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In vitro JAK kinase activity and inhibition assays.

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Summary

The discovery that a range of myeloproliferative diseases and leukemias are associated with Janus Kinase (JAK) mutations has highlighted the importance of JAK/STAT signaling in disease and sparked a renewed interest in developing JAK inhibitors. *In vitro* kinase assays are the most direct and quantitative method to assess mutant forms of JAK for altered enzymatic properties as well as verifying and quantifying the affinity and efficacy of potential inhibitors. Here, we describe protocols for heterologous expression and purification of JAK kinases from insect cells, assays to determine the activity of these purified kinases and finally inhibition assays to determine the effectiveness of potential inhibitors.

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1. Introduction

Cytokine binding to a specific, cognate cell-surface receptor initiates an intracellular signaling cascade that is driven by activation of a family of receptor-bound tyrosine kinases known as JAKs (Janus Kinases)(1). Once activated, JAKs phosphorylate STATs (Signal Transducers and Activators of Transcription), transcription factors normally sequestered in the cytoplasm(2). Activated STATs dimerise and translocate into the nucleus, where they upregulate transcription of a suite of cytokine-responsive genes(3-5).

There are four mammalian JAKs, JAK1-3 and TYK2, each consisting of four domains(6-8). The N-terminal FERM domain binds to the appropriate membrane-bound receptor whilst the C-terminal kinase (catalytic) domain phosphorylates substrate proteins. Between these are a non-canonical SH2 domain and a pseudokinase domain. Although full-length JAKs are difficult to express using recombinant systems, the kinase domain can be easily expressed and purified using the baculovirus expression system in *Spodoptera frugiperda* (*Sf*21) insect cells.

A number of different leukemias and myeloproliferative disorders are caused by JAK mutations (9-21). A hallmark of these diseases is that JAK is mutated in such a way as to render it constitutively active. This can be either via **point mutation** or by **oncogenic fusion** of JAK. The end result is that the cell's normal cytokine signaling pathways are activated even in the absence of cytokine (22). This aberrant activation then leads to dysregulated proliferation and disease. Given this there is intense interest in characterizing the enzymatic properties of mutant forms of JAK (Turnover rate, substrate affinity and ATP affinity) as well developing and quantifying small molecule inhibitors of JAKs.

In vitro kinase assays are a valuable method to gain mechanistic insights into both kinase activity and, where inhibitors exist, inhibition. These assays are the only method available to quantify enzymatic parameters such as K_M and k_{cat}. Here, we describe protocols to measure kinase activity (and inhibition) using a radiometric assay. We find this assay to be the most robust and quantitative method to measure kinase activity and inhibition.

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2. Materials

1. Plasmid pFastBac HTb (Life Technologies, Carlsbad, CA, USA)

2. Equipment and reagents for PCR (thermocycler, deoxynucleotide triphosphates, high-fidelity and Taq polymerases, DNA templates, oligonucleotide primers)

3. Restriction enzymes (BamHI, NotI, alkaline phosphatase)

4. Equipment for DNA electrophoresis (Tris-acetate buffer, agarose, gel tanks, ethidium bromide, UV transilluminator)

5. Competent *E. coli* cells for cloning (DH10B or TOP10)

6. Luria broth (LB)-agar plates containing 100μg/mL ampicillin

7. LB media containing 100μg/mL ampicillin

8. DNA miniprep kit

9. Big Dye Terminator sequencing reagents (Life Technologies/Applied Biosystems) and oligonucleotide primers for sequencing

10. DH10Bac chemically-competent cells (Life Technologies)

11. 15 mL round bottomed polypropylene and polystyrene tubes (BD)

12. Super broth (35g tryptone, 20g yeast extract, 5g NaCl, 2.5mL 2M NaOH to 1L with deionized water)

13. LB-Bac agar plates (LB-agar containing 50 μg/mL Kanamycin, 10 μg/mL Tetracycline, 7 μg/mL Gentamicin, 0.1 μg/mL BluoGal, 40 μg/mL IPTG)

14. LB-Bac media (Luria-Bertani broth containing 50 μg/mL Kanamycin, 10 μg/mL Tetracycline, 7 μg/mL Gentamicin)

15. Solution I (15 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 100 μg/mL RNase A)

16. Solution II (0.2 M NaOH, 1% v/v SDS)

17. Solution III (3 M sodium acetate-acetic acid pH 5.5)

18. Isopropanol

19. 70% ethanol

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20. TE: 10mM Tris-HCl, 1mM EDTA, pH 8

21. *Spodoptera frugiperda* (*Sf*9 or *Sf*21) insect cells (LifeTechnologies)

22. Sf900 II SFM insect cell media (LifeTechnologies)

23. 1L Schott bottles and 2.8L Fernbach flasks

24. Humidified 27°C incubators: stationary and shaking

25. Equipment for cell culture (Class II Biosafety cabinet, microscope for cell inspection and counting, hemocytometer, slide and coverslip)

26. Tissue culture-treated six-well plates (e.g. Corning catalog number 3516)

27. Insect cell transfection reagent (e.g. LifeTechnologies CellFectin II)

28. 50mL polypropylene Falcon tubes

29. Vented cap 75cm^2 and 150 cm^2 cell culture flasks

30. Cytobuster reagent (Merck, Whitehouse Station, NJ, USA)

31. Equipment for SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

32. Equipment and reagents for Western blotting (transfer apparatus, PVDF membrane, milk powder, anti-His primary antibody, horseradish peroxidase conjugated secondary antibody, chemiluminescent reagent, device for imaging blot or film and developer)

33. Lysis Buffer A: 200 mM NaCl, 20 mM HEPES-HCL pH7.5 , 5 mM imidazole-HCl buffer pH 7.5, 5% v/v glycerol, 5 mM 2-mercaptoethanol

34. Phenylmethylsulfonyl fluoride (PMSF) and EDTA-free Complete Protease Inhibitor cocktail tablets (Roche Applied Science, Penzberg, Germany)

35. Wash Buffer B: 200 mM NaCl, 20 mM HEPES-HCl pH 7.5, 35 mM imidazole-HCl pH 7.5, 5% v/v glycerol, 5 mM 2-mercaptoethanol

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36. Elution Buffer C: 200 mM NaCl, 20 mM HEPES-HCl pH 7.5, 250 mM imidazole –HCl pH 7.5, 5% v/v glycerol, 5 mM 2-mercaptoethanol

37. Sonicator

38. Peristaltic pump

39. 1mL Ni^{2+} immobilized metal affinity chromatography (IMAC) cartridge

40. 10x kinase buffer: 200 mM Tris pH 8.0, 1 M NaCl, 1 mM DTT, 40 mM $MgCl_2$, 1 mg/mL BSA.

41. 3x kinase buffer (60 mM Tris pH 8.0, 300 mM NaCl, 0.3 mM DTT, 3 mM ATP, 12 mM $MgCl_2$, 0.3 mg/mL BSA, 3 mM peptide substrate).

42. γ-^{32}P-ATP (3000 Ci/mmol, 10 mCi/mL)

43. Tyrosine kinase substrate peptide (e.g STAT5b: RRAKAADGYVKPQIKQVV)

44. P81 phosphocellulose paper (Whatman, GE Healthcare)

45. Phosphorimager device

46. Liquid scintillation counter

3. Methods

The methods described below outline: (1) expression of JAK kinase in insect (*Spodoptera frugiperda*) cells (2) Affinity purification of the expressed JAK kinases, (3) Assay of JAK activity (4) Inhibition Assay of potential JAK inhibitors.

3.1. Expression of JAK in Sf21 cells

3.1.1. Preparation of expression construct.

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This section describes a convenient procedure for cloning each JAK into the pFastBac HTb vector (LifeTechnologies) by PCR, restriction digestion and ligation.

1. The kinase domain boundaries used for each of the four JAKs are as follows (*see* **Note 1**):

JAK2 836-1132 (Genbank: Protein, AAH54807; cDNA, BC054807)

TYK2 875-1183 (Genbank: Protein, AAH94240; cDNA, BC094240)

JAK1 861-1153 (Genbank: Protein, EDL30874; cDNA, AK141210)

JAK3 807-1124 (Genbank: Protein, AAC50950; cDNA, U09607)

Each kinase domain can be conveniently cloned into pFastBac HTb *via* 5' BamHI and 3' NotI restriction sites, as encoded by the following oligonucleotides (mismatches in uppercase):

Jak1 5' (BamHI site underlined) CGCGGATcCacaacagaggtggaccccactc

Jak1 3' (NotI site underlined) ATAAGAATGCGGCCGCttattttaaaagtgcttcaaatccttc

Jak2 5' (BamHI site underlined) CGCggATcCtttgaagacagggaccctacacag

Jak2 3' (NotI site underlined) ATAAGAATGCGGCCGCtcacgcagctatactgtcccgg

Jak3 5' (BamHI site underlined) CGCggATcccagctctatgcctgccaagac

Jak3 3' (NotI site underlined) ATAAGAATGCGGCCGctatgaaaaggacagggagtggtg

Tyk2 5' (BamHI site underlined) CGCggATcCtcggctgtgaactcagactcacc

Tyk2 3' (NotI site underlined) ATAAGAATGCGGCCGCtcagcacacgctgaacacggaag

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Standard PCR protocols should be employed to amplify each cDNA from their respective template using a high-fidelity DNA polymerase (such as Phusion Hot Start, Finnzymes) and then cloned into the pFastBac HTb vector using standard procedures. Insert DNA sequences should be verified by Big Dye Terminator sequencing before proceeding.

3.1.2. Preparation of bacmids.

This section describes the transformation of the pFastBac expression construct into DH10Bac (LifeTechnologies) or DH10MultiBac (ATG Biosynthetics, Merzhausen, Germany) chemically-competent cells to generate bacmids by recombination. Further details of procedures are described in the Bac-to-Bac (LifeTechnologies) or MultiBac Turbo (ATG Biosynthetics) product manuals.

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1. Aliquot 20-100μL DH10Bac or DH10MultiBac chemically-competent cells, thawed on ice, into pre-cooled 15 mL round-bottomed polypropylene tubes, and incubate with 1 μL of 1 ng/µL pFastBac HTb:JAK miniprep DNA on ice for 30 minutes.

2. Heat shock cells for 45 seconds in a 42°C water bath before returning to ice for 2 minutes.

3. Add 0.8 mL Super broth to transformed cells and recover for 4-5 hours at 37°C, 220 rpm before plating 0.3 mL on LB-Bac plates. Culture plates for 36-48 hours at

37°C to allow sufficient time for blue pigment to develop in bacmid recombination-negative colonies.

4. Streak selected white colonies on fresh LB-Bac plates and incubate for 36-48 hours to ensure chosen colonies are not contaminated with blue colonies.

5. Inoculate 2.5mL LB-Bac media with an isolated white colony and culture for 16 hours at 37°C, 220 rpm.

6. Transfer 1.5mL of culture to a microfuge tube and pellet cells at 13000 x*g*, 1 minute using a benchtop microfuge.

7. Completely remove supernatant and resuspend pellet in 0.3mL ice-cold Solution I by gently pipetting up and down. It is crucial to avoid vortexing at any stage during bacmid purification, since the large bacmid DNA is prone to fracturing.

8. Lyse cells by adding 0.3 mL room temperature Solution II. Mix by gentle inversion and incubate for <5 minutes at room temperature.

9. Add 0.3 mL ice-cold Solution III. Mix by inversion and incubate on ice for 10 minutes.

10. Centrifuge in a benchtop centrifuge (maximum speed, 10 minutes) to eliminate insoluble material.

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11. Pipette 0.75 mL of lysate supernatant into a pre-cooled microfuge tube containing 0.75mL isopropanol. Mix by inversion and rest on ice for 10-15 minutes.

12. Pellet bacmid DNA by centrifugation in a benchtop microfuge at 4°C (maximum speed, 30 minutes).

13. Remove and discard supernatant using a pipette (rather than decanting), taking care not to disturb the DNA pellet.

14. Add 0.5 mL ice-cold 70% ethanol to the tube. Invert tube gently before centrifuging for 5 minutes in a benchtop microfuge.

15. Using a pipette, remove supernatant, again taking care to avoid disturbing the DNA pellet.

16. Repeat 0.5mL 70% ethanol wash and spin. Carefully remove supernatant using a pipette again. Spin briefly to collect residue and remove by pipette.

17. Dry DNA pellet at room temperature for 10-15 minutes. Add 100μL 1x TE pH 8.0. Since the bacmid is prone to shear damage, do not pipette up and down, but allow pellet to dissolve in TE at room temperature. Typically, 1.5 mL of culture will yield 100 μL of 300-800 ng/μL bacmid DNA. Bacmid DNA should be stored at 4°C or aliquotted and stored at -80°C to avoid DNA fractures that may arise from freeze-thaw cycles.

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18. Verify the presence of the JAK cDNA in the bacmid by performing an analytical PCR reaction using 0.5 μL bacmid DNA as the template with the M13 reverse primer and a JAK-specific forward primer.

3.1.3. Preparation of baculoviruses.

This section describes the introduction of bacmid DNA into *Spodoptera frugiperda* (*Sf*9 or *Sf*21) cells and the subsequent preparation of high infectivity baculoviruses.

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1. *Spodoptera frugiperda Sf*9 or *Sf*21 cells can be purchased from LifeTechnologies and maintained in commercially-sourced, complete, serum-free culture media, such as Sf900-II SFM (LifeTechnologies) or ESF921 (Expression Systems) media. Insect cells are grown in the absence of antibiotics and should be manipulated only in a Biosafety Cabinet under sterile conditions with strict adherence to aseptic technique. All vessels and apparatus used for insect cell culture should be sterile.

2. 50-300 mL *Sf*21 cell cultures, at a cell density of 0.3-4x10^6 cells/mL, should be grown in 1L Schott bottles (or equivalent) in a humidified 27°C incubator shaking at 130rpm. Typically, a culture at 0.3x10^6 cells/mL will reach a density of 3x10^6 cells/mL after 3 days.

3. For *Sf*21 transfection, 0.9x10^6 cells in a 2 mL volume of Sf900-II SFM should be adhered to each well of a tissue culture-treated six-well plate (such as Corning Costar catalog number 3516) for 25-30 minutes in a humidified 27°C incubator.

4. To prepare the bacmid for transfection, add 1μg of Bacmid DNA to 100μL of Sf-900 II SFM medium in a 15 mL polystyrene tube. For each transfection, mix 6μL of an appropriate transfection reagent, such as CellFectin II (LifeTechnologies) or GeneJuice (Merck), with 100μL of Sf-900 II SFM medium in a separate 15mL polystyrene tube, and add 106μL to each DNA-medium mix. Tap tube gently to mix and incubate at room temperature for 30-45 minutes.

5. Add 0.8 mL Sf900-II SFM media to each bacmid:transfection reagent mix.

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6. Well-by-well, aspirate media from each well of a six-well plate and replace with a bacmid mix. It is advisable to add the bacmid mix by slowly dropping on to the cell monolayer to avoid detaching the adhered cells.

7. After 5-6 hours in a humidified 27°C incubator, aspirate the supernatant from each cell monolayer and replace with 2 mL Sf900-II SFM. This should be done well-by-well to avoid cells drying out. Care should be taken to add the fresh media dropwise to avoid detaching cells from the monolayer. Most importantly, care should be taken to avoid cross-contamination between wells.

8. Incubate for 4 days in a humidified 27°C incubator.

9. Harvest P1 virus by pipetting the supernatant from each well of the six-well plate to labeled 15mL polystyrene tubes. Centrifuge at 500 x*g*, 5 minutes at room temperature to remove debris. The supernatant should be transferred to a new 15 mL polystyrene

tube and stored in the dark at 4°C. Under these storage conditions, the P1 virus will retain infectivity for >6 months.

10. High-infectivity virus should be prepared by two amplification passages. Firstly, P2 virus should be prepared by adhering 10^7 *Sf*21 cells (in a 20 mL volume of Sf900-II SFM media) to a T75 vented cell culture flask (such as Corning) for 25-30 minutes at 27°C before adding 100 μL P1 virus.

11. After 3 days in a humidified incubator at 27°C, decant supernatant from T75 flask into a 50 mL Falcon tube. Centrifuge at 500 x*g* for 5 minutes at room temperature to eliminate debris. Decant supernatant into a fresh 50 mL Falcon tube and store at 4°C in the absence of light.

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12. Prepare P3 virus by adhering 2x10^7 *Sf*21 cells to T150 vented cell culture flasks (such as Corning) in 30 mL Sf900-II SFM media for 20-30 minutes at 27°C. To each flask, add a further 20 mL Sf900-II SFM and 50 μL P2 virus. Return to humidified 27°C incubator.

13. Harvest P3 virus after 3 days by decanting supernatants from T150 flasks into 50 mL Falcon tubes and centrifuging for 5 minutes at 500x*g* to eliminate debris. Transfer supernatants to sterile vessels for storage at 4°C and wrap in aluminium foil to exclude light. P3 virus stored under these conditions will retain infectivity for >6 months.

3.1.4. Determination of optimal conditions for protein expression.

This section describes a procedure to empirically determine the volumetric ratio of P3 virus to number of *Sf*21 cells for optimal protein expression.

1. P3 virus titrations are performed in six-well plates (such as Costar catalog number 3516). 10^6 *Sf*21 cells in 2mL Sf900-II SFM are adhered for 25-30 minutes at 27°C.

2. Titrate P3 virus by adding 5, 20, 50, 100 μL P3 virus to successive wells. Place plate in a humidified 27°C incubator.

3. After 48 hours, aspirate supernatant and lyse infected monolayer with 0.3mL ice-cold Cytobuster reagent (Novagen). Place six-well plate on ice and pipette reagent up and down over cell monolayer to ensure complete lysis.

4. Transfer lysates to 1.5 mL microfuge tubes and spin at 4°C, >13000rpm to pellet insoluble material.

5. Prepare 10 μL samples of lysate supernatants and pellets (resuspended in 0.3 mL water) and resolve proteins using standard reducing SDS-PAGE.

6. Transfer proteins from SDS-PAGE gel to PVDF membrane using standard techniques and perform Western blot analysis with an anti-His tag antibody. Typically, we observe optimum soluble JAK yields for infections using 50 μL P3 virus per 10^6 *Sf*21 cells.

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3.1.5. Protein expression.

1. Culture 400-500 mL *Sf*21 cells to a density of 2.5-3.5 x 10^6 cells/mL in a 2.8L Fernbach flask at 85-90 rpm, 27°C (*see* **Note 2**).

2. Based on the ratio of P3 baculovirus per 10^6 *Sf*21 cells determined to give optimal protein expression in section 3.1.4, calculate and add an appropriate volume of P3 baculovirus to the Fernbach flask.

3. Allow infection and JAK expression to occur for 46-50 hours at 27°C in a humidified shaking (85-90 rpm) incubator.

4. Pellet cells by centrifugation at 500x*g* for 5 minutes.

5. Snap-freeze pellets in liquid nitrogen and store at -80°C until use.

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3.2. Purification of JAK from Sf21 cells

Note: The cloning strategy outlined in 3.1.1 results in an N-terminal His_6 tag. This section describes the purification of His_6 tagged JAKs (*see* **Note 3**).

1. Thaw cell pellets in a waterbath at room temperature. Resuspend in Lysis Buffer A (supplemented with 1 mM Phenyl-methyl-sulfonyl-fluoride (PMSF) and EDTA-free Complete Protease Inhibitor cocktail (Roche)), using 5 mL of lysis buffer for every pellet from 100 mL *Sf*21 culture. Pool cell suspension in a single 50 mL Falcon tube.
2. Lyse the cell suspension by sonication using 6 x 10s bursts with a 10s rest between each burst. Use a moderate power level and perform in an ice/water bath to ensure the temperature of the lysate is maintained at 4°C.
3. Pellet the insoluble material by centrifugation (>20,000 x*g*, 30 minutes, 4°C). Clarify supernatant by passing through a syringe-driven 0.45 μm-filter and collecting the filtered material.
4. Connect a 1 mL Ni^{2+} IMAC cartridge to a peristaltic pump, such as a Gilson Minipuls3, set up in a cold room (*see* **Note 4**). Wash cartridge with 5-10mL Lysis Buffer A at a flow rate of 1 mL/minute. Suitable Ni^{2+} cartridges include HiTrap Chelating SP (GE Healthcare) charged with 0.1 M $NiCl_2$, HisTrap HP (GE Healthcare) or NiMAC (Novagen).
5. Pass clarified lysate over Ni^{2+} IMAC cartridge *via* peristaltic pump at a flow rate of ~1mL/minute.
6. Wash the Ni^{2+} IMAC cartridge with 7-10 mL of Lysis Buffer A *via* peristaltic pump at 1 mL/minute flow rate.
7. Repeat wash with 7-10 mL Wash Buffer B.

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8. Elute His_6-JAK by passing 5-6 mL of Elution Buffer C through Ni^{2+} IMAC cartridge *via* peristaltic pump at a flow rate of ~1 mL/minute and collecting eluate in a 50mL Falcon tube containing 25mL Lysis Buffer A (*see* **Note 5**)

9. Concentrate the supernatant to 0.5 mL by ultrafiltration using a 10 kDa MWCO spin concentrator (*see* **Note 6**).

10. Further purify by gel filtration using a Superdex 200 10/300 column. Use Tris Buffered Saline (pH 7.5) as the running buffer (*see* **Note 7**).

11. Analyze fractions by SDS-PAGE, pool desired fractions, snap-freeze in liquid nitrogen and store at -80°C until required for use.

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3.3. JAK activity assays

The first protocol describes the determination of K_M and V_{max} for the purified kinases. These assays use a synthetic peptide that contains only a single tyrosine as a substrate,. Ideally this will be based on known JAK substrates (for example the STAT5b(693-708) peptide: RRAKAADG<u>Y</u>VKPQIKQVV). *See* **Note 8.** These reactions contain two substrates: ATP and peptide. To determine $K_M^{peptide}$ and $V_{max}^{peptide}$ the ATP concentration will be held constant and in excess (2 mM) whilst the peptide concentration is varied (covered in 3.3.1). Conversely, to determine K_M^{ATP} and V_{max}^{ATP} the peptide concentration will be held constant at (4 mM) and in excess whilst the ATP concentration is varied (covered in 3.3.2).

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3.3.1 JAK activity assay (To determine $K_M^{peptide}$ and $V_{max}^{peptide}$) .

This protocol results in 10 individual reactions, each with a different peptide substrate concentration. Each reaction will be performed in a single well of a microtitre plate in a total volume of 15 μl with the aid of a multichannel pipette. Therefore, care must be taken with all pipetting steps and the reactions designed such that 5 μl is the lowest volume pipetted into the final reaction. Even with these precautions, and a state-of-the-art multichannel pipette, volumetric errors are likely to be the biggest source of deviation. It is recommended that these reactions be performed in duplicate so that any errors can be estimated. The final composition of each reaction will be as follows:

30 mM Tris-HCl pH 8.0

100 mM NaCl

0.1 mg/mL BSA

2 mM ATP (*see* **Note 9**)

4 mM $MgCl_2$ (*see* **Note 10**)

1 uCi γ-^{32}P-ATP

0.1 mM DTT

20 nM JAK enzyme

0-4 mM peptide substrate

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1. Prepare 10x kinase assay buffer

2. Prepare 1 ml of 30 mM ATP using ATP-Na_2 salt (powder) stored at -20°C. This must be performed immediately prior to the assay set up.

3. Prepare 100 µl of 12 mM peptide by dissolving lyophilized peptide in 30mM Tris-HCl, pH 8.0. Ensure that the pH of the final solution is 7-9 (*see* **Note 11**).

4. Using a 96 well microtitre plate, make a series of 10 2-fold serial dilutions (using 30 mM Tris-HCl, pH 8.0) of peptide substrate. The final volume in each well should be 5 µl (*see* **Note 12**).

5. Prepare sufficient mastermix for 12 reactions (18 µl 10x kinase buffer, 12 µl 30 mM ATP, 1.2 µl γ-^{32}P-ATP, 28.8 µl $H_2$0). From this step you must exercise radioactive safety precautions and perform all manipulations behind a perspex screen. Add 5 µl of mastermix to peptide substrate.

8. The reaction is started by the addition of enzyme (*see* **Note 13**). Therefore enzyme (JAK, 60 nM) should be pre-aliquotted into 10 empty wells in the microtitre plate and allowed to equilibrate to room temperature. Start all reactions simultaneously by transferring 5μl of JAK into wells containing substrate/mastermix with a multichannel

pipette. Mix well by pipetting up and down 10 times. Cover plate with clear tape to prevent evaporation.

9. Allow the reaction proceed for 10 minutes then, using a multichannel pipette, spot 4 μl of each reaction onto P81 phosphocellulose paper and immediately place paper into 100 ml 5% H_3PO_4. The paper should be marked so that the location of each spot will be known.

10. Take a second time-point at 20 minutes by repeating step 9 (*see* **Note 14**).

11. Wash P81 paper 3 more times using 100 ml 5% H_3PO_4, 20 mins each time. Perform a final 10 second wash in Acetone and allow paper to air-dry

12. Expose P81 paper to a phosphorimager plate overnight and scan.

13. Excise each spot individually and measure incorporated radioactivity by scintillation counting (*see* **Note 15**).

14. Plot Incorporated radioactivity vs [Substrate] and fit to the Michaelis-Menten equation using software such as Sigmaplot.

15. Measure total radioactivity by scintillation counting of 4 μl of a 1:100 dilution of the remaining reaction mixture. The equation:

$$\frac{\text{Incorporated radioactivity}}{\text{Total radioactivity (x100)}} \times [\text{ATP}]\ (2\times10^{-3}\ \text{M}) \times \text{Reaction volume}\ (15\times10^{-6}\ \text{L})$$

yields the total amount of phosphoproduct formed (in moles, *see* **Note 16**). Dividing this number by the number of moles of JAK enzyme present in the reaction and again by reaction time, in seconds, yields the absolute turnover rate (s^{-1}) or k_{cat} when the substrate concentration is saturating.

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3.3.2 JAK activity assay (To determine K_M^{ATP} and V_{max}^{ATP}) .

This is essentially identical to 3.3.1 except that the ATP concentration is varied (0-2mM) whilst the substrate peptide concentration is held constant (4mM). Note that the specific activity of ATP (in terms of Ci/mmol) per reaction remains constant.

1. Follow 3.3.1, steps 1-4

2. Prepare 20 μl of 6 mM ATP (containing 1 μl of γ-^{32}P-ATP) and make a series of 2-fold serial dilutions using a 96–well microtitre plate. The final dilution should contain 20mM Tris-HCl pH 8.0 and 0 mM ATP. From this step you must exercise radioactive safety precautions and perform all manipulations behind a perspex screen

3. Follow 3.3.1, step 6.

4. Prepare sufficient mastermix for 12 reactions (18 μl 10x kinase buffer, 42 μl 17 mM peptide). Add 5 μl of mastermix to wells containing substrate.

5. Follow 3.3.1, steps 8-13

6. Plot Incorporated radioactivity vs [Substrate] and fit to the Michaelis-Menten equation using software such as Sigmaplot.

7. Follow 3.3.1, step 15

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3.4 JAK inhibition assays

This protocol describes the determination of IC_{50} for potential JAK inhibitors (*see* **Note 17**). These assays are similar to those detailed in the previous sections except that each reaction is performed using a constant concentration of ATP and substrate and a variable concentration of inhibitor. The final composition of each reaction will be as follows:

30 mM Tris-HCl pH=8.0

100 mM NaCl

0.1 mg/mL BSA

2 mM ATP (*see* **Note 18**)

4 mM $MgCl_2$

1 uCi γ-^{32}P-ATP

0.1 mM DTT

20 nM JAK enzyme

1 mM peptide substrate

Variable inhibitor concentration

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1. Add 0.1 µl γ-^{32}P-ATP per reaction to 3x kinase buffer (*see* **Notes 19, 20**).
2. Using a 96 well microtitre plate, make a series of 10 2-fold serial dilutions (using 30 mM Tris-HCl, pH 8.0) of inhibitor. The final volume in each well should be 5 ul. (*see* **Note 21**).
3. Add 5 µl of kinase buffer from (1) to each well.

4. The reaction is started by the addition of enzyme. Therefore enzyme (JAK, 60nM) should be pre-aliquotted into 10 empty wells in the microtitre plate and allowed to equilibrate to room temperature. Start all reactions simultaneously by transferring 5 μl of JAK into wells containing substrate/mastermix with a multichannel pipette. Mix well by pipetting up and down 10 times. Cover plate with clear tape to prevent evaporation.

5. Follow 3.3.1, steps 9-12

6. Plot Incorporated radioactivity vs log(Inhibitor Concentration). The inflection point of the curve yields IC_{50}.

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4. Notes

1. We routinely prepare the kinase domains of all four JAKs using the described method. The poor expression and low yields of soluble, full length JAKs preclude their preparation by the same method at present.

2. Expressing active JAK results in relatively poor yields (ca. 0.5 mg/L). A standard method to increase yield is to add a JAK inhibitor to the expression media, for example the ATP-competitive inhibitor CMP-6 (Merck). Whilst this increases yield by 10-fold it is, in our hands, impossible to remove post-purification and will therefore interfere with downstream assays.

3. A modified vector that encodes an N-terminal Glutathione-S transferase (GST) can also be used and the GST-JAK expressed under identical conditions to those

described above. In that case use standard GST purification protocols to purify the GST-JAK fusion protein.

4. A column bed can be prepared from Ni-NTA resin in place of a prepacked cartridge. Care should be taken to avoid exposure of the His_6-JAK to the high imidazole concentration of Elution Buffer C for long periods of time, such as when gravity flow is used for elution.

5. We have observed that high concentrations of imidazole may compromise JAK solubility and immediate dilution of the eluate limits protein precipitation.

6. ATP, like ATP-competitive inhibitors helps to stabilize JAK by binding to the active site and inducing a more "closed" conformation. In the absence of ATP, JAK is liable to aggregate upon concentration. We find this especially problematic with JAK1. Hence, at this point, ATP and $MgCl_2$ can be added to JAK (final concentrations 1 mM, 2mM respectively) to help stabilize it.

7. After gel-filtration chromatography JAK should be >95% pure. If further purification is required then anion-exchange chromatography using a Mono-Q column can be employed. In this case Tris-HCl pH 8.5 should be used as the running buffer and the enzyme eluted with a gradient of 0-500 mM NaCl.

8. The two arginine residues are placed at the N-terminus so that the peptide will be positively charged at acidic pH and thus easily separated from nucleotides and free

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phosphate by adsorption onto P81 phosphocellulose paper. They can also be placed at the C-terminus if preferred.

9. We have observed that JAK begins to lose activity in concentrations of ATP greater than 2 mM

10. It is likely that JAK binds ATP concurrently with two magnesium ions, therefore the ATP:Mg^{2+} ratio should be kept at 1:2.

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11. Be careful to ensure the pH is correct, we observe significant residual Trifluoracetic acid and Acetonitrile in peptides from a variety of suppliers which will inhibit JAK at high concentrations. Lyophilisation of the peptide from purified water will usually resolve this problem.

12. In our hands the use of a microtitre plate and multichannel pipette yields the greatest accuracy and allows the assay to be performed in a high-throughput fashion. However, many other formats also yield acceptable results.

13. Thaw and dilute JAK immediately prior to performing the assay(s). We see significant loss of activity over 24-48 hours.

14. Performing two time-points allows the researcher to determine whether activity is linearly proportional to time. This condition is necessary to determine kinetic parameters such as K_M and k_{cat}. It is not strictly necessary for determining the IC_{50} of inhibitors.

15. Scintillation counting allows the incorporation of radioactivity to be correlated to an absolute quantitation of product formation however this information is only required for determination of k_{cat} and not K_M and can hence be omitted if desired.

16. The x100 factor is as a result of diluting the 4 μl of reaction mix by 100 before measuring via scintillation counting.

17. Proteins can be tested for inhibitory activity using this assay, as well as small molecules. We assay the inhibitory activity of SOCS (Suppressors of Cytokine Signalling) proteins using this methodology. In this case the assay is a useful technique to determine whether a protein is a direct inhibitor of JAK.

18. Do not exceed 2mM ATP if 4 mM $MgCl_2$ is used. We observe inhibition of enzyme activity when higher ATP concentrations are used. This may be due to ATP rather than MgATP binding to the active site which forms a non-productive complex.

19. If the JAK inhibitor being tested is ATP-competitive then a lower concentration of ATP (100 μM) can be used which will yield more sensitive results

20. By keeping the ATP and substrate concentrations saturating and ensuring that timepoints are taken where product formation is linear with time the IC50 values obtained using this methodology will be a good approximation of the true Ki value, at least for a non-competitive inhibitor. However in many instances an IC_{50} determination will be sufficient and the concentrations of ATP and substrate can be varied at will.

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21. The presence of DMSO is very often necessary to ensure small molecule inhibitors are soluble. If this is required ensure that the concentration of DMSO in the final reaction is < 5%.

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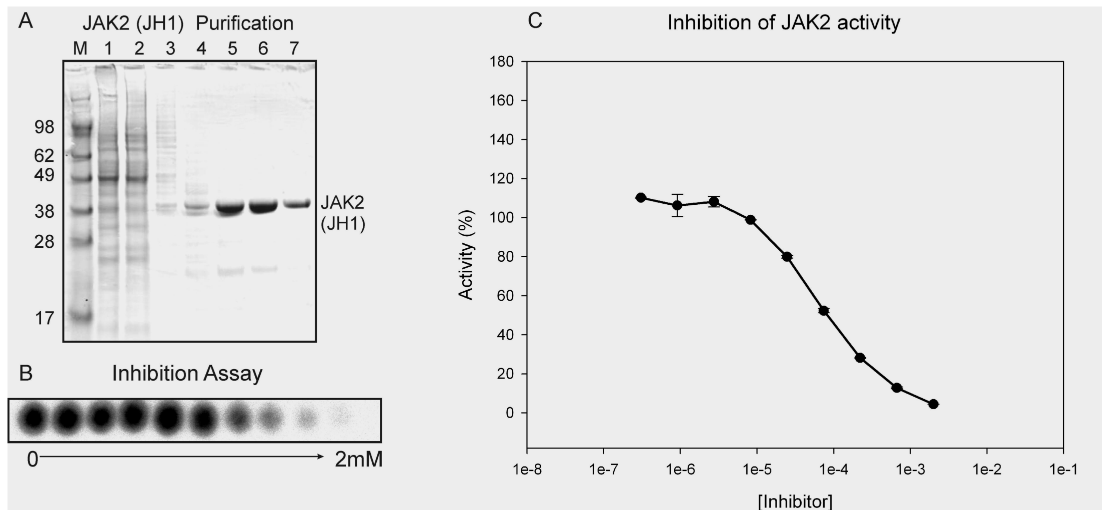

Figure 1. Purification of JAK2 kinase domain and its use in *in vitro* inhibition assays. (A) SDS-PAGE analysis of JAK2 (JH1 domain) purified from insect cells. Lanes 1-4: whole cell lysate, soluble cell lysate, imidazole wash and imidazole elution fractions. Lanes 5-7 Gel filtration fractions. (B) *In vitro* Inhibition assay of JAK2 using ADP performed according to 3.4. The ADP concentration was varied from 0-2mM (left to right). (C) Analysis of the Inhibition assay performed in (B). The IC_{50} of the inhibitor used (ADP) can be read off the plot as the concentration required to give 50% inhibition (100µM in this case).

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