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HIV-1 Accessory Proteins: Vpu and Vif

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Abstract

HIV-1 Vif and Vpu are accessory factors involved in late stages of viral replication. Vif regulates viral infectivity by preventing virion incorporation of APOBEC3G and other members of the family of cytidine deaminases, while Vpu causes degradation of CD4 and promotes virus release by functionally inactivating the host factor BST-2. This chapter described techniques used for the characterization of Vif and Vpu and their functional interaction with host factors. Many of the techniques are, however, applicable to the functional analysis of other viral proteins.

Keywords

Vif; Vpu; Accessory protein; Restriction factor; Virus–host interactions

1 Introduction

The primary goal of a virus is the infection of host cells in order to replicate its genome and to produce progeny virions for the infection of new target cells. Some viruses cause long-lasting chronic infections, while others replicate in fast, lytic cycles. However, replication of all viruses depends to a large degree on specific host factors, from the recognition of specific cell surface receptors required for virus entry into a target cell to the packaging of cellular factors into virions. HIV penetrates target cells through fusion with the host plasma membrane. This is followed by partial uncoating and reverse transcription of the viral RNA and subsequent integration of the double-stranded cDNA into the host genome. The integrated provirus then serves as template for the synthesis of viral proteins, which ultimately assemble into progeny virions that are released from the infected host cell. We are far from fully understanding all of the complex virus–cell interactions that take place during a single replication cycle; however, such interactions occur at virtually every step during the replication cycle. Recent years have brought rapid progress in the identification and characterization of novel host factors supporting or restricting HIV replication. In particular, the recent identification of host restriction factors such as Trim-5α, APOBEC3G, and BST-2/tetherin has significantly advanced our understanding of HIV cell tropism. The molecular mechanisms that dictate host restrictions, however, remain poorly understood.

Primate immunodeficiency viruses, including HIV-1, are characterized by the presence of viral accessory genes that encompass vif, vpr, vpx, vpu, and nef. The vif, vpr, and nef genes are expressed in most HIV-1, HIV-2, and SIV isolates. In contrast, the vpu gene is found only in HIV-1 and some SIV isolates. The vpx gene, on the other hand, is absent in HIV-1 but is common to HIV-2 and most SIV strains. Current knowledge indicates that none of the primate lentiviral accessory proteins has enzymatic activity. Instead, it seems that these

proteins interact with cellular ligands to either act as adapter molecules to redirect the normal function of host factors for virus-specific purposes or to inhibit a normal host function by mediating its degradation or by causing intracellular mislocalization/sequestration. Such functions are consistent with the host-specific nature noted for most of the accessory proteins.

Here we described techniques used for the characterization of the viral accessory proteins Vif and Vpu and their functional interaction with host factors. However, many of the techniques are applicable to the functional analysis of other viral proteins.

2 Materials

2.1 Buffers

Binding buffer—0.1 % BSA in PBS.

Wash buffer—50 mM Tris 7.4*.

300 mM NaCl.

0.1 % Triton X-100.

(* adjust pH in final mixture).

Transfer Buffer—43 g glycine.

9.1 g Tris.

(Dissolve in 2,400 ml H₂O; add 600 ml of methanol).

20× TN buffer—48.4 g Tris base.

362.0 g NaCl.

(Add H₂O to almost 2 l, *adjust pH with HCl to pH 7.4*, then bring to 2 l with H₂O).

1× TN–TN buffer—50 ml 20× TN.

3 ml Tween-20.

0.5 ml NP-40 (now sold under the name IGEPAL CA-630). 947 ml H₂O.

Final composition of TN–TN buffer: 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.3 % Tween-20, 0.05 % IGEPAL CA-630/.

1× TN–T buffer—50 ml 20× TN.

3 ml Tween-20 (100 %).

947 ml H₂O.

Final composition of TN-T buffer: 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.3 % Tween-20.

Saturated ammonium sulfate—At 0 °C, a saturated ammonium sulfate solution is 3.9 M. To prepare a saturated solution of ammonium sulfate, weigh in 550 g of ammonium sulfate and add H₂O to 1,000 ml (this is about 4.1 M). Dissolve ammonium sulfate by heating to 55 °C. The ammonium sulfate should be completely dissolved. Let solution cool to room temperature. Some of the ammonium sulfate should precipitate back out. The supernatant represents the saturated ammonium sulfate solution.

Sodium-phosphate Buffer pH 6.5 (1 M)—Prepare 1 M solutions of Na₂HPO₄ (Dibasic) and NaH₂PO₄ (Monobasic) add NaH₂PO₄ to Na₂HPO₄ until desired pH is achieved.

2.2 RT Cocktail

- 60 mM Tris-HCl pH 7.8.
- 75 mM KCl.
- 5 mM MgCl₂.
- 0.1 % NP40 (Igepal).
- 1.04 mM EDTA.
- 5 µg/ml polyA (Pharmacia 27-4110-01; prepare 10 mg/ml stock solution).
- 0.16 µg/ml oligo-dT (Pharmacia 27-7858-03 prepare 1 mg/ml stock solution).

2.3 Substrate Oligonucleotide (See Note 2)

5' ATTATTATTA TTATACCCAA TTCTTTATTT ATTTATTT AT TT 3'

5x Deaminase buffer

200 mM Tris pH 8.0	200 µl (1 M)
200 mM KCl	200 µl (1 M)
250 mM NaCl	50 µl (5 M)
25 mM EDTA	50 µl EDTA (0.5 M)
5 mM DTT	10 µl DTT (0.5 M)
Triton X-100	10 µl X-100 (10 % → final: 0.1 %)
10 % glycerol	100 µl (100 %)
	380 µl H ₂ O (to 1 ml)

10x UDG Buffer

- 600 mM Tris-HCl, pH 8.0.
- 10 mM EDTA.
- 10 mM DTT.

- 1 mg/ml BSA.

5× Transcription Buffer

- 200 mM Tris pH 7.5.
- 10 mM Spermidine.
- 30 mM MgCl₂.

10× Nucleotide Mix

- 5 mM ATP.
- 5 mM UTP.
- 5 mM CTP.
- 5 mM GTP.

In vitro transcription reaction

- 5 µl linearized template DNA (1 µg/µl).
- 21 µl H₂O.
- 5 µl 10× Nucleotides.
- 5 µl DTT.
- 10 µl 5× Transcription buffer.
- 2 µl RNasin (Promega).
- 2 µl SP6 RNA polymerase (Boehringer 10 U/µl).

3 Methods

3.1 Analysis of BST-2/Tetherin Cell Surface Expression by FACS

BST-2/Tetherin is a transmembrane protein expressed at the surface of many cell types. BST-2 expression is associated with impaired particle release by a variety of viruses, incl. human and simian immunodeficiency viruses, Ebola, etc. [1]. This is due to the tethering of budding virions to the plasma membrane of virus-producing cells. Several viruses, incl. HIV-1, HIV-2, and Ebola have developed strategies to bypass the inhibitory effect of BST-2 by down-regulating BST-2 from the cell surface. The effect of viral factors on cell surface expression of BST-2 can be determined by FACS.

1. If testing suspension cultures proceed to **step (6)**. If cells are grown in monolayers go to **step 2**.
2. Wash cells once with cold PBS.
3. Wash cells once with cold PBS containing 20 mM EDTA.
4. Incubate cells for 15 min at 4 °C in 3–4 ml of PBS containing 20 mM EDTA.
5. Break up cell aggregates by pipetting up and down several times.

6. Transfer cells to 15 ml centrifuge tube and incubate on ice for 15 min.
7. Pellet cells in table top centrifuge (5 min, 1,450 rpm).
8. Wash cells once with 4 ml of 1 % BSA in PBS.
9. Suspend cells in 1 ml of 1 % BSA in PBS and transfer to 1.5 ml reaction tube.
10. Pellet cells in minifuge (20 s, 8,000 rpm).
11. Suspend cells in 50 μ l of 1 % BSA in PBS.
12. Add mouse IgG (1 mg/ml) to block nonspecific IgG binding sites on cells.
13. Incubate 10 min at 4 °C.
14. Add 10 μ l of BST-2-specific antibody (diluted 1:100 in PBS containing 1 % BSA); carefully mix.
15. Incubate 30 min at 4 °C.
16. Wash cells twice with 1.5 ml of 1 % BSA in PBS.
17. Resuspend cells in 100 μ l of 1 % BSA in PBS.
18. Add 8 μ l of second antibody (e.g., APC-conjugated donkey anti-rabbit; Jackson ImmunoResearch) prediluted 1:40 in 1 % BSA in PBS.
19. Incubate 30 min at 4 °C in the dark.
20. Wash cells twice with 1.5 ml of 1 % BSA in PBS.
21. Resuspend cells in 500 μ l of 1 % paraformaldehyde in PBS.
22. Analyze samples.

3.2 Pulse/Chase Analysis to Assess Protein Stability or Measure Rate of Virus Release

All proteins produced by a cell have a finite lifespan and are eventually degraded by dedicated proteases to prevent the accumulation of defective or misfolded proteins. The average lifespan of a given protein can range from minutes to multiple hours. In many cases, protein degradation has the added purpose of regulating cellular and/or viral mechanisms. For instance, the HIV-1 accessory protein Vpu can induce the degradation of the host cell surface receptor CD4 [2]. CD4 is an inherently stable protein with a half-life of more than 8 h. However, in HIV-infected cells, the presence of Vpu reduces the half-life of CD4 to about 15 min. Thus, Vpu accelerates the turnover of CD4 by about 30-fold. Most experimental approaches to determine the stability of HIV proteins involve the transfection of adherent cell lines (e.g., 293 T, HeLa). We found it advantageous to perform the labeling of transfected cells in solution rather than on monolayers. The reasons are threefold: (a) cell suspensions can be kept in very small volumes minimizing the amounts of radiolabeled amino acids required without compromising on high specific activity of the isotope; (b) handling times can be minimized, which is a critical factor especially for short pulse and/or chase times; (c) RPMI-based labeling medium can be used for both adherent and non-adherent cell types.

Generally, the half-life of proteins is measured by pulse-labeling cells with [^{35}S] methionine or methionine/cysteine and then “chasing” the samples for a specified time after removal of unincorporated radioactivity (i.e., incubation of cells in medium containing excess of unlabeled amino acids) followed by immunoprecipitation of the proteins. We generally use a mixture of methionine and cysteine since it is less expensive than purified methionine. To improve incorporation of radiolabeled amino acids into proteins, cells are “starved” (i.e., incubated in medium lacking methionine and/or cysteine) prior to addition of the labeled amino acids. In our experience, radiolabeled amino acids are used up by the cells within 10–20 min; therefore extending the labeling period beyond 20 min without adding more labeled amino acids generally does not improve the results. Also, when using short labeling times (i.e., 5 min or less), it is imperative to work fast. Therefore, if multiple parallel samples are to be processed, it may be necessary to stagger the samples. Individual samples are stored on dry ice until all samples have been collected and can be processed for immunoprecipitation. If multiple chase time points are to be determined we found it most effective to distribute the pulse labeled cells into aliquots immediately after the pulse. This minimizes the number of pipetting steps and avoids potential errors due to cell clumping. For immunoprecipitation of labeled proteins, we generally pre-adsorb the desired antibody to the matrix (Protein A or Protein G Sepharose) and then expose the cell lysates to the immobilized antibodies on a rotating platform.

1. If cells are adherent (i.e., monolayer cultures), detach cells using a cell scraper. We generally do not wash the cells prior to detachment since in many instances transfected cells detach and float in the culture. For a standard pulse/chase analysis with four to five individual time points we use approximately 5×10^6 cells.
2. Transfer cells to a 15 ml centrifuge tube and pellet in table top centrifuge (5 min, 450 rpm).
3. Suspend cells in 5 ml of labeling medium (i.e., RPMI lacking methionine and/or cysteine) and incubate in a 37 °C water bath for 20 min.
4. Pellet cells and suspend in 1 ml of labeling medium; transfer cells to 1.5 ml screw cap tube (Note: a screw cap tube should be used to minimize the risk of contamination caused by accidental opening of the lid when using snap cap tubes).
5. Pellet cells and suspend in 200 μl of labeling medium.
6. Add 30 μl of [^{35}S] methionine/cysteine (10 mCi/ml). This will result in a high specific activity (~ 1.3 mCi/ml) in the labeling reaction.
7. Incubate in 37 °C water bath for desired time (pulse).
8. Prepare screw cap tubes, one for each time point, containing 1 ml of complete RPMI (i.e., containing 10 % FBS and the full complement of amino acids). Place tubes with lids off into 37 °C water bath, except for the first tube (zero time point), which is placed on ice.
9. After pulse, pellet cells in minifuge (20 s, 8,000 rpm) and remove supernatant (discard into radioactive waste container).

10. Suspend cells in complete RPMI (200 μ l per chase time point; i.e., 1 ml for five time points) and immediately distribute 200 μ l ea. into prepared tubes. Note: start by adding cells to the zero time point (sample on ice) and then to the pre-warmed tubes beginning with the last time point. Pellet the zero time point sample (20 s, 8,000 rpm); discard supernatant into radioactive waste and store cells on dry ice. Total handling time from the end of the pulse should be less than 2 min.
11. Close all tubes of the 37 °C chase samples and mix cells occasionally by inverting tubes. Incubate at 37 °C for the specified time period.
12. Remove 1 tube ea. at the appropriate time points, pellet cells, remove supernatant, and store on dry ice. After all samples have been collected continue to next step.
13. Prepare cell lysates as follows: remove samples from dry ice and bring to room temperature by briefly placing in waterbath.
14. Suspend cell pellet in 200 μ l of lysis buffer. We generally employ a Triton X-100 buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5 % Triton X-100), but other lysis buffers can be tested.
15. Briefly vortex samples and incubate on ice for 5–10 min.
16. Briefly vortex again and then pellet insoluble material in minifuge (3 min, 13,000 rpm).
17. Transfer supernatant to new tube (sample can be frozen and stored at this point) or add directly onto antibody-conjugated Sepharose beads for immunoprecipitation.

3.3 Immuno-precipitation

Immunoprecipitation relies on the ability of Protein A or Protein G to bind immunoglobulins or antigen/antibody complexes with high affinity. Protein A or Protein G chemically coupled to Sepharose beads are commercially available (e.g., Sigma Aldrich) and allow for easy recovery of immobilized immune complexes by low-speed centrifugation.

Immunoprecipitation of proteins is a two-step process. In the first step, antibodies are bound to Protein A- or Protein G-Sepharose beads. Excess antibodies are then washed off and the protein of interest is incubated with the Protein A/G–antibody complexes in binding buffer. Bound immune complexes are then extensively washed, antigens are eluted, and prepared for further analysis. All incubations are at 4 °C on a rotating platform to keep Sepharose beads suspended. To accommodate large sample sizes, supernatants are aspirated using a pipet tip connected to a vacuum trap. Binding buffer and wash buffer are distributed using a repeater pipetter.

1. Wash Protein A or Protein G Sepharose beads once with PBS.
2. Distribute into 1.5 ml screw cap tubes to achieve 40–50 μ l of packed beads.

Note: we dilute beads 1:10 in PBS and aliquot 500 μ l of suspension using wide-bore tips. Also, screw cap tubes (with O-rings) should be used to prevent accidental opening of the tubes, which could result in radioactive contamination.
3. Pellet beads (20 s, 10,000 rpm) and suspend in 1 ml of 0.1 % BSA in PBS.

4. Add antibody (dilution to be determined for each antibody).
5. Incubate samples on a rotating wheel for 1 h at 4 °C.
6. Pellet beads and remove supernatant containing unbound antibody.
7. Wash beads once with wash buffer. Note: adjust pH of wash buffer after adding all of its components since addition of detergent affects the pH of the solution!
8. Suspend beads in 1 ml of 0.1 % BSA in PBS.
9. Add 200 µl of antigen (e.g., metabolically labeled cell extracts).
10. Incubate on a rotating wheel for 1 h at 4 °C.
11. Wash beads 3× with 1 ml of wash buffer.
12. Suspend beads in 100 µl of sample buffer (2 % SDS, 62.5 mM Tris pH 6.8, 5 % 2-mercaptoethanol, 5 % glycerol, 0.001 % bromophenol blue) and heat in heat block for 5–10 min with occasional vortexing to suspend the beads.
13. Separate samples by SDS-PAGE followed by fluorography (if metabolically labeled samples are involved) or by immunoblotting (for IP/western).

3.4 Immunoblotting

Immunoblotting is a widely used method to detect proteins and analyze their size and abundance in cell extracts. Combined with cell fractionation methods, immunoblotting can be used to identify the subcellular localization of proteins. Our immunoblot protocol uses Tris/NP40 (Igepal)/Tween buffers that produce clean and reproducible results with low background. Note: Overloading gels with too much protein or using primary and secondary antibodies at too high concentrations can result in significant background. If blot looks “dirty,” diluting the primary antibody will often dramatically improve the results. Some of our custom made antibodies are used at dilutions of up to 30,000.

3.5 Preparation of Whole Cell Lysates

1. If using adherent cells, remove cells from flask with a cell scraper.
Note: do not wash cells prior to harvesting to avoid loss of loosely attached or unattached (floating) cells. Transient expression of proteins can cause cells to lose adherence.
2. Transfer cells to centrifuge tube and pellet in table top centrifuge at 1,300–1,500 rpm for 5 min; discard supernatant (unless it is a virus-containing supernatant that will be processed for virus pelleting).
3. Suspend cell pellet in 1 ml of PBS and transfer to 1.5 ml screw cap tube.
4. Pellet cells in minifuge (20 s, 10,000 rpm); discard supernatant.
5. Suspend cell pellet in PBS (200 µl per 5×10^6 cells).
6. Add an equal volume of 2× sample buffer and solubilize proteins by heating at 95 °C for 5–10 min with occasional vortexing.

7. Use 20–30 μ l per sample/lane for gel electrophoresis.

3.6 Transfer of Proteins to PVDF Membrane

1. After gel electrophoresis, soak gel for 30 min in transfer buffer on rocker platform.
2. Prepare “sandwich” as follows.
  - Soak sponge pad in transfer buffer and lay on transfer grid.
  - Soak Whatman paper and add on top.
  - Remove gel from rocker platform and place on wet Whatman paper.
  - Note: Make sure there are no air bubbles trapped between gel and filter paper.
  - Cut PVDF membrane to size, hydrate in methanol, then soak in transfer buffer.
  - Place PVDF membrane on gel; avoid air bubbles!!
  - Soak a second piece of Whatman paper in transfer buffer and place on top of PVDF membrane.
  - Soak second sponge pad in transfer buffer and place on top of second Whatman paper.
  - Complete sandwich by closing transfer grid and mounting assembly into transfer tank. (Note: PVDF membrane must face the anode (positive pole) of the transfer unit).
  - Fill tank with transfer buffer and transfer protein over night at 80 mA constant current. Alternatively, transfer can be done at 50 V constant voltage for 2 h.

3.7 Antibody Incubation

1. After protein transfer to PVDF membrane is complete, remove membrane from transfer unit and block membrane with 5 % nonfat dry milk in 1 $\times$ TN buffer for 30 min on rocker platform.
2. Wash membrane briefly with 1 $\times$ TN-TN, then 1 $\times$ TN-T buffer.
3. Add 30 ml of TN-T buffer and appropriate amount of primary antibody (e.g., 3 μ l of antibody for a 1:10,000 dilution) and incubate filter on rocker platform for 1 h.
4. Wash membrane 5 min each with 1 $\times$ TN-TN, then 1 $\times$ TN-T.
5. Add 30 ml of TN-T buffer and appropriate amount of second antibody (e.g., HRP-conjugated anti-rabbit IgG).
6. Incubate 1 h on rocker platform.
7. Wash membrane twice for 5 min each with TN-TN, then TN-T (four washes total!).

8. Incubate membrane with detection reagent (e.g., 1 min with Amersham Chemiluminescence reagent).
9. Drip off excess substrate and place membrane between a clear plastic sheet protector.
10. Expose to Kodak BioMax Light film.

3.8 Preparation of VSVg Pseudotyped Virus Stocks

Pseudotyping is the processing of expanding the host range of a virus by incorporating a heterologous envelope protein. A popular envelope protein used to expand the host range of HIV is the G protein of Vesicular Stomatitis Virus (VSVg). The HIV Env glycoprotein does not interfere with the packaging of VSVg. Therefore VSVg pseudotyping can be used to expand the host range and increase the infectivity of HIV-1 for a single round of replication to produce virus that can then spread to CD4+ cells via its own Env protein.

1. Transfect subconfluent HeLa cells with proviral DNA (e.g., NL4-3) together with pCMV-VSVg DNA. A plasmid ratio of 5:1 or 10:1 (provirus:VSVg vector) works well. Harvest virus 48 h after transfection.

Note: harvesting the virus 48 h after transfection will allow the first round of virus to reinfect HeLa cells and produce a boost of virus during the second cycle or replication. Virus titer after 48 h is ~10 times higher than after 24 h.

2. Centrifuge supernatant (3 min, 1,300 rpm) to remove residual cells.
3. Filter cell-free sup through 0.45 µm syringe filter (use filters with low protein binding).
4. Remove and save a small aliquot for RT assay.
5. Determine reverse transcriptase activity.
6. If concentration of virus is desired, pellet virus in SW41 rotor in ultracentrifuge at 35,000 rpm for 75 min at 4 °C.
7. Resuspend virus in RMPI without FCS and store samples at –80 °C.

3.9 Sucrose Step Gradient Purification of Virions

Historically, purification of HIV-1 virions was done by centrifuging the virus through linear 20–60 % sucrose gradients. HIV-1 virions have an approximate density of ~1.165 g/ml, which corresponds to the density of ~38 % sucrose. In 20–60 linear sucrose gradients, HIV-1 virions will therefore accumulate approximately in the middle of the gradient. To harvest virions from the gradients, 10–15 fractions are collected either from the top or the bottom of the gradient. The disadvantage of this procedure is the large number of samples that are collected and need to be analyzed in subsequent steps. A simpler, yet efficient, way of separating virion-associated and soluble viral proteins is to subject the virus-containing supernatants to a sucrose step gradient centrifugation. Step gradients consist of a cushion of 60 % sucrose, which is overlaid with a buffer of 20 % sucrose. Since the density of HIV virions is higher than 20 % sucrose but lower than 60 % sucrose, virions will accumulate at

the interphase of 20 % and 60 % sucrose. Soluble proteins will not enter the 20 % sucrose buffer and remain at the top of the gradient. Therefore, only three fractions need to be collected and analyzed: (a) the top fraction containing soluble proteins; (b) the 20 % sucrose buffer zone (should not contain viral proteins); and (c) the interphase of 20 %/60 % sucrose (easily visible by eye; contains intact virus and viral cores).

1. Concentrate viral supernatants by pelleting in SW41 rotor (90 min, 35,000 rpm).
2. Suspend viral pellet in 1 ml of PBS or RPMI (without FBS added).
3. Prepare step gradient as follows.
  - a. Add 2.0 ml of 60 % sucrose (in PBS) to bottom of SW55 centrifuge tube.
  - b. Overlay with 2.1 ml of 20 % sucrose (in PBS).
4. Add 0.5 ml of concentrated virus to top.
5. Centrifuge for 60 min at 35,000 rpm.
6. Collect three fractions of 1.1 ml ea. from top of gradient.
  - a. Optional: add 0.1 ml of protease inhibitor cocktail to each fraction.
7. Mix 90 μ l of each fraction with 30 μ l of 4 $\times$ sample buffer and heat at 95 $^{\circ}$ C for 5–10 min.
8. Samples are ready for analysis.

3.10 Purification of Viral Cores (Detergent Stripping)

HIV-1, like all retroviruses, contains a viral capsid that is surrounded and protected by a lipid membrane derived from the plasma membrane of the virus-producing host cell. The viral capsid is formed by the viral capsid protein after it is cleaved from the Gag precursor protein. The capsid encloses the viral RNA genome as well as enzymes critical for early steps of the viral replication cycle, i.e., reverse transcriptase and integrase. In addition, the HIV-1 accessory proteins Vif and Vpr localize to the viral core as do the antiviral host factors APOBEC3G and APOBEC3F. In fact, core association of these host factors is critical for their antiviral activity [3]. To discriminate between virus encapsidation and core association, viruses can be stripped of their membranes by treatment with detergent followed by step gradient centrifugation. To minimize the time of which virions are exposed to detergent, virus is not directly mixed with detergent but pelleted through a layer of Triton X-100.

1. Concentrate viral supernatants by pelleting in SW41 rotor (90 min, 35,000 rpm).
2. Suspend viral pellet in 1 ml of PBS or RPMI (without FBS added).
3. Prepare step gradient as follows.
  - a. Add 2.0 ml of 60 % sucrose (in PBS) to bottom of SW55 centrifuge tube.
  - b. Overlay with 2.1 ml of 20 % sucrose (in PBS).
  - c. Overlay with 0.1 ml of PBS (control sample) or 0.1 ml of 1 % Triton X-100 (core sample).

4. Add 0.5 ml of concentrated virus to top.

Note: add virus to gradients without Triton X-100 first to minimize time that virions are exposed to detergent.

5. Centrifuge for 60 min at 35,000 rpm.
6. Collect three fractions of 1.1 ml ea. from top of gradient.
(Optional: add 0.1 ml of protease inhibitor cocktail to each fraction)
7. Mix 90 µl of each fraction with 30 µl of 4× sample buffer and heat at 95 °C for 5–10 min.
8. Samples are ready for analysis.

3.11 Reverse Transcriptase (RT) Activity Assay

Retroviruses encode a reverse transcriptase (RT) whose function it is to reverse transcribe the RNA genome into a double-stranded cDNA upon infection of a target cell. RT is encoded with the Gag-Pol precursor protein and is released from its precursor by proteolysis during virus maturation. Virus-associated reverse transcriptase activity can be conveniently determined in a quantitative in vitro assay that measures the incorporation of radiolabeled dNTPs into a DNA molecule, which is synthesized from a DNA primer (oligo-dT) using an RNA template (poly-A). Synthesized DNA molecules will bind to DEAE paper, while mono-nucleotides will not bind and can be washed off. Thus, radiolabeled DNA immobilized on DEAE paper is directly proportional to the enzymatic activity, which is directly proportional to the number of virions in a sample. The RT assay represents a fast and reliable way of quantifying viruses in culture supernatants.

1. Add 1 µl of [³²P]-dTTP (10 µCi) per ml of RT cocktail just before use, mix by vortexing.
2. Aliquot 50 µl of cocktail into 96-well round bottom plate.
3. Add 10 µl of virus sample; mix by pipetting up and down several times.
Note: for larger sample numbers use multichannel pipetter.
4. Incubate 90 min at 37 °C.
5. Spot 10 µl of sample onto DEAE paper (e.g., Whatman DE81).
6. Air-dry filter.
7. Wash three times for 5–10 min with 2× SSC.
8. Wash once with EtOH (200 proof) to dehydrate filter.
9. Dry filter.
10. Analyze samples in liquid scintillation counter or expose to PhosphorImager plate.

3.12 Luciferase Assay

Traditionally, virus replication can be measured by determining the virus-associated reverse transcriptase activity released into the supernatant of an infected culture. However, in many instances, viruses are infectious for a single round only and can therefore not establish a spreading infection. In such instances, single-cycle infectivity assays have been developed. There are different systems with different readouts. However, the general principle of the assays is very similar and relies on the activation of an indicator gene that is under the transcriptional control of a Tat-inducible LTR-promoter. Here we describe the virus-induced activation of a firefly luciferase gene in TZM-bl cells [4]. TZM-bl is a HeLa-derived cell line expressing CD4 and CCR5 and is susceptible to infection by both X4- and R5-tropic HIV. The luciferase gene is under the control of the HIV-1 LTR.

1. Plate 5×10^4 TZM-bl cells in 1.5 ml per well in a 24-well plate. For triplicate samples prepare three wells per sample. Incubate overnight at 37 °C in a 5 % CO₂ incubator.
2. The following day, add 50–200 µl virus-containing supernatant.
3. Incubate cells for 48 h at 37 °C in a 5 % CO₂ incubator.
4. Remove culture medium and add 300 µl of 1× Promega lysis buffer.
5. Place plate at –80 °C for at least 30 min.
6. Thaw plate and complete cell lysis by pipetting up and down several times.
7. Transfer lysate to 1.5 ml reaction tube and store on ice (for long-term storage, store lysate at –80 °C).
8. Aliquot 5 µl of each sample into non-transparent white 96-well plate.
9. Add 25 µl of Promega Steady Glo substrate and analyze in plate reader luminometer.

3.13 Cytidine Deamination Assay (to Measure Catalytic Activity of APOBEC3G)

APOBEC3G was recently identified as a potent antiviral host factor that potently inhibits virus replication by editing viral genomes and/or inducing degradation of the viral cDNA [5]. APOBEC3G is a member of a family of proteins with cytidine deaminase activity. APOBEC3G as well as the related APOBEC3F target single-stranded DNA and catalyze the conversion of cytidine residues to deoxyuridine. Deoxyuridines are then transcribed into adenine, leading to G to A mutations on the coding strand in HIV-1. Alternatively, deoxyuridines can be recognized by a DNA repair pathway that results in the hydrolysis of uracil by Uracil-DNA Glycosylase (UDG) and could lead to fragmentation of the viral cDNA. Deaminase activity can be measured in vitro by exposing single-stranded 5' [³²P] end-labeled DNA oligonucleotides containing APOBEC target sites to cytidine deaminase followed by alkaline hydrolysis of the UDG-treated sample. The cleaved product is separated from uncleaved probe on urea-containing acrylamide gels (sequencing gels).

3.14 5' [³²P] End-Labeling of DNA Oligonucleotide

- Use ~3–5 pmol of synthetic oligonucleotide.

5 µl oligo (0.5–1 pmol/µl).

- 10× PNK buffer (supplied with enzyme).

5 µl [³²P] γ-ATP.

16 µl H₂O.

1 µl T4 Polynucleotide kinase.

- Incubate 30 min, 37 °C.
- Stop reaction by adding 1 µl EDTA (0.5 M).
- Remove unincorporated ATP using a Sephadex spin column.
(Use Roche mini quick spin oligo columns [Cat # 1814397]).
- Follow manufacturer's recommendations.
- Store purified oligonucleotide at –20 °C.

3.15 Deamination Reaction

1. Dilute deaminase buffer 1:5 to obtain the working concentration.
2. Combine purified 1 µl of [³²P] end-labeled oligo with 50 µl of sample containing APOBEC3G. If sample is “dry” (e.g., virus pellet) suspend in 50 µl deaminase buffer (40 mM Tris pH 8.0, 40 mM KCl, 50 mM NaCl, 5 mM EDTA, 10 % (v/v) glycerol, 1 mM DTT).
3. Incubate 4 h, 37 °C.
4. Purify (desalt) oligonucleotide on a G25 quick spin column (Roche).

3.16 UDG Treatment

1. Combine 44 µl of desalted uridylated oligo.
2. 5 µl 10× UDG buffer.
3. 1 µl UDG (Roche # 1269062).
4. Incubate 2 h, 37 °C.
5. Stop reaction by adding 6 µl of 1.65 M NaOH.
6. Incubate 5 min, 37 °C to hydrolyze apurinic/apyrimidinic sites.
7. Neutralize sample by adding 6 µl 1.8 M HCl.
8. Combine 5 µl of the reaction mix with FA buffer (90 % formamide, 10 % glycerol, 0.01 % bromophenol blue).
9. Heat sample (95 °C, 5 min).
10. Separate on urea–20 % PAGE.

Note: bromophenol blue (BB) runs at position ~8 in 20 % gels.

Run BB to about 2/3 of the gel.

3.17 In Vitro Transcription and Translation

In vitro transcription/translation is a convenient way to study proteins in a cell-free environment and without a need for specific antibodies. There are commercial kits available that allow the coupled transcription/translation of proteins in a single step. Here we describe a protocol where mRNA is produced by in vitro transcription and then used for the translation of proteins using micrococcal nuclease-treated rabbit reticulocyte lysate. The advantage of this system is that it allows more easily to manipulate the mRNA ratios used for translation and to use defined mixtures of different mRNAs.

The system described here is based on SP6 RNA polymerase driven gene expression from pSP64 or pSP65 vectors. These vectors contain multiple cloning sites that allow gene transcription in sense and anti-sense orientation (Fig. 1). SP6 RNA polymerase produces run-off transcripts. Therefore, plasmid DNA must be linearized at a site downstream of the stop codon of the desired gene prior to in vitro transcription.

3.17.1 Linearize Template DNA

- Linearize 20 µg of plasmid DNA with an appropriate restriction enzyme.
- Check that linearization is complete by analytical agarose gel electrophoresis.
- Purify linearized DNA by extraction with phenol and chloroform followed by precipitation with ethanol.
- Suspend DNA in H₂O at 1 µg/µl.

(See Note 3).

3.17.2 In Vitro Translation

- 40 µl Reticulocyte lysate (Promega).
- 10 µl [³⁵S]-methionine.
- µl RNA from in vitro transcription reaction.
- Incubate at 30 °C for 60 min or longer.
- Optional: stop reaction by adding RNase A (5 µg/ml final concentration).
- Use 10 µl aliquot of for immunoprecipitation.

(See Note 4).

3.18 Generation of Custom Antisera in Rabbits

Antibody-based detection methods such as immunoblotting, immunoprecipitation, or indirect immunofluorescence have become invaluable assays in modern molecular biology, biochemistry, and immunology. Some antibodies are commercially available. However, in many instances (e.g., newly identified viral or cellular factors), antibodies are unavailable. To work around this problem, proteins are often tagged with high affinity epitopes which

can be identified by commercial antibodies. Epitope-tagging is very popular in modern research but has limitations. For instance, it will not allow analysis of endogenous proteins. Also, epitope-tagged proteins can differ in their biophysical and functional properties from the authentic untagged protein. We have developed a simple, yet efficient, system to produce custom antibodies. The process involves expression of the desired protein (or portions of it) in *E. coli*, enrichment of the protein by step-wise fractionation of bacterial lysates, and gel purification of the recombinant protein. The resulting product can be used for immunization of rabbits. Typically, we produce up to 200 ml of high-titered antiserum from a single rabbit at a cost of less than \$10.00 per ml serum. The bacterial expression vector pPLc24 and the inducible strain *E. coli* 537 can be made available upon request for noncommercial purposes.

3.19 Expression of Recombinant Protein in *E. coli*

Expression of heterologous proteins in *E. coli* occurs as an in-frame fusion to the N-terminal 99 amino acids of the polymerase of the bacteriophage MS2 which is under the transcriptional control of the lambda P_L promoter. The cloning vector pPLc24 provides *Bam*HI/*Hind*III sites for the directional cloning of the insert (*see* Fig 2). Induction of protein synthesis in *E. coli* occurs through heat-inactivation of the lambda repressor protein expressed from a separate plasmid in the inducer strain (*E. coli* 537). The resulting proteins form highly insoluble inclusion bodies that can be enriched by step-wise extraction of bacterial lysates with urea. This strategy produces protein that is typically more than 90 % pure. Additional purification is achieved by preparative SDS-PAGE.

3.19.1 Cloning of Desired Gene for Expression in *E. coli*

1. PCR amplify the desired gene using primers encoding for a 5' *Bam*HI and a 3' *Hind*III site. Note: the 5' *Bam*HI site (GGATCC) must be placed such that the underlined sequence (GAT) is in-frame (and not separated by a stop codon) with the amplified gene. Also, the 3' primer should encode a stop codon that is placed in-frame with the amplified gene and located upstream of the *Hind*III site.
2. Clone the *Bam*HI/*Hind*III-digested PCR product into *Bam*HI/*Hind*III-digested pPLc24. Use the ligation mix to transform competent *E. coli* 537 bacteria (*see step 3*).
3. Transformation of *E. coli* 537: The *E. coli* 537 strain contains a kanamycin (Km) selectable plasmid encoding the heat-labile lambda repressor gene, which at permissive temperature (28 °C) inhibits expression of the recombinant protein. To prevent early induction that could result in the death of transformed bacteria, cells need to be incubated at 28 °C! This includes the incubation of the bacteria prior to plating. Because of the lower temperature, incubation of transformed bacteria is 2 h at 28 °C prior to plating. Plate bacteria on ampicillin (Amp; 100 µg/ml) and kanamycin (30 µg/ml) double-selection plates. Note: Double-selection plates are required to maintain the heat-labile lambda repressor gene encoded by a Km-selectable vector in *E. coli* 537 cells and to select for bacteria containing the Amp-selectable vector pPLc24. Incubate plates in a bacterial incubator at 28 °C.

Colonies may be very small after overnight incubation and additional incubation may be required before colonies can be picked.

3.19.2 Test Induction

1. Pick 12 colonies and inoculate 5 ml of LB medium containing Amp (100 µg/ml) and Km (30 µg/ml). Incubate cultures in bacterial shaker-incubator at **28 °C!!** for 24 h. This is the overnight culture.
2. Prepare fresh 10 ml tubes containing 2 ml ea. of LB medium without antibiotics; preheat in water bath to 42 °C. Add 0.5 ml of the overnight culture to pre-warmed medium. Transfer tubes to bacterial shaker-incubator and incubate for 2 h at **42 °C!!** with vigorous shaking to ensure good aeration. Note: Incubation at 42 °C will inactivate the heat-labile lambda repressor and induce protein synthesis.
3. Transfer 1.5 ml of induced culture and 300 µl of uninduced overnight culture (Note: induced culture was diluted 1:5) to 1.5 ml screw cap tubes.
4. Pellet bacteria in minifuge, discard supernatant, and suspend pellet in 50 µl of water. Add 50 µl of sample buffer and heat samples at 95 °C for 5–10 min with occasional vortexing until sample is no longer viscous. Pellet insoluble material (1 min, 13,000 rpm in minifuge) and load samples onto SDS-PAGE. The concentration of the acrylamide is dictated by the predicted size of the protein. Typically, a 12.5 % SDS-PAGE is appropriate.
5. Stain gel with Coomassie brilliant blue (see next section).

3.19.3 Coomassie Staining of Gels

1. Prepare staining solution by dissolving 0.6 g of Coomassie brilliant blue in 500 ml of MeOH (Note: use glass beaker to avoid permanent staining of plastic equipment). Add 100 ml glacial acetic acid and adjust final volume to 1,000 ml with deionized water.
2. Stain gel in staining solution on rocker platform for 30 min at room temperature.
3. Remove staining solution and briefly rinse gel with water.
4. Destain gel using destaining solution (5 % MeOH, 7.5 % acetic acid). Stained proteins will become visible within a short period after addition of the destaining solution. However, complete destaining will require multiple changes of the destaining solution over 1–2 days.
5. Successful expression of recombinant protein can be assessed by comparing the protein pattern of the induced and uninduced cultures (*see* Fig. 3a, lanes 1 and 2).
6. Prepare glycerol stocks of positive cultures from the appropriate uninduced overnight cultures and store at –80 °C. Note: the expression-based screening employed here bypasses the purification of DNA clones. Therefore, it is important to maintain a frozen stock of the transformed bacteria.

3.20 Preparative Production of Recombinant Protein

1. Grow 100 ml overnight culture of positive candidates at 28 °C.
2. Preheat 2 × 200 ml of LB medium (without antibiotics) to 42 °C using a 500 ml Erlenmeyer flask.
3. Add 50 ml of overnight culture to each of the flasks and incubate at 42 °C with vigorous shaking.
4. Remove 7.5 ml of induced culture to serve as an induction control. Pellet bacteria and suspend in 250 µl of water. Add 250 µl of sample buffer and process as above (beginning with step (b4)) to verify that protein induction was successful.
5. Pellet the remaining bacteria and store pellet at –20 °C until induction control is completed.

3.21 Purification of Recombinant Protein

1. Thaw bacterial pellet from step (d5) and suspend in 20 ml of PBS.
2. Transfer to 50 ml oakridge centrifuge tube (to allow subsequent centrifugation at 15,000 rpm).

Note: all subsequent steps are performed on ice unless indicated otherwise.
3. Sonicate bacteria. Note: for best results pellet bacteria between cycles (30 min, 15,000 rpm at 4 °C) and direct the force of the ultrasound directly towards the bacterial pellet. Complete bacterial lysis is ideal but not critical.
4. Pellet bacterial lysate and remove supernatant. Supernatant represents *fraction 1*.
5. Suspend the remaining pellet in 1 M urea (in H₂O) by sonication. Repeat pelleting/sonication cycle three times. At this point the color of the pellet should have changed from the original color and the pellet size should be significantly smaller. Bacteria typically do not withstand sonication in 1 M urea and should be quantitatively lysed at this point.
6. Pellet lysate and remove supernatant. Supernatant represents *fraction 2*.
7. Suspend the remaining pellet in 10 ml of 8 M urea (in H₂O) by sonicating. Repeat **steps 5** and **6**. Supernatant represents *fraction 3* and will contain the bulk of the recombinant protein.
8. Solubilize the remaining pellet in 10 ml of sample buffer. This is *fraction 4*.

3.22 Quality Control of Protein Purification

Run SDS-PAGE with uninduced sample (corresponding to 300 µl of culture; *see step 6*), induced culture (100 µl sample from step (d5)); 50 ml of fraction 1 and 30 µl ea. of fractions 2 to 4 (corresponding to 1.5 ml of induced culture), mixed with an equal volume of sample buffer. Stain gel with Coomassie brilliant blue (see section (c)). A representative result is shown in Fig. 3b.

3.23 Preparative Gel Electrophoresis and Protein Extraction

1. Further purification of the recombinant protein can be achieved by preparative SDS-PAGE. To maximize capacity, gels up to 7.5 mm thick can be prepared using multiple spacers and a comb in the stacking gel is omitted. A typical 16 cm wide and 1.5 mm thick gel with 16 wells can hold approx. 100 μ l of sample. By using five spacers and neglecting the comb we can load up to 10 ml (i.e., $5 \times 20 \times 100 \mu$ l) of fraction 3 onto a single assembly.
2. To identify the protein, the gel is stained briefly (5 min or less) in Coomassie brilliant blue until bands become visible. The gel is then rinsed with water and the dominant band, representing the recombinant protein is cut out with a scalpel. If multiple bands appear and it is not clear which one represents the desired recombinant protein, all potential candidates are cut out and separately extracted.
3. To extract the protein from the acrylamide, cut the acrylamide strip into small blocks and transfer to 50 ml centrifuge tube. Cover a small plastic pipette (1 or 2 ml) at the end with parafilm and use it to crumble the acrylamide. Add 10–20 ml of PBS and press mix through a 30 or 60 ml syringe without needle attached. Repeat several times until no big acrylamide junks remain. If mix is too dry, add more PBS. At the end there should be a layer of PBS above the swollen acrylamide paste. Note: Because the gel was briefly stained, the extract will appear blue. The blue color serves a convenient tracer for the protein.
4. Incubate acrylamide at 55–60 °C overnight.
5. Transfer sample to 200 ml filter flask and apply vacuum to extract liquid from acrylamide. Remove vacuum and add 10 ml of PBS to acrylamide. Let sit for 5 min; then stir with a parafilm-wrapped pipette (avoid damaging the membrane) and reapply vacuum. Repeat this step until the acrylamide is completely white and no blue tracer remains in the acrylamide. Note: If the protein aggregates and cannot be extracted from acrylamide with PBS, add SDS (0.1–1 %) to PBS.
6. Concentrate protein in speed vac starting with 10 5 ml tubes. Punch small hole in lid of tube to allow liquid to evaporate. Add more protein extract as sample dries down. If protein/SDS in sample starts to precipitate, suspend the sample by warming to 37 °C, pool all tubes, and dialyze protein against PBS (4 h at room temperature). Check protein concentration by SDS-PAGE followed by Coomassie staining using a BST standard (1–30 μ g) as reference. Concentrate the recombinant protein until a final concentration of 0.5–1 mg/ml is achieved. Store concentrated protein at –80 °C.
7. Use purified recombinant protein for immunization and subsequent boosting of a rabbit. Approximately 250–300 μ g of protein in 500 μ l mixed with an equal volume of adjuvant have produced high-titered antisera.

3.24 Purification of IgG from Immune Serum

Custom antibodies are generally provided as whole serum containing IgG as well as large amounts of serum albumin. In some cases it is necessary to purify the immunoglobulin away

from serum albumin or other serum proteins. The method described here uses a batch process that allows for the fast and efficient purification of IgG from plasma. The protocol consists of three steps that include concentration of protein by ammonium sulfate precipitation, removal of serum albumin and other serum proteins by binding the DEAE cellulose, and a second ammonium sulfate precipitation followed by dialysis of the purified IgG against PBS. The protocol is designed for 5 ml of rabbit serum but it can be scaled up or down as needed.

3.24.1 First Ammonium Sulfate Precipitation

1. Place 5 ml of serum into a 50 ml centrifuge tube.
2. Add 5 ml of saturated ammonium sulfate drop-wise while vortexing at low speed.
3. Incubate on ice for 10 min.
4. Pellet in Sorvall SS35 rotor (or similar) at 10,000 rpm, 15 min.
5. Suspend pellet in 4 ml PBS (adjust total volume to 5 ml).
6. Add 5 ml of saturated ammonium sulfate as above.
7. Incubate on ice for 10 min.
8. Pellet in Sorvall SS35 rotor (or similar) at 10,000 rpm, 15 min.
9. Suspend pellet in 4 ml 50 mM Na-phosphate buffer (pH 6.5).
10. Dialyze overnight at 4 °C against 4 l of 50 mM Na-phosphate buffer (pH 6.5).

3.24.2 Ion Exchange Chromatography (Batch Procedure)

1. Equilibrate DEAE Cellulose (Whatman, DE52) with 50 mM Na-phosphate buffer (pH 6.5).
2. Transfer 10 g (wet weight) to 50 ml centrifuge tube.
3. Wash DEAE cellulose twice with 20 ml of 50 mM Na-phosphate buffer (pH 6.5); pellet by centrifugation for 5 min at 7,000 rpm between each wash.
4. Add dialyzed serum and mix on rocker platform (or rotating wheel) for 1 h at room temperature.
5. Pellet DEAE cellulose by centrifugation for 10 min at 8,000 rpm.
6. Recover supernatant and transfer to fresh 50 ml centrifuge tube.
7. Wash DEAE cellulose once with 5 ml of 50 mM Na-phosphate buffer (pH 6.5).
8. Recover supernatant and combine with first supernatant.
9. Centrifuge combined supernatants for 10 min at 8,000 rpm to pellet carry-over DEAE cellulose.
10. Recover supernatant and filter through 0.45 µm syringe filter into fresh centrifuge tube.

3.24.3 Second Ammonium Sulfate Precipitation

1. Determine total volume of filtrate and drop-wise add equal volume of saturated ammonium sulfate.
2. Incubate 10 min on ice.
3. Pellet by centrifugation at 10,000 rpm for 15 min.
4. Discard sup.
5. Suspend pellet in 3 ml PBS (total volume will be ~4 ml) and dialyze against PBS overnight at 4 °C.
6. Determine protein concentration and purity of sample by analytical SDS-PAGE.
7. Use a dilution series of a BSA standard as a reference (e.g., 1, 2, 5, 10, 20 µg BSA) and load several volumes of purified IgG (e.g., 1, 3, 10 µl).
8. Stain gel with Coomassie brilliant blue.

4 Notes

1. For IP/western it is recommended that samples are heated in buffer lacking mercaptoethanol. This will prevent the reduction of IgG disulfide bridges and minimize background caused by cross-reactivity of antibodies used for immunoblotting with the IgG from the immunoprecipitation.
2. The underlined CCC motif is the preferred target site of APOBEC3G, while the TTC sequence is preferred target of APOBEC3F. Incubation of this template to APOBEC3G or APOBEC3F will result in deamination at these sites and produce cleavage products of different size subsequent to UDG treatment.
3. It is important to mix reagents at room temperature (otherwise spermidine will irreversibly aggregate DNA!); it is recommended to add the individual components in the order listed above.
  - a. Incubate sample at 40 °C for 1–2 h.
  - b. Add 150 µl H₂O to increase volume.
  - c. Phenol extract once.
  - d. Chloroform extract twice.
  - e. Add Na-acetate (3 M) to 0.3 M final concentration.
  - f. Add 1 ml EtOH (200 proof).
  - g. Incubate 10–20 min on dry ice.
  - h. Pellet RNA (10 min, 14,000 rpm Eppendorf).
  - i. Dry pellet and suspend in 50 µl H₂O.
  - j. Use 5 µl RNA for a standard 50 µl in vitro translation reaction.

4. Samples can also be analyzed directly without prior immunoprecipitation. In this case, proteins should be precipitated with trichloroacetic acid first to remove unincorporated radiolabel.

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Fig. 1.

pSP64 and pSP65 are available from Promega Corp and allow the cloning of a desired gene in sense and anti-sense orientation into a multiple cloning site (MCS) downstream of the SP6 RNA polymerase promoter. For in vitro transcription, DNA must be linearized downstream of the inserted gene using one of the available unique restriction sites

Fig. 2.

pPLc24 is a vector constructed for the expression of proteins in *E. coli* for the purpose of antibody production. Protein expression is under the control of the lambda leftward promoter (lambda PL). Desired proteins are expressed as fusion to residues 1–99 of the bacteriophage MS2 polymerase (MS2-Pol) by cloning the desired gene in-frame with the MS2 pol gene. Protein expression is induced by heat-inactivation of a heat-labile lambda repressor protein that is expressed in the *E. coli* 537 strain from a kanamycin-selected plasmid. Transformants must be grown under double selection (ampicillin and kanamycin)

Fig. 3.

Expression and purification of recombinant protein in *E. coli*. **(a)** Protein expression of cloned genes is done by expression screening and comparison of uninduced (*lane 1*) and induced cultures (*lane 2*). Induced recombinant protein is identified by the *arrow*. Recombinant protein expressed with the pPLc24 vector system is in inclusion bodies. Recombinant protein is insoluble in PBS (*lane 3*) and 1 M urea (*lane 4*) but is extracted by 8 M urea (*lane 5*). **(b)** Quality control and quantitation of purified recombinant protein. A BSA standard (1–30 μ g) is loaded on the *left*. Various volumes of the concentrated purified antigen (1–15 μ l) are loaded on the *right*. The estimated concentration of the protein shown here is ~2 mg/ml