 - b. Elute with a 0–2 M NaCl (0%–100% buffer B3) steep linear gradient for 10 min.
 - c. Finally, elute with 100% buffer B3 for 10 min.

A typical chromatogram for YT-Indy cell granules is shown in Figure 3.37.4A.

AAD esterolytic activity (GzmB, see Support Protocol 4) is found in elution peak 3 and 4 while antibacterial activity is mainly found in the peak 4. Since measuring GNLY antibacterial activity takes at least 12 hr, we recommend pooling the GzmB-containing fractions with the last elution peak without testing for GNLY before further purification on the S-column.

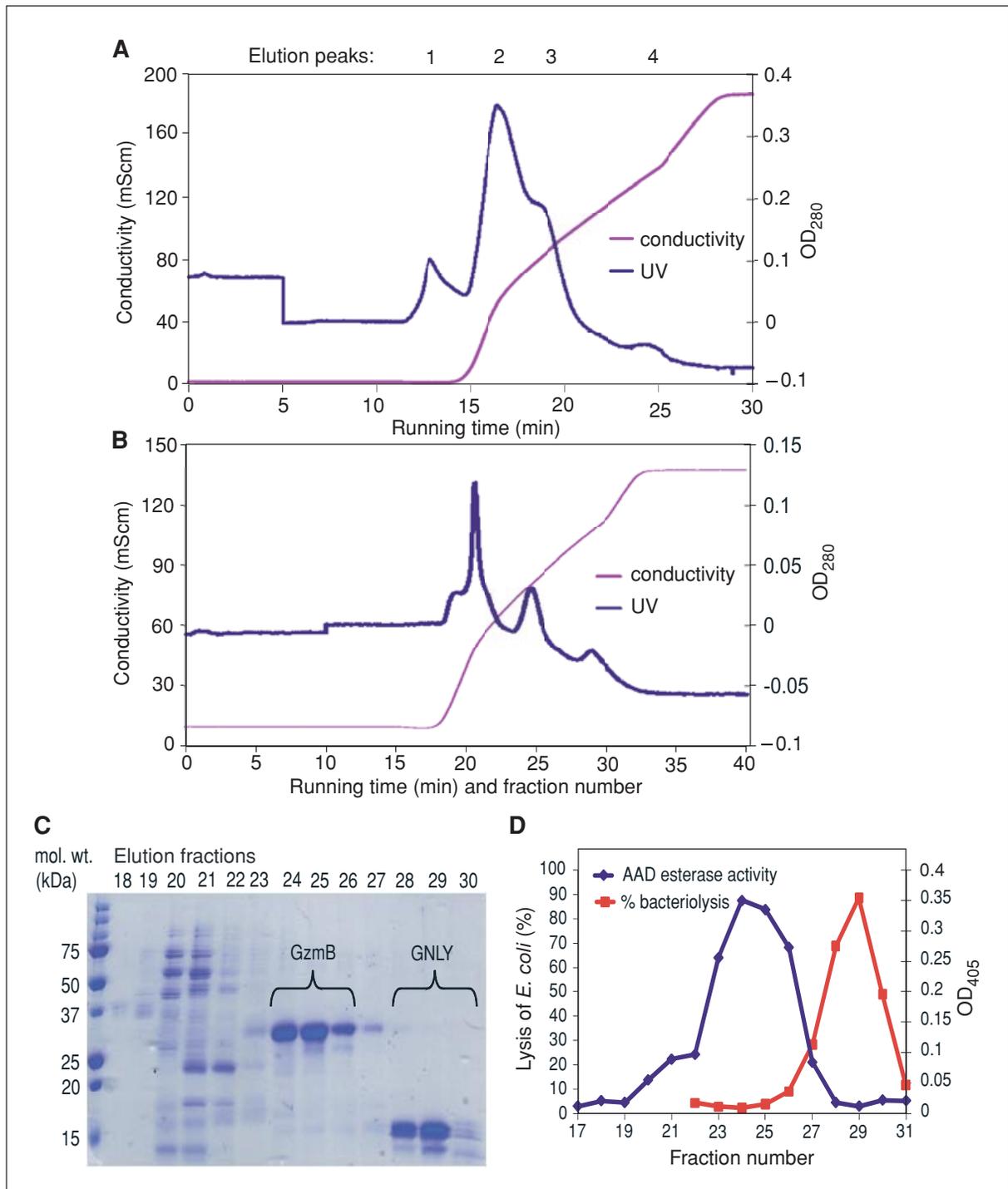


Figure 3.37.4 GzmB and GNLY purification from YT Indy cells. A Chromatogram (OD_{280} and conductivity) of the protein elution from the heparin column is shown in (A), and from the SP Sepharose column in (B). The eluted fractions from the SP Sepharose column were further analyzed by electrophoresis on a 12.5% polyacrylamide gel and by Coomassie blue staining (C) by AAD esterase assay and bacteriolytic activity (CFU assay) (D). For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb0337>.

- Concentrate the pooled fractions to a final volume ≤ 1 ml using an ultracentrifugation concentrator with a molecular cutoff ≤ 10 kDa.

If granules containing GzmA and GzmB are used as starting material, the eluate fractions should also be screened for BLT activity (see Support Protocol 5).

- Equilibrate the SP Sepharose column with 10 CV of buffer A4.
- Dilute the pooled and concentrated fractions of the heparin column 20-fold in buffer A4.
- Manually load the sample on the S-column at 1 ml/min.

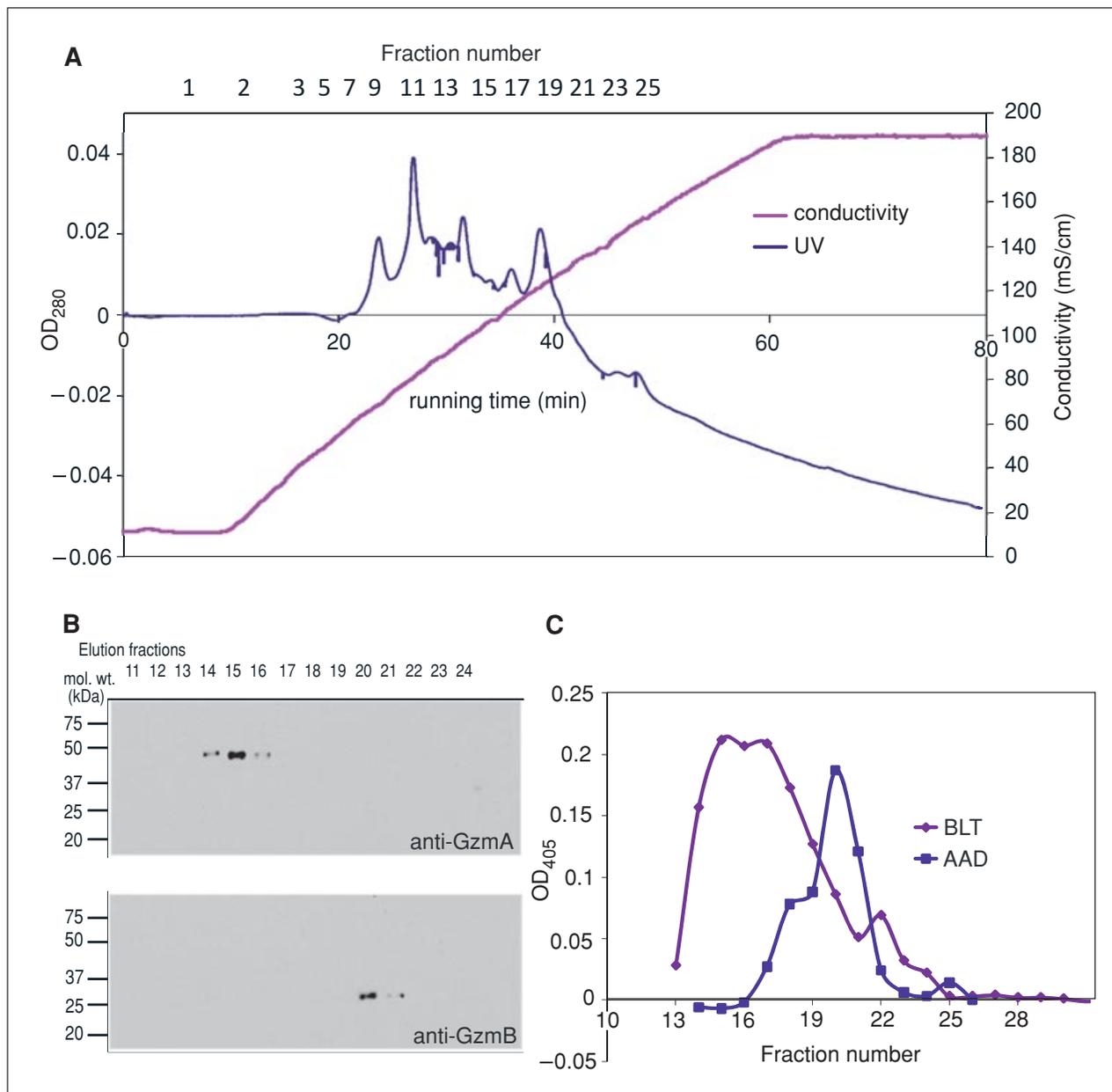


Figure 3.37.5 GzmA and GzmB purification from human LAK cells. The chromatogram (OD₂₈₀ and conductivity) of the protein elution from the Mono-S column is shown in (A). The fractions were analyzed by electrophoresis on a 12.5% polyacrylamide gel and immunoblotting using anti-GzmA and anti-GzmB antibodies (B). The elution fractions were further screened for esterase activity (BLT and AAD) (C). Note there is BLT activity in fractions that stained negative for GzmA (fractions 17 and 18). The source of the esterase activity is most likely GzmK (not specifically tested in this purification). For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb0337>.

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19. Wash the column with buffer A4 until the UV baseline is reached (2 to 3 CV).

Substitute a MonoS column if granules contain GzmA and GzmB to separate these.

Separate granzymes and GNLY on SP Sepharose column

20a. Use the following program at a flow rate of 1 ml/min to elute granzymes and GNLY from the SP Sepharose column using the following program:

- i. Wash with an additional 2 CVs of buffer A4.
- ii. Elute with a 0–1 M NaCl (0%–100% buffer B4) linear gradient for 20 min.
- iii. Finally, elute with 100% buffer B4 for 10 min. GzmB elutes at ~60%–70% of buffer B4, GNLY at ~90%–100% buffer B4 (Fig. 3.37.4B).

21a. Analyze elution fractions using SDS-PAGE (UNIT 6.1) and stain with Coomassie brilliant blue (UNIT 6.6; Fig. 3.37.4C). Test purity by immunoblotting (UNIT 6.2). Perform activity testing (Support Protocols 3 to 9; Fig. 3.37.4D).

22a. Separately pool and concentrate fractions containing GzmB (28 kDa) or GNLY (15 and 10 kDa) to a final volume of ~250 μ l before dividing into 5- μ l aliquots and storing at -80°C .

Separate GzmA and GzmB on Mono-S column

20b. If GzmA and GzmB-containing granules are used as starting material, perform the purification on a Mono-S column to allow better resolution of the cationic granule proteins.

21b. Apply the following program at a flow rate of 0.5 ml/min for elution:

- i. Wash 20 min with buffer A4.
- ii. Elute with a 0.05–1 M NaCl (0%–100% buffer B4) linear gradient for 60 min.
- iii. Finally, elute with 100% buffer B4 for 20 min.

GzmA elutes at ~50% to 60% and GzmB at 60% to 70% of buffer B4 (Fig. 3.37.5A).

Be aware that GzmK (found in LAK cells as well as RNK granules) elutes also in this salt concentration range and exhibits BLT activity (see Support Protocol 4; Fig. 3.37.5B). The eluting fractions should therefore be carefully screened using SDS-PAGE under reducing and non-reducing conditions to distinguish between monomer- and dimer-containing fractions (GzmA is a dimer and GzmK a monomer under non-reducing conditions), as well as immunoblotting using GzmA, B, and K antibodies (Fig. 3.37.5C).

22b. Proceed to step 23.

23. Determine the protein concentration with the BCA protein assay kit.

24. Test protein activity as described in Support Protocols 5 and 6.

IMMUNOSTAINING ASSAY FOR PERFORIN AND/OR GRANZYME EXPRESSION

Immunostaining of cells can be used to assess expression of perforin and granzymes.

Materials

Poly-L-lysine (Sigma)

Cultures of CTL or NK cells

Phosphate-buffered saline (PBS; Cellgro, cat. no. 21-031-CV)

4% (w/v) PFA in PBS, pH 7.4

Permeabilization buffer: 0.2% (v/v) Triton X-100 in PBS

Blocking buffer: 10% (v/v) FBS in PBS

Mouse anti-human PFN, clone Pf80/164 (Mabtech); 1:600 dilution for microscopy

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Mouse anti-human GzmB, clone GB11 (Caltag Laboratories); 1:300 dilution for microscopy

Mouse anti-human GzmA, clone CB9 (BD Pharmingen); 1:300 dilution for microscopy

Mouse anti-granulysin (BD Pharmingen)

Incubation buffer: PBS/0.05% (v/v) Triton X-100

Fluorochrome-conjugated secondary antibody (Molecular Probe)

Vectashield mounting medium containing DAPI (Vector Laboratories)

18-mm circle coverslips

96-well plates

Centrifuge, adaptors for plates

Fluorescence microscope

1. One day before staining, prepare Poly-L-lysine-coated 18-mm circle coverslips in 12-well plates.
2. Wash CTL or NK cells in 10 ml PBS and resuspend in 0.5 ml PBS.
3. Add 50,000 cells to the coated coverslips or into a 96-well plate for flow cytometry analysis, centrifuge 3 min at $500\times g$, room temperature, and remove supernatants.
4. Fix the cells 20 min in PBS containing 2% PFA at room temperature.
5. Wash the cells three times, each time with 1 ml PBS and permeabilize for 5 min in 1 ml permeabilization buffer.
6. Wash twice, each time in 1 ml PBS and add 1 ml blocking buffer for 30 min.
7. Wash one time in 1 ml PBS.
8. Add the required dilution of primary antibody (see Materials list) for PFN, GzmB, GzmA, or GNLY to the cells and incubate 1 hr at room temperature in 0.2 ml incubation buffer.
Use incubation buffer to achieve the appropriate antibody dilutions.
9. Wash the cells three times, each time with 1 ml incubation buffer and incubate 1 hr at room temperature with fluorochrome-conjugated secondary antibodies (diluted 1:500 in incubation buffer or according to the manufacturer's protocol) in 0.2 ml incubation buffer.
10. Wash cells three times, each time in 1 ml PBS and mount in a small drop (~ 10 to $20\ \mu\text{l}$) Vectashield mounting medium before fluorescence microscopy analysis (see Fig. 3.37.1A).

SUPPORT PROTOCOL 2

FLOW CYTOMETRY ASSAY FOR PERFORIN AND/OR GRANZYME EXPRESSION

As an alternative to microscopy, flow cytometry can be used to screen for PFN, granzyme, and GNLY expression.

Materials

Jurkat (ATCC), LAK cells, *or* YT-Indy cells from Basic Protocol 1 (step 10)

4% (w/v) PFA in PBS, pH 7.4

FACS buffer (see recipe)

Phosphate-buffered saline (PBS; Cellgro, cat. no. 21-031-CV)

Saponin (Sigma)

Mouse anti-human PFN, clone δG9 (BD Pharmingen) for flow cytometry

Mouse anti-granulysin (BD Pharmingen) for flow cytometry
Mouse anti-GzmA (clone CB9, BD Pharmingen)
Mouse anti-GzmB (clone GB11, Catlag)
Secondary antibody

Microcentrifuge tubes *or* 96-well V-bottom plates
Centrifuge
Flow cytometer

1. Dispense $1-5 \times 10^5$ cells in microcentrifuge tubes or in 96-well V-bottom plates. Centrifuge cells 3 min at $400 \times g$, room temperature, to pellet cells.
2. Resuspend the cells in 50 μ l of PBS containing 2% paraformaldehyde. Incubate 20 min at room temperature.
3. Wash the cells with 1 ml FACS buffer (microcentrifuge tubes) or twice with 200 μ l FACS buffer (96-well plate), centrifuging the cells 5 min at $400 \times g$, room temperature, each time, and aspirating the supernatants.
4. Resuspend the cells in 50 μ l PBS containing 0.1% (w/v) saponin to permeabilize the cells for intracellular staining.
5. Add 1 μ l anti-GzmA, 5 μ l anti-GzmB, 2.5 μ l anti-perforin, or 2.5 μ l anti-GNLY antibody. Incubate 30 min at room temperature in the dark.
6. If a secondary antibody is required, wash the cells as in step 3, and resuspend in 50 μ l PBS containing 0.1% (w/v) saponin and the appropriate dilution of fluorochrome-conjugated secondary antibody.
7. Wash the cells as in step 3, and resuspend the cells in 100 μ l FACS buffer.

The cells are now ready for flow cytometry analysis (Fig. 3.37.1B).

8. Using a nonstained control sample, determine the optimal Forward Scatter and Side Scatter settings and adjust the fluorophore channels to baseline levels. Using stained samples, measure the fluorescence intensity of effector molecule-stained cells compared to the appropriate isotype control-stained samples.

MEASURING PERFORIN ACTIVITY BY HEMOGLOBIN RELEASE ASSAY

Perforin induces membrane damage by calcium-dependent multimerization to form pores in target cell membranes. Hemoglobin release from sheep red blood cells is commonly used to detect perforin activity during purification.

Materials

Sheep red blood cells (Rockland Immunochemicals)
Hanks' balanced salt solution (HBSS), without calcium, magnesium, and phenol red, ice cold
HBSS + 4 mM CaCl_2 , ice cold
FPLC fractions of perforin purification (Basic Protocol 2)
Saponin (Sigma)
96-well round-bottom microplate
96-well flat-bottom microplates
Microplate reader

1. Wash sheep red blood cells three times, each time with 50 ml cold HBSS and resuspend at 1% (v/v) in HBSS containing 4 mM CaCl_2 .

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2. Transfer 100 μ l of red blood cell solution to a well of a 96-well round-bottom microplate.
3. Add 10 to 20 μ l of each FPLC fraction to the well.
4. Incubate the plate 20 min at room temperature.
5. Centrifuge the plate 5 min at $400 \times g$, 4°C .
6. Transfer 50 μ l of cell-free supernatant from each well to a 96-well flat-bottom microplate.
7. Detect the hemoglobin release into supernatant by reading absorbance at 412 nm (Fig. 3.37.3B).
8. Calculate the percent release relative to the maximal hemolysis determined by adding 0.01% (v/v) saponin to the red blood cells as (experimental hemolysis – spontaneous hemolysis)/(maximal hemolysis – spontaneous hemolysis) \times 100.

**SUPPORT
PROTOCOL 4**

MEASURING PERFORIN ACTIVITY BY PROPIDIUM IODIDE UPTAKE

Propidium iodide uptake is usually used to determine the sublytic level of PFN for a given cell type to establish the conditions for efficient delivery of granzymes for apoptosis assays.

Materials

Target cells
 Buffer C (see recipe)
 FPLC fractions from perforin purification (Basic Protocol 2)
 Buffer P (see recipe)
 An5 buffer
 Propidium iodide (PI; Sigma)
 37°C incubator
 Flow cytometer

1. Wash 5×10^4 target cells of interest in 0.5 ml buffer C and resuspend in 30 μ l of the same buffer.
2. Make dilutions of purified perforin in 30 μ l of buffer P.
The diluted form at this stage is at a concentration of $2 \times$.
3. Add 30 μ l of the diluted perforin to 30 μ l of cells from step 1.
Because different PFN dilutions/concentrations need to be tested, it is convenient to use a 96-well V-bottom microplate for this assay.
4. Incubate 15 min at 37°C .
5. Wash cells once with 100 μ l An5 buffer.
6. Resuspend cells in 50 μ l An5 buffer with 2 $\mu\text{g/ml}$ propidium iodide.
7. Measure PFN-induced necrosis immediately by flow cytometry by measuring PI uptake (Fig. 3.37.6).

A PFN concentration that causes $\sim 5\%$ to 20% cell death is considered a sublytic dose that is ideal for granzyme delivery and induction of apoptosis (Keefe et al., 2005). It is also important to note that the sublytic dose of PFN varies from cell to cell and must be determined for each experiment.

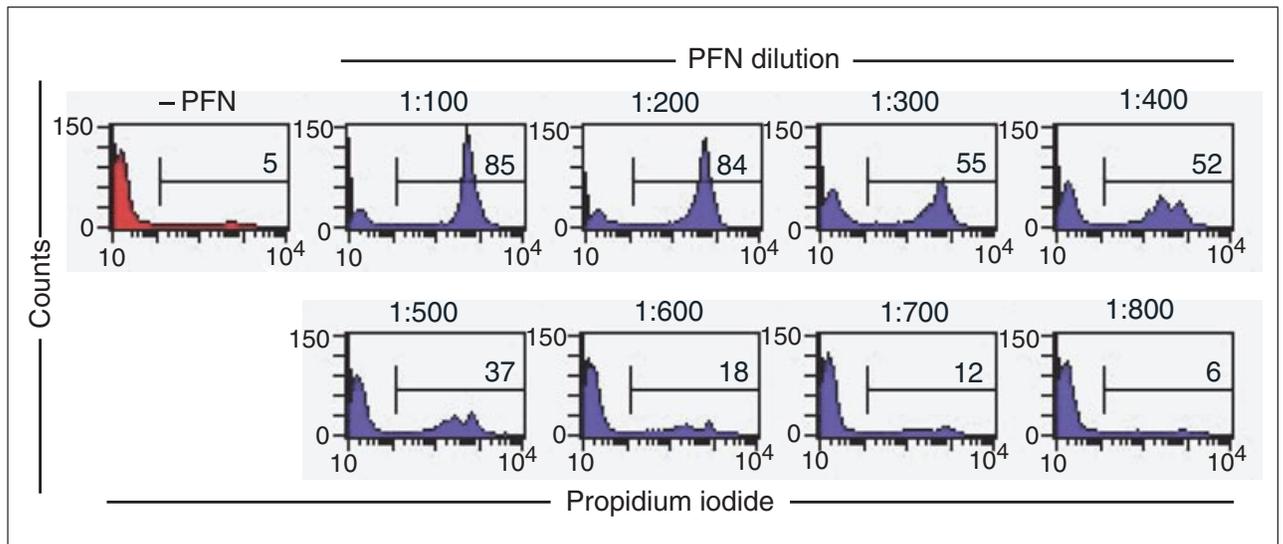


Figure 3.37.6 Measuring PFN activity by flow cytometry by propidium iodide uptake. HeLa cells were treated with different dilutions of purified native rat PFN for 15 min at 37°C before staining with propidium iodide (PI) and analysis by flow cytometry. Percentage of PI positive cells is indicated. In this experiment, the sublytic PFN concentration is between 1:600 and 1:700.

MEASURING GzmB ACTIVITY

The GzmB activity is measured by assessing its proteolytic activity. The measurement of peptide substrate (AAD) hydrolysis is commonly used during the purification of GzmB. GzmB-induced apoptosis can also be detected by various methods (see Support Protocols 9 and 10).

Materials

AAD assay buffer (see recipe)
 FPLC fractions of GzmB purification (Alternate Protocol 2)
 96-well flat-bottom plates
 Microplate reader

1. Make fresh AAD assay buffer. Make sure to mix thoroughly.
2. Combine 5 μ l of FPLC fractions or control buffer (for blank control) with 200 μ l of AAD assay buffer in a 96-well flat-bottom plate.
3. Incubate the plate for 5 min at 37°C.
4. Measure OD_{405 nm} using a microplate reader.

Fractions with OD > 0.1 are considered positive for GzmB activity (see Fig. 3.37.4D).

MEASURING GzmA ACTIVITY

GzmA activity is measured by assessing its proteolytic activity. The measurement of peptide substrate (BLT) hydrolysis is commonly used during the purification of GzmA. GzmA-induced apoptosis can also be detected by various methods (see Support Protocols 9 and 10).

Materials

BLT assay buffer (see recipe)
 FPLC fractions for GzmA purification (Alternate Protocol 2)

**SUPPORT
 PROTOCOL 5**

**SUPPORT
 PROTOCOL 6**

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96-well flat-bottom plate
37°C incubator
Microplate reader

1. Freshly add BLT and DTNB to make BLT assay buffer. Make sure to mix thoroughly.
2. Combine 5 µl of FPLC fractions or control buffer with 200 µl of BLT assay buffer in a 96-well flat bottom plate.
3. Incubate the plate for 5 min at 37°C.
4. Measure OD_{405 nm} using a microplate reader.

Fractions with OD_{405 nm} >0.2 are considered positive for GzmA activity. These fractions may be pooled and concentrated using the Centricon MWCO 10 kDA concentrating units (Fig. 3.37.5C).

SUPPORT PROTOCOL 7

MEASURING GNLY-MEDIATED ANTIBACTERIAL ACTIVITY BY COLONY-FORMING UNIT ASSAY

The effect of GNLY on bacteria can be measured by quantifying bacterial numbers by colony formation on agar plates.

Materials

Bacteria: *E. coli*, *Listeria*, *Salmonella*, or whatever lab-strain is available
LB medium and LB agar plates (APPENDIX 2A)
Colony forming and turbidimetry unit assay buffer (see recipe)
Fractions for GNLY purification (Alternate Protocol 2)
37°C incubator

NOTE: NaCl concentrations higher than 50 mM interfere with GNLY activity.

1. Grow bacteria to mid-log phase (OD_{600 nm} = 0.5 equaling ~10⁸/ml viable bacteria) in LB medium in a bacterial culture shaker at 37°C.
2. Wash bacteria three times, each time in 1 ml colony forming and turbidimetry unit assay buffer and dilute to 5 × 10⁵ bacteria/ml.
3. Prepare serial dilutions (between 2 µM and 0.06 µM) of GNLY in 25 µl of colony forming and turbidimetry unit assay buffer. Use buffer only as the negative control.

Serial dilutions of antibiotics or of human beta-defensin 1 (Innavogen) can serve as positive control.

4. Add 25 µl of bacterial dilutions (equaling ~1.25 × 10⁴ viable bacteria) to each reaction, mix gently, and incubate 1 hr at 37°C. Include a no-bacteria control (assay buffer only).
5. Add 250 µl of LB medium to each reaction and prepare 10-fold serial dilutions from each reaction.
6. Plate 50 µl of the dilutions on LB agar plates and incubate at 37°C overnight prior to colony counting.
7. Calculate the percent lysis using the following equation: % specific lysis = CFU_{GNLY-treated} / CFU_{buffer-treated} × 100 (Fig. 3.37.4D).

MEASURING GNLY-MEDIATED ANTIBACTERIAL ACTIVITY BY TURBIDIMETRY

The effect of GNLY on bacteria can also be measured by quantifying bacterial numbers by the change in turbidity of liquid cultures.

Materials

Bacteria: *E. coli*, *Listeria*, *Salmonella*, any non-virulent bacterial lab-strain

LB medium (APPENDIX 2A)

Colony forming and turbidimetry unit assay buffer (see recipe)

Fractions for GNLY purification (Alternate Protocol 2)

96-well transparent flat-bottomed plates

37°C incubator

Microplate reader (with ability to monitor kinetics at 37°C and discontinuous shaking; Spectra Max 340PC, Molecular Devices)

1. Grow bacteria to mid-log phase ($OD_{600} = 0.5$ equaling $\sim 10^8$ /ml viable bacteria) in LB medium
2. Wash bacteria three times in 1 ml colony forming and turbidimetry unit assay buffer and dilute to 10^5 bacteria/ml
3. Prepare serial dilutions of GNLY in 25 μ l of colony forming and turbidimetry unit assay buffer in a flat-bottomed, transparent 96-well plate. Use buffer only as a negative control.

Serial dilutions of antibiotics or human beta-defensin 1 (Innavogen) can serve as positive control.

4. Add 25 μ l of bacterial dilutions (equaling $\sim 2.5 \times 10^3$ viable bacteria) to each well, mix gently, and incubate 1 hr at 37°C.
5. Add 150 μ l of LB medium to each well. Monitor $OD_{600\text{ nm}}$ in the plate reader while shaking at intervals for 12 hr or until the steady-state of the bacterial growth curve is reached.

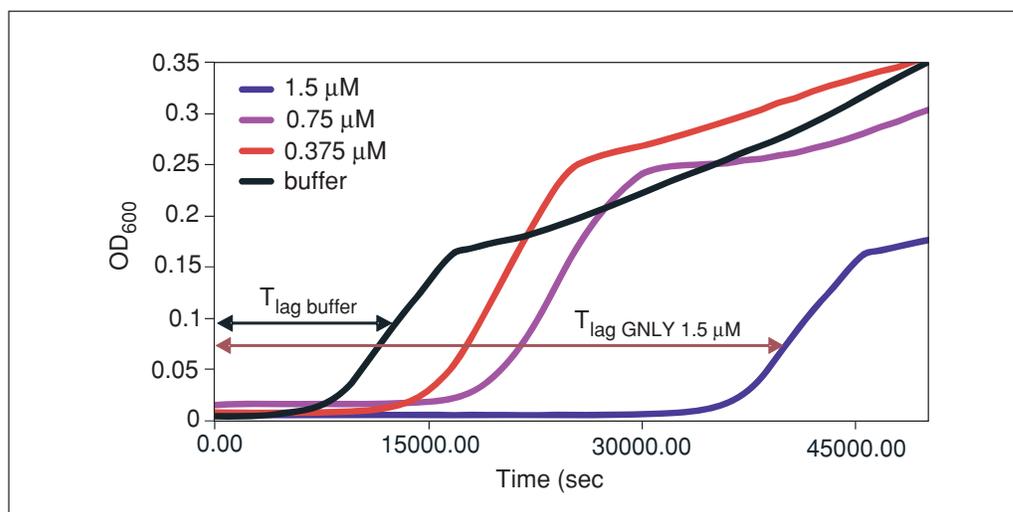


Figure 3.37.7 Measuring the antibacterial activity mediated by GNLY using turbidimetry. Typical bacterial growth curves from buffer- as well as GNLY-treated *E. coli* are shown. A possible read-out from these curves is to measure the time until a certain threshold OD is reached (T_{Lag}) and compare control versus test curves. For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb0337>.

6. Analyze the bacterial growth curves.

The length of the lag-phase (T_{Lag}) of the bacterial growth curves (Fig. 3.37.7) reflects the initial bacteria count and can therefore be used to quantify the antibacterial activity of GNLY.

GRANZYME-MEDIATED CHROMIUM RELEASE APOPTOSIS ASSAY

To measure the activity of granzymes in cells, purified granzymes and PFN are added and cell death can then be monitored by a number of assays. Here, we provide the most sensitive measure of total cell death (apoptosis + necrosis), which is a chromium release assay.

Materials

Target cells

K10 medium: RPMI 1640 + 10% (v/v) FBS

^{51}Cr -labeled Na_2CrO_4 (NEN BioLabs), 5 $\mu\text{Ci}/\mu\text{l}$

Hanks' balanced salt solution (HBSS)

Buffer C (see recipe)

Purified granzymes and perforin

Buffer P (see recipe)

96-well V-bottom plate

37°C incubator

TopCount scintillation counter and LumaPlate 96 (PerkinElmer)

1. Harvest target cells and resuspend the cells in 1 ml K10 medium and label with 100 μCi of ^{51}Cr for 1 hour at 37°C.
2. Wash the labeled target cells three times, each time in 10 ml HBSS.
3. Resuspend cells in buffer C at the same density used to determine the sublytic perforin concentration (see Support Protocol 4).

Generally, 10^4 – 10^5 cells per well in 30 μl are dispensed in triplicate in a 96-well V-bottom plate.

4. Dilute perforin in 30 μl buffer P to 2 \times the sublytic dose previously determined.
5. Dilute the purified granzyme to 2 \times the final concentration in the same tube of buffer P and add 30 μl to cells.

Include perforin only and granzyme only controls. Additionally, include buffer only controls (spontaneous release) and cells in 1% NP-40 (maximal release).

6. Incubate the plate for 1 to 4 hr at 37°C.
7. Pellet cells and cell debris by centrifuging the plate for 5 min at 400 \times g, room temperature.
8. Remove 30 μl of supernatant, taking care not to disturb the cell pellet, and add to a LumaPlate 96.
9. Allow the supernatant to dry several hours or overnight before measuring ^{51}Cr release into the supernatant using a TopCount counter.

Calculate the percent release relative to the maximal release determined by adding NP40 to the target cells as $(\text{experimental release} - \text{spontaneous release})/(\text{maximal release} - \text{spontaneous release}) \times 100$.

GRANZYME-MEDIATED ANNEXIN-V/PROPIDIUM IODIDE (PI) ASSAY

To measure the activity of granzymes in cells, purified granzymes and PFN are added and cell death can then be monitored by a number of assays. Here we provide a flow cytometry–based assay that distinguishes apoptotic cells (generally Annexin V⁺) from necrotic cells (generally Annexin V⁻).

Materials

Target cells
Buffer C (see recipe)
Buffer P (see recipe)
Purified granzymes and perforin
An5 buffer (see recipe)
Annexin-V-APC (Caltag)
Propidium iodide (PI)

96-well V-bottom plates
37°C incubator
Centrifuge with plate-adaptor

1. Wash cells once with 5 ml buffer C.
2. Resuspend cells and dispense 10⁵ cells/well of 96-well V-bottom plates in 30 μl buffer C.
3. Dilute perforin in 30 μl buffer P to 2× the sublytic dose previously determined.
4. Dilute the purified granzyme to 2× the final concentration in the same tube of buffer P and add 30 to cells.

Make sure to include PFN only, granzyme only, and buffer-only treated cells.

5. Incubate the treated cells for 30 to 60 min at 37°C.
6. Add 100 μl An5 buffer to each well.
7. Centrifuge the plate 3 min at 500 × g, room temperature, in a centrifuge containing a plate-adaptor.
8. Resuspend the cells in 100 μl An5 buffer containing APC-conjugated Annexin V (1:33 dilution) and incubate 10 min at room temperature in the dark.
9. Wash the cells twice, each time in 100 μl An5 buffer.
10. Resuspend the pellet in An5 buffer containing 2 μg/ml propidium iodide and analyze cells by flow cytometry (Fig. 3.37.8).

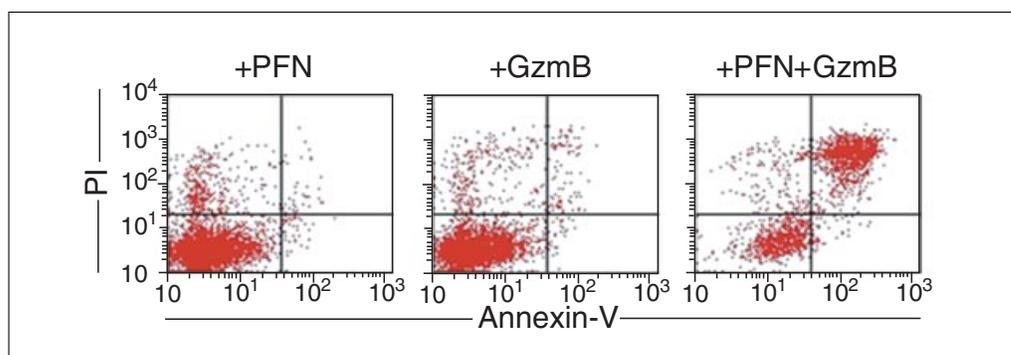


Figure 3.37.8 Analysis of PFN and GzmB-mediated apoptosis by Annexin-V/PI assay. HeLa cells were treated for 1 hr at 37°C with sublytic rat PFN and purified human GzmB before staining with Annexin-V and propidium iodide. Cells were analyzed by flow cytometry. For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb0337>.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

AAD assay buffer

H₂O containing:

50 mM Tris·Cl, pH 7.5 (APPENDIX 2A)

0.2 mM Boc-Ala-Ala-Asp-Thiobenzyl ester (AAD; MP Biomedicals), 20 mM stock solution in DMSO (Sigma)

0.22 mM of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma), 0.55 M stock solution in DMSO

Prepare fresh

An5 buffer

H₂O containing:

10 mM HEPES, pH 7.5

140 mM NaCl

2.5 mM CaCl₂

Store up to 6 months at room temperature

BLT assay buffer

H₂O containing:

50 mM Tris·Cl, pH 7.5

0.2 mM N α -benzylozycarbonyl-L-lysine thiobenzyl ester (BLT; Sigma), 0.5 M stock solution in DMSO

0.22 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma), 0.55 M stock solution in DMSO

Prepare fresh

Buffer C

Hanks' balanced salt solution (HBSS)

10 mM HEPES, pH 7.5

4 mM CaCl₂

0.4% (w/v) bovine serum albumin (BSA)

Store up to 1 month at 4°C

Buffer P

Hanks' balanced salt solution (HBSS)

10 mM HEPES, pH 7.5

Store up to 1 month at 4°C

Colony forming and turbidimetry unit assay buffer

H₂O containing:

20 mM HEPES, pH 7.4

10 mM NaCl

Store up to 1 month at 4°C

Continuous 40% adjusted Percoll gradient

Relaxation buffer (see recipe) containing:

40% (w/v) Percoll (Sigma)

250 mM sterile sucrose

10 mM HEPES pH 7.2

7 mM HCl

Prepare fresh

Elution buffer B1

Equilibration buffer A1 (see recipe) containing 1 M imidazole, pH 7.5. Store up to 1 month at 4°C.

Elution buffer B2

H₂O containing:
20 mM Tris·Cl, pH 7.2 (APPENDIX 2A)
1 mM EGTA
Store up to 1 month at 4°C

Elution buffer B3

H₂O containing:
20 mM Tris·Cl, pH 7.2 (APPENDIX 2A)
0.1 mM EGTA
2 M NaCl
Store up to 1 month at 4°C

Elution buffer B4

H₂O containing:
20 mM bis-Tris, pH 5.8
1 M NaCl
Store up to 1 month at 4°C

Equilibration buffer A1

Distilled H₂O containing:
1 M NaCl
20 mM HEPES, pH 7.2
10% (w/v) Betaine (Sigma)
Store up to 1 month at 4°C

Equilibration buffer A2

H₂O containing:
20 mM Tris·Cl, pH 7.2 (APPENDIX 2A)
1 mM EGTA
2 M NaCl
Store up to 1 month at 4°C

Equilibration buffer A3

H₂O containing:
20 mM Tris·Cl, pH 7.2 (APPENDIX 2A)
0.1 mM EGTA
Store up to 1 month at 4°C

Equilibration buffer A4

H₂O containing:
20 mM bis-Tris, pH 5.8
50 mM NaCl
Store up to 1 month at 4°C

FACS buffer

Phosphate-buffered saline (PBS; Cellgro, cat. no. 21-031-CV)
2% (v/v) fetal bovine serum (FBS; heat-inactivated)
Store up to 6 months at 4°C

HE buffer

H₂O containing:

150 mM NaCl
10 mM HEPES, pH 7.5
2 mM EDTA, pH 8.0 (APPENDIX 2A)
Store up to 1 month at 4°C

HHH buffer

Hanks' balanced salt solution (HBSS) containing:

10 mM HEPES, pH 7.2
100 U/ml heparin (Sigma)
Prepare fresh

K10 medium

RPMI-1640 medium supplemented with:

10% (v/v) fetal bovine serum (FBS; heat-inactivated)
100 U/ml penicillin (from a 20× stock)
100 μg/ml streptomycin (from a 20× stock)
6 mM HEPES
1.6 mM L-glutamine
50 μM 2-mercaptoethanol
Store up to 1 to 2 months at 4°C

Relaxation buffer

H₂O containing:

10 mM KCl
3.5 mM MgCl₂
10 mM PIPES pH 6.8
1.25 mM EGTA pH 8.0
1 mM ATP (Sigma)

Relaxation buffer can be prepared in advance without ATP. This buffer can be stored 1 to 2 days at 4°C prior to addition of the ATP. Once ATP is added the buffer must be discarded after each use.

Solubilization buffer

H₂O containing:

1 M NaCl
20 mM sodium acetate, pH 4.5
2 mM EDTA, pH 8.0 (APPENDIX 2A)
Store up to 6 months at 4°C

COMMENTARY

Background Information

Cytotoxic T lymphocytes and NK cells deliver their effector molecules via specialized secretory lysosomes. These lysosomes, or cytotoxic granules, are dual-function organelles, containing both lysosomal proteases, which function at acidic pH, and secretory products such as perforin, which function at neutral pH when they are released from the killer cells to induce target cell death. Indeed, when cytotoxic T lymphocytes and natural killer cells form an immune synapse with a

specifically recognized target cell destined for elimination, cytotoxic granules move to the immune synapse where the cytotoxic granule membrane fuses with the killer cell membrane, releasing the granule contents into the synaptic cleft (Voskoboinik and Trapani, 2006; Chowdhury and Lieberman, 2008). These cytotoxic granules contain perforin (PFN), needed to deliver the granzymes into the target cell (Keefe et al., 2005), and a family of highly homologous serine proteases named granzymes (Gzms), whose major job is to

induce programmed cell death to eliminate virus-infected and tumor cells (Stinchcombe et al., 2001). By activating apoptosis instead of necrosis, inflammation is minimized to focus on the intended target and minimize effects on bystander cells. Cytotoxic granules of human NK cells and cytotoxic T cells also contain granzysin (GNLY), a protein with antimicrobial activity against a broad spectrum of microbial pathogens. Isolation of cytotoxic granules and further purification of native PFN, granzymes, and/or GNLY allows researchers to study how the granzymes initiate programmed target cell death pathways, how PFN induces internalization of granzymes into the target cell cytosol, and how GNLY induces the death of microbial pathogens.

The protocols described in this unit are based on isolation of cytotoxic granules from CTL and NK cells by nitrogen cavitation as originally developed by Borregaard (Borregaard et al., 1983). CTL and NK cells are resuspended in relaxation buffer designed to mimic the conditions found in the cytosol of neutrophils, facilitating the separation of cytoplasmic organelles from nuclei. Cells are lysed by nitrogen cavitation and cytotoxic granules are purified over a Percoll density gradient. Although purified granules can be used for some studies, further FPLC purification of effector molecules is often necessary for other purposes. Active recombinant mouse GzmB and human GzmA can also be expressed and purified (Martinvalet et al., 2008b). A method was also developed to express and purify active recombinant GNLY (Ernst et al., 2000). However, active recombinant PFN is difficult to purify and even purified native PFN is tricky to work with (as discussed above). For that reason, many researchers substitute a bacterial pore-forming protein such as streptolysin O for PFN as the delivery agent to introduce granzymes into target cells. However, we feel that using native PFN is a more physiologically relevant system. Thus, isolation of cytotoxic granules is a reliable source of active native PFN, granzymes, as well as GNLY. For perforin isolation, IMAC (immobilized metal affinity chromatography) procedure is preferred. The advantage of cobalt IMAC for PFN purification is that there are few other co-purifying proteins, and this method allows better separation between granzymes and PFN (Winkler et al., 1996). Both GzmB and GNLY are purified by a 3-step method adapted from Shi et al. (2000), taking advantage of hydrophobic interactions of PFN with phenyl Sepharose to separate PFN from the other

granule components, followed by two consecutive cation-exchange columns (heparin and SP Sepharose or MonoS) to separate the more basic granule components.

Critical Parameters

The two-step cytotoxic granule isolation protocol described here is particularly suitable for use of granules and/or purified effector proteins in studies that aim to define the different mechanisms of target cell death by the CTL/NK cells granule pathway. Cytotoxic granules can also be isolated using a protocol described by Millard et al. (1984). This method includes heparin in the cell lysis buffer to optimize granule yield, and DNase I to digest DNA released from nuclei damaged during the cell lysis step. However, the use of these granules for further studies of granzyme-mediated cell death is not recommended because DNase I added during purification can contribute to nonspecific DNA fragmentation. By comparison, cytotoxic granule extracts by the Borregaard method are free of non-specific nuclease activity (Davis et al., 2003). Before any cytotoxic granule purification, LAK, CTL, or NK cells activation state should be tested by a PFN and/or granzyme expression assay.

One of the most important variables that affect the success of granule isolation is the cell lysis by nitrogen cavitation. Indeed, as mentioned before, the yield of granules is markedly influenced by the cavitation process and excessive cavitation ruptures the cytotoxic granules.

The other important step is the continuous Percoll density gradient. The number of cell equivalents loaded into a preformed 20-ml Percoll continuous gradient should not exceed 10^{10} in 5 ml. When more cells are used, the users are encouraged to prepare additional tubes of Percoll gradient. Moreover, users are strongly encouraged to test harvested granule fractions for activity. Usually granules are harvested by withdrawing the bottom 5 ml from each tube. The highest granule titer is in the first 4 ml, but it is sometimes necessary to harvest 7 ml in order to fully recover granules. Isolated cytotoxic granules must be properly stored in solubilization buffer at -80°C .

At present, rat RNK-16 cells are the best source of high-quality and high-yield native PFN. The use of YT-Indy NK cells also offers a reliable source of human PFN that avoids *in vivo* propagation, but with a much lower yield. Because NK cell lines expand slowly, it can be difficult to reach the number of cells necessary for purification of high-yield protein. One of the critical steps before

chromatography purification of PFN from cytotoxic granules is granule solubilization. At least two freeze/thaw cycles are necessary to solubilize granules fully. Best results are obtained with a freeze cycle at -80°C overnight, followed by thawing for 2 hr on ice, and a freeze/thaw cycle with liquid nitrogen. After buffer exchange with a desalting column it is also crucial to note that PFN is no longer in a Ca^{2+} -chelated buffer and can be inactivated by traces of Ca^{2+} . Hence, the FPLC step must be performed as quickly as possible. Moreover, EGTA must be added into each fraction collector tube before the FPLC run to prevent PFN inactivation by traces of Ca^{2+} . Concentrated purified PFN must be stored at -80°C . PFN must be handled on dry ice, and PFN remaining after an assay can be saved at -80°C , pooled, and activity retitered before using.

The best results for isolating GzmB and GNLY using YT-Indy cells as the source of granules are usually obtained if the protocol is completed within 1 day. Furthermore, to optimize the yield, it is critical that the NaCl concentration of the loading samples for the cation exchange columns not exceed 100 mM; the granzymes and GNLY will otherwise not bind to the heparin or S-column. To improve activity in subsequent experiments using purified GzmB and GNLY, it is necessary to concentrate the proteins to $>20\ \mu\text{M}$. High NaCl concentration (almost 1 M) in the eluate fractions will interfere with protein activity. Concentrated, purified granzymes must be stored at -80°C . It is also important to note that measuring purified GzmA activity with BLT is highly sensitive but might not be as specific as substrate cleavage. We highly recommend to test purified GzmA for cleavage of its substrate SET (Beresford et al., 2001) either using recombinant SET (Martinvalet et al., 2008a) or whole-cell lysate (Bovenschen et al., 2009).

Anticipated Results

Cytotoxic granule preparation depends on the amount of starting material. Typically, preparation of cytotoxic granules from RNK-16 harvested from ten F344 rats yields 50 to 100 μg of purified native PFN. We also usually purify about 500 μg GzmB and about 300 μg GNLY from 3×10^9 YT Indy cells and about 100 to 150 μg GzmA from 7.5×10^9 LAK cells. Although these protocols are well established and characterized, users should take care to assess their purified PFN, granzymes, or GNLY for contaminants by SDS-PAGE and silver or Coomassie staining and/or by prob-

ing with antibodies specific for PFN, GzmB, GzmA, or GNLY.

Time Considerations

The protocols for isolation of cytotoxic granules from human LAK cells or YT-Indy cells (Basic Protocol 1) or from rat RNK-16 cells (Alternate Protocol 1) involve several steps and require about 1 day. The in vitro growth of LAK/YT-Indy or in vivo amplification of RNK-16 cells require about 3 weeks to reach the desired number of cells. Extra time should also be allotted to characterize PFN and Gzms expression in the cells used for granule extraction, either by microscopy (6 hr; Support Protocol 1) or by flow cytometry (2 hr; Support Protocol 2).

Purification of the effector proteins (PFN, GzmA, GzmB, GNLY) by FPLC requires an additional day to complete (Basic Protocol 2 and Alternate Protocol 2). Although analysis of the activity of purified proteins does not require more than a few hours, this analysis must be performed before pooling positive fractions and adds extra time to the overall procedure. During purification of effector proteins, measuring PFN activity in isolated fractions by hemoglobin release assay (Support Protocol 3) requires ~ 1 hr. Then, measurement of PFN activity in concentrated, pooled positive fractions and determination of the sublytic concentration can be easily performed by propidium iodide uptake (Support Protocol 4) in ~ 30 min. Similarly, measuring GzmB (Support Protocol 5) or GzmA (Support Protocol 6) activity by AAD or BLT assays, respectively, can be performed in 30 min. Finally, measuring GNLY activity by colony-forming assay (Support Protocol 7) or turbidimetry (Support Protocol 8) requires ~ 16 hr (which includes an overnight incubation).

Purified effector proteins (PFN/GzmA/GzmB) can then be used to induce apoptosis of target cells. The chromium release assay described in Support Protocol 9 necessitates ~ 6 hr for completion, whereas measuring Gzm-induced cell death by Annexin-V/PI staining (Support Protocol 10) can be performed in 2 hr.

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