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Functional analysis of West Nile virus proteins in human cells

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Summary

West Nile Virus (WNV) lineage 2 strains have been responsible for large outbreaks of neuroinvasive disease in the United States and Europe between 1999–2012. Different strains in this lineage have previously been shown to produce either severe or mild neuroinvasive disease in mice [1]. Phylogenetic and amino acid comparisons between highly or less virulent lineage 2 strains have demonstrated that the nonstructural (NS) gene(s) were most variable [1,2,3]. However, the roles of some of the NS proteins in virus life cycle are unknown. The aim of this chapter is to describe simple computational and experimental approaches that can be used to: (1) explore the possible roles of the NS proteins in virus life cycle; and (2) test whether the subtle amino acid changes in WNV NS gene products contributed to the evolution of more virulent strains. The computational approaches include methods based on: (1) sequence similarity, (2) sequence motifs, and (3) protein membrane topology predictions. Highlighted experimental procedures include: (1) isolation of viral RNA from WNV infected cells; (2) cDNA synthesis & PCR amplification of WNV genes; (3) cloning into GFP Expression Vector; (4) bacterial transformation; (5) plasmid isolation and purification; (6) transfection using activated dendrimers (Polyfect); (7) immunofluorescence staining of transfected mammalian cells.

Keywords

WNV; computation; protein topology; sequence similarity; cloning; transfection; immunofluorescence

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²Some commercial PCR buffers may already conveniently include MgCl₂. If this is the case, do not add more MgCl₂. Excess of MgCl₂ will decrease the fidelity of the polymerase, which may produce unwanted gene products or promote misincorporation of nucleotides.

1. Introduction

The West Nile virus (WNV) genome consists of a single-stranded, positive-sense RNA of approximately 11-kb that encodes a single polyprotein precursor, which is processed by cellular and viral-encoded proteases into three structural proteins and seven nonstructural (NS) proteins [4]. The roles of the NS proteins in the WNV life cycle are not clearly characterized, except for NS2B-3, which is the viral protease (Pro), [5], and NS5, which serves as the soluble RNA-dependent RNA polymerase (RdRp) [6]. Although the NS proteins have been shown to be components of the virus replication complex [5], defining the individual roles of the NS proteins in viral pathogenesis has been one of the major challenges in the flavivirus field.

Phylogenetic analysis between highly or less virulent WNV strains has demonstrated that certain amino acid changes in the NS gene(s) were associated with large outbreaks of neuroinvasive disease in the United States and Europe [1,2,3]. However, the exact mechanistic roles of these changes and their contributions to WNV pathogenesis are still unknown. Here, we describe computational and experimental approaches that can be utilized to investigate the functional role of WNV NS proteins in virulence and pathogenesis.

Computational methods are techniques that bioinformatics researchers use to assign biological or biochemical roles to proteins. Proteins with comparable sequences are usually homologous and may thus have similar functions. The proteins will be annotated using the sequences of similar proteins of other genomes in the databases. If a newly sequenced gene is found to be similar to a previously sequenced gene, then an evolutionary relationship can be inferred and the function of the new gene is likely to be the same, or at least similar, to the function of the known gene.

Once a protein sequence has been obtained, whether it is the sequence of a single cloned fragment or of an entire WNV genome, then a computer based method can be used to look for special sequence features associated with the genes. In this analysis the protein databases are searched to determine if the test sequence is identical or similar to any genes that have already been sequenced. NCBI BLAST (an acronym for Basic Local Alignment Search Tool) has programs used to generate alignments between a protein sequence, referred to as a “query” and protein sequences within a database, referred to as “subject” sequences. This protocol will include the programs and databases that are currently available online.

Experimental approaches involve cloning the NS genes and expressing the resulting plasmids in human cells. With the methods outlined in this section, any WNV genes can be amplified, fused to GFP and be easily expressed in human cells, such as HEK293T, or other highly transfectable cell lines. Fusing WNV genes to GFP also allows for the visualization of the individual protein when antibodies directly against the WNV protein are not readily available. This information should allow any laboratory skilled in computer analysis and molecular virology methods to establish gene fusion expression system for studies of differences in protein localization, intracellular membrane association, protein secretion, and cytokine and chemokine induction profiling. This system can also be useful for studying other viruses that require high-level biocontainment or are inherently difficult to grow.

2. Materials

2.1. Computational methods

No special materials or equipment, other than a computer with high quality Internet connection, are required to analyze nucleotide and/or protein sequence databases.

2.2. Isolation and Purification of WNV RNA

1. Vero cells (ATCC, Manassas, VA, Catalog Number CCL-81)
2. 1X M199 media supplemented with heat-inactivated fetal bovine serum (FBS), sodium bicarbonate, L-glutamine and penicillin-streptomycin (*Refer to Note*¹)
3. 1X Dulbecco's phosphate-buffered saline (DPBS)
4. 6-well plates
5. RNA isolation kit (Qiagen, Valencia, CA, Catalog Number 52904)
6. Humidified 5% CO₂, 37°C incubator

2.3. cDNA Synthesis and PCR Amplification of WNV Genes

1. Nuclease-free sterile water
2. 10X Buffer
3. 25 mM MgCl₂ (*Refer to Note* 2)
4. 10 mM dNTP mix
5. 10 µM Forward primer in sterile water
6. 10 µM Reverse primer in sterile water
7. 5 U/µL *Taq* DNA polymerase
8. Automated thermal cycler
9. cDNA Synthesis Kit
10. Isolated WNV RNA
11. 0.2 ml PCR tubes
12. *Optional*: Gel extraction kit (Qiagen, Valencia, CA, Catalog Number 28704)

2.4. Cloning Into GFP Expression Vector, Bacterial Transformation and Plasmid Isolation

1. GFP Fusion TOPO® TA Expression Kit (Life Technologies Grand Island, NY, Catalog Number K4820-01) (*Refer to Note*³)
2. LB agar plates containing 50 to 100 µg/mL of ampicillin

¹To make 1000 mL of 1X M199 media, combine the following and then filter: 100 mL 10X M199, 50 mL inactivated FBS, 29.2 mL 7.5% sodium bicarbonate, 10 mL L-glutamine, 10 mL penicillin-streptomycin (5,000 Units/mL) and dH₂O up to 1000 mL. Store at 4°C.

³Using these kits, GFP will be fused to the C-terminal end of the amplified NS gene product.

3. LB medium containing 50 to 100 µg/mL of ampicillin
4. 42°C water bath or electroporator
5. 37°C non-shaking incubator
6. 37°C shaking incubator
7. Plasmid DNA Maxi Kit (Qiagen, Valencia, CA, Catalog Number 12162)
8. Chemically competent TOP10 *E. coli* (provided in kit) or electrocompetent *E. coli*
9. Inoculation loops
10. *Optional*: Plasmid Mini Prep Kit (Qiagen, Valencia, CA, Catalog Number 27104)

2.5. Transfection Using Activated Dendrimers (Polyfect)

1. Human Embryonic Kidney 293T cells (HEK293T) (ATCC, Manassas, VA, Catalog Number CRL-11268)
2. Autoclaved round glass cover slips, 12 mm in diameter
3. Poly-L-Lysine (PLL; molecular weight 70,000 – 150,000) solution: Dissolve PLL in sterile water to attain final concentration up to 100 µg/mL
4. Plasmid DNA (from Miniprep or Maxiprep)
5. Complete Dulbecco's Modified Eagle's Medium, serum free (DMEM)
6. Complete DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (5,000 Units/mL)
7. Polyfect Transfection Reagent (Qiagen, Valencia, CA, Catalog Number 301105)
8. 24-well plates
9. Sterile Eppendorf 1.6 mL microcentrifuge tubes
10. Humidified 5% CO₂, 37°C incubator

2.6 Immunostaining of Transfected Proteins

1. Dulbecco's Phosphate-Buffered Saline (DPBS), 1X
2. Phosphate-Buffered Saline (PBS), 1X
3. Paraformaldehyde (PFA) fixative: 3.7% PFA solution in 1X PBS
4. Triton X-100 solution: 0.4% Triton in 1X PBS
5. 5% bovine serum albumin (BSA) solution: Dissolve 0.5 g BSA in 10 mL 1X PBS
6. Sharp-pointed forceps
7. Primary antibody against host or viral protein of interest

8. Fluorochrome-conjugated secondary antibody specific to host species of primary antibody
9. Mounting medium with DAPI
10. *Optional:* Anti-GFP antibody

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1. Sequence Similarity Based Methods

3.1.1 Protein BLAST

1. Visit the National Center for Biotechnology Information (NCBI) Blast website (www.ncbi.nlm.nih.gov) homepage and examine the following options (Fig. 1).
2. Click on the protein blast program “blastp” to run.
3. Enter the query FASTA protein sequence of interest to the query box and click on BLAST button at the bottom (*Refer to Note*⁴).

3.1.2. Translated BLAST—If only the nucleotide sequence of the amplified PCR product or cloned fragment is obtained, ‘Translated BLAST’ can be utilized. The translated blast search employs a genetic code to translate either the “query,” database “subjects,” or both, into protein sequences, which are then aligned as in “blastp.” The translations are conducted in the three forward as well as the three reverse reading frames so that no possible translation is missed.

There are three varieties of translated BLAST search available: “tblastn,” “blastx,” and “tblastx.” In the first variant, “tblastn,” a protein sequence query is compared to the six-frame translations of the sequences in a nucleotide database. In the second variant, “blastx,” a nucleotide sequence query is translated into six reading frames, and the resulting six-protein sequences are compared, in turn, to those in a protein sequence database (*Refer to Note*⁵). In the third variant, “tblastx,” both the “query” and database “subject” nucleotide sequences are translated in six reading frames, after which 36 (6×6) protein “blastp” comparisons are made (*Refer to Note*⁶). For more tutorials, visit the Wheeler D, Bhagwat M. (2007) Example-Driven Web-Based BLAST Tutorial. Humana Press Inc. <http://www.ncbi.nlm.nih.gov/books/NBK1734/>

⁴BLAST “query” sequences are given as character strings of single letter amino acid codes, preceded by a definition line, beginning with a “>” symbol and this format is known as FASTA. The algorithm reveals the database sequence that most closely matches the sequence of interest. How similar the query sequence is to other WNV isolates will also help determine possible evolutionary changes.

⁵A common use of the “tblastn” and “blastx” programs is to help annotate coding regions on a nucleotide sequence; they are also useful in detecting frame-shifts in these coding regions.

⁶The “tblastx” program provides a sensitive way to compare transcripts to genomic sequences without the knowledge of any protein translation; however, it is very computationally intensive.

3.2. Sequence Motif-Based Methods

The development of protein domain databases such as Pfam *by Sanger Institute* (<http://pfam.xfam.org/search>), MOTIF *by GenomeNet, Japan* (<http://www.genome.jp/tools/motif/>), Motif Scan *by MyHits, SIB, Switzerland* (http://myhits.isb-sib.ch/cgi-bin/motif_scan), and others allow one to find known domains within a query sequence, providing evidence for likely functions. Within protein domains, shorter signatures known as motifs are associated with particular functions, and motif databases such as PROSITE ('database of protein domains, families and functional sites') can be searched using a query sequence. Motifs can, for example, be used to predict subcellular localization of a protein. Short signal peptides direct certain proteins to a particular location such as the mitochondria, and nucleus and various tools exist for the prediction of these signals in a protein sequence.

3.2.1. Searching motif databases

1. Visit this website <http://molbiol-tools.ca/Motifs.htm>.
2. Pick one site from the list of protein domain databases. As an example, click the link for the "Pfam" site to reach the Pfam search engine.
3. Paste the protein sequence of interest into the query box to have it searched for matching Pfam families and press submit (*Refer to Note 7*).

3.3. Protein Membrane Topology Prediction Based Method

The Protein Membrane Topology Prediction Method integrates features for secondary structures, transmembrane helices, globular regions, coiled-coil regions, structural switch regions, protein-protein and protein-DNA binding sites, sub-cellular localization, domain boundaries, cysteine bonds, metal binding sites and disulphide bridges.

1. Visit this website <http://www.bioinformatics.utep.edu/BIMER/tools/transmembrane.html>.
2. Pick one site from the list under "Predictions of Transmembrane Domains in Protein Sequences."
3. As an example, click on the highlighted "SOSUI" on the search sites to visit the "SOSUI" search engine.
4. Enter a title or comment for the sequence on the first rectangular box. Enter the sequence of interest with one-letter symbol to the query box or by copy and paste (*Refer to Note 8*).
5. To execute the query press the button that says "Exec" (*Refer to Note 9*).

⁷Sequence lengths must be less than 10,000 residues. Only residue symbols are allowed in the sequence and sequences containing other characters will not be accepted. Sequence search results will show the detailed description of the results.

⁸Sequence length should not be less than 20 amino acids or more than 5000 amino acids.

⁹The SOSUI result will provide the following: total protein sequence length, average of hydrophobicity, and a table detailing the total number and length of helices, the positions of the transmembrane helices in the sequence, and the type (primary or secondary). In addition, a display option will show the hydropathy profile and helical wheel diagram of predicted segments.

3.4. Protein Expression Analysis

3.4.1. Isolation and Purification of WNV RNA

1. Seed the Vero cells at 10^6 cells per well of a 6-well plate in 1X M199 medium containing 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified 5% CO₂ atmosphere for 24 hours.
2. Discard the old culture medium from each well.
3. Infect the cells with 1 mL of the WNV strain of interest at a multiplicity of infection of 1 (MOI-1) and incubate the cells at 37°C in a humidified 5% CO₂ atmosphere for 1 hour.
4. Discard the medium. Rinse cells with 1X DPBS and remove remaining DPBS with a pipet. Add 3 mL fresh growth medium per well and incubate the cells in the incubator for 48 hours.
5. Collect the supernatant and follow the commercial viral RNA isolation kit protocol (QIAamp Viral RNA Mini Kit) or any similar commercial kit to isolate and purify viral RNA from the infected cells.

3.4.2. Designing Primers For PCR-Based Cloning Into GFP-Expression Vectors

1. Prior to designing primers to amplify the gene, review the sequence of the GFP-expression vector (Life Technologies Grand Island, NY, Catalog Number K4820-01).
2. Using the NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov/nucleotide>), find a full-length genome of the WNV strain of interest to design the forward and reverse primers for WNV genes.
3. The forward primer will simply be the first 18–30 nucleotides of the gene. Incorporate the Kozak sequence containing the ATG start codon at the beginning of the 5' end of the forward primer (*Refer to Note*¹⁰).
4. To design the reverse primer use the reverse complementary sequence of the last 18–30 nucleotides of the gene. Insert an extra guanine nucleotide at the beginning of the 5' region of the reverse primer to successfully clone the resulting PCR product in frame into the GFP expression vector.
5. Although these forward and reverse primers can be designed manually, use online tools such as Primer-BLAST to ensure that the characteristics of the primer (primer length, annealing temperature, melting temperature, GC content etc.) are all optimized (*Refer to Note*¹¹).

¹⁰The Kozak sequence (ex. ACCACCATG or similar) is not included in the expression vector and needs to be added in the forward primer for proper translation.

¹¹Refer to this article for more information: Ye, J. et al. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. doi:10.1186/1471-2105-13-134.

6. Once the primer design is complete, submit and order the primer sequences online (*Refer to Note* ¹²).

3.4.3. cDNA Synthesis & PCR Amplification of WNV Genes

1. Use any commercial cDNA Synthesis Kit to synthesize cDNA using the RNA extracted from WNV-infected cells (*Refer to Note* ¹³).
2. Prepare a standard 25 μ L reaction in a PCR tube on ice. Refer to Table 1.
3. Gently mix the reaction and briefly spin down in microcentrifuge.
4. Run the PCR sample according to the cycling conditions in Table 2 (*Refer to Note* ¹⁴).
5. The size of the amplified PCR product can be verified using agarose gel electrophoresis (*Refer to Note* ¹⁵).
6. If there appears to be multiple bands on the gel, scale up to a 50 μ L PCR reaction and use a commercial gel extraction kit to excise the DNA corresponding to the expected size of the fragment.
7. Adenylate the gel extracted PCR product. Using the 10 μ L PCR reaction in Table 3, perform an extra extension cycle at 72°C with *Taq* Polymerase and the purified DNA fragment (*Refer to Note* ¹⁶).

3.4.4 Cloning Into GFP Expression Vector, Transformation into E. Coli, and Plasmid Isolation—

The following protocol takes advantage of TOPO[®] cloning technology to fuse PCR products to a GFP vector. After cloning and verification of the correct orientation, the resulting plasmid can be purified and used directly for transfection into mammalian cells [7,8,9].

1. Follow the overall protocol included in the GFP TOPO[®] TA Expression Kit. (https://tools.thermofisher.com/content/sfs/manuals/gfptopo_man.pdf)
2. Perform “TOPO[®] cloning reaction and Transformation” on pg. 9–10 of the TOPO[®] manual (*Refer to Note* ¹⁷).

¹²Custom DNA Oligos can be ordered online from Integrated DNA Technologies (IDT), Invitrogen (Thermo-Fisher Scientific), Sigma-Aldrich or any similar vendor.

¹³Using the reverse primer of the WNV gene of interest is advantageous since it creates gene-specific cDNA. However, random hexamer primers may also be used to prime the complete length of the WNV RNA sequence if the resulting cDNA will be used repeatedly to amplify different WNV genes.

¹⁴Amplification parameters may vary depending on the thermal cycler, the buffers, and the melting temperatures of the primers. If the Forward and Reverse primers have different melting temperatures, use the following formula to quickly calculate the annealing temperature for the PCR as a starting point:

$$\text{Annealing Temperature} = [(T_m \text{ of Forward Primer} - 5) + (T_m \text{ of Reverse Primer} - 5)] / 2$$

Further adjustment of annealing temperatures may be needed to optimize the amplification of the PCR product.

¹⁵The concentration of agarose should be adjusted depending on the expected size of the PCR fragment. Use higher concentrated agarose gels for smaller DNA fragments and lower concentrations (<2%) for larger fragments >3 kb in size.

¹⁶Adenosine overhangs must be added to the gel extracted PCR products since it may have been lost during the gel extraction process.

¹⁷When setting up the TOPO[®] cloning reaction for transformation into chemically competent *E. coli*, use as much fresh PCR product as possible. In addition, as little as 0.5 μ L of the GFP TOPO[®] vector can be added. Sterile water is not a necessary component for the

3. After transformation, incubate the plate at 37°C overnight (*Refer to Note* ¹⁸).
4. Verify the correct orientation of the inserted gene by using the methods under “Analysis of Positive Clones” on pg. 12 of the TOPO[®] manual. Streak a patch plate to preserve colonies (*Refer to Note* ¹⁹).
5. Once a positive clone has been identified, streak the positive transformant on LB agar plates with ampicillin and incubate at 37°C overnight.
6. Prepare a starter culture by using an inoculating loop to transfer bacteria from the colony into 5 mL of LB containing ampicillin. Shake at approximately 300 rpm at 37°C overnight for 12–16 hours (*Refer to Note* ²⁰ and *Note* ²¹).
7. Dilute the starter culture 1/1000 into a larger volume of 100 to 500 mL of LB broth containing ampicillin (*Refer to Note* ²²). Shake at approximately 300 rpm at 37°C overnight for 12–16 hours.
8. Harvest the bacteria cells by centrifugation at 6,000 rpm for 20 minutes at 4°C.
9. Follow the commercial Qiagen Maxi Kit protocol or any similar commercial Maxi Prep Kit to isolate and purify the plasmid from bacterial cells.
10. Send the plasmid DNA for sequencing and store purified plasmid in 1X TE Buffer at –20°C or below.

3.4.5. Transient Expression Using Activated Dendrimers (Polyfect)—This following method of transfection has been optimized for a variety of adherent cell lines such as COS-7, Hela, HEK293, HEK293T and CHO cells in 24-well plates. The quick incubation times, high cell viability and low toxicity of the transfection reagent makes it ideal for transfecting GFP fusion proteins into mammalian cells [7,8].

1. Place 1 autoclaved glass cover slip/well in a sterile 24-well plate.
2. Add approximately 300 µL of PLL solution to cover the surface of the well (*Refer to Note* ²³).

TOPO[®] reaction and reactions simply with PCR product, salt and vector can yield positive colonies. For larger PCR products >1 kb, incubate the TOPO[®] reaction at room temperature for 30 minutes.

¹⁸Incubate the plates for less than 18 hours to avoid the formation of satellite colonies.

¹⁹Restriction analysis or sequencing of PCR products can be conducted to verify the sequence and orientation of the inserted gene. Alternatively, positive transformants can be analyzed with colony PCR using, for example, a T7 Forward primer (provided in kit) with a WNV Reverse primer. The PCR cycling conditions to verify the orientation of the insert is generally the same as those used when amplifying the PCR product. However, note that the extension time should be slightly longer to accommodate the extra base pairs between the insert and the priming site on the vector.

²⁰*Optional:* If only a small amount of plasmid DNA (<20 µg) is needed, the bacteria can be harvested after 16 hours. Harvest the bacterial cells in the starter culture by centrifugation at 5,000 rpm for 5 minutes at room temperature. Perform Mini Prep to obtain plasmid DNA, which may be used for subsequent transfection or for sequencing. Plasmid DNA can be quickly isolated using Mini Prep kits, but may not be as pure as those extracted using Maxi Kits.

²¹When the insert is larger (>3 kb), shortening the incubation time of the starter culture at 37°C to 6–8 hours before diluting it in the larger culture volume may help prevent overgrowth of culture and inactivation of ampicillin.

²²Because the GFP Fusion TOPO[®] vector contains a pUC origin of replication which promotes high copy numbers in bacterial cells, any volume between 100 and 500 mL can be used, depending on how much plasmid needs to be purified for downstream applications. Also, use a flask with a volume at least 3–4 times the volume of culture to allow for proper aeration and bacterial growth.

²³Ensure that all of the coverslips do not float to the surface and are completely inundated by the PLL solution. PLL is added to the coverslips to promote attachment of the cells to the coverslips. If the cover slips begin floating to the surface of the PLL solution, use a sterile pipet tip to gently push the coverslips back to the bottom of the well.

3. Incubate cover slips with PLL solution for 1 hour at 37°C.
4. Remove PLL solution from each well and rinse three times with sterile water.
5. Use PLL-coated coverslips for seeding immediately or store plate at 4°C (*Refer to Note* ²⁴).
6. Seed 7×10^5 cells per well in 24-well plates containing PLL-coated cover slips. Grow overnight in a CO₂ incubator at 37°C to 80% confluency.
7. On the day of transfection immediately prior to preparing transfection complexes, aspirate old media and replace each well with 320 µL of fresh, pre-warmed DMEM containing serum and antibiotics (*Refer to Note* ²⁵).
8. In a sterile Eppendorf tube, dilute 2 µg of DNA with DMEM without any serum, proteins or antibiotics to a total volume of 100 µL. Mix and spin down solution to remove any drops of DNA or media from the top of the tube (*Refer to Note* ²⁶ and *Note* ²⁷).
9. Add 20 µL of Polyfect Transfection Reagent to the diluted DNA. Immediately mix by pipetting up and down approximately 5 times to thoroughly mix the DNA and the transfection reagent.
10. Incubate samples for 10 minutes at room temperature (20–25°C) to allow for complex formation.
11. Add 600 µL of DMEM containing serum and antibiotics to tube containing the transfection complexes. Mix by pipetting up and down at least twice to terminate complex formation.
12. Dropwise add 180 µL of the transfection complexes + DMEM to each well to transfect a total of 4 wells in the 24-well plate. Gently swirl the dish to promote even distribution of the complexes in the well.
13. Incubate cells with the transfection complexes at 37°C and 5% CO₂ for 24 hours to allow for gene expression (*Refer to Note* ²⁸).

3.4.6 Detection of Proteins In Mammalian Cells Using Immunofluorescence—

Using the following protocol for immunofluorescence involves minimizing primary and secondary antibody solutions to conserve valuable antibodies. The separate permeabilization and blocking steps combined with thorough washing steps minimizes background to attain clear fluorescent microscopy images [7].

1. At 24 hours after transfection, remove culture medium and wash cells once with 1X DPBS. Remove DPBS with vacuum.

²⁴PLL-coated cover slips in 24-well plates are best when used immediately, but can be stored at 4°C for approximately two months.

²⁵Add DMEM slowly to the side of the well to avoid dislodging the cells from the cover slip.

²⁶It is crucial to use DMEM that does not contain any serum, proteins or antibiotics when diluting the plasmid DNA. The presence of antibiotics and serum in the media at this step will interfere with transfection complex formation and reduce transfection efficiency.

²⁷Diluting 2 µg of DNA in DMEM for a total volume of 100 µL can be used to transfect 4 wells of a 24-well plate. If only 2 wells of a 24-well plate need to be transfected, the total volume of diluted DNA, as well as the volumes for the transfection reagent and DMEM containing serum, should be reduced in half.

²⁸Changing media containing the transfection complexes is not necessary after 4–6 hours.

2. Fix cells with 3.7% PFA solution for 5–10 minutes.
3. Vacuum off fixative and wash twice with 1X PBS (5 min/wash).
4. Permeabilize with 0.4% Triton X-100 for up to 10 minutes (*Refer to Note* ²⁹).
5. Vacuum off detergent and wash three times with 1X PBS (5 min/wash).
6. Block cells with 5% BSA at room temperature for 45 minutes to 1 hour.
7. Vacuum off BSA and wash once with 1X PBS (5 min/wash).
8. Prepare a 50 μ L primary antibody solution as follows: Dilute primary antibody in 2% BSA and 1X PBS. Vortex well and spin down before use (*Refer to Note* ³⁰).
9. Cut a piece of parafilm and pipet the 50 μ L drop of primary antibody onto the parafilm. Using sharp-pointed forceps, carefully pick up the coverslip from the well and place the cover slip, face down, on top of the droplet (*Refer to Note* ³¹).
10. Incubate cells with the primary antibody for 1 hour at room temperature.
11. Using the forceps, carefully move the coverslip from the parafilm into the 24-well plate. Ensure that the cells on the coverslip (face down on the parafilm) are now faced up in the well.
12. Wash three times with 1X PBS (5 min/wash) on a horizontal shaker.
13. Prepare a 50 μ L secondary antibody solution as follows: Dilute secondary antibody in 2% BSA and 1X PBS. Vortex well and spin down before use.
14. Repeat above steps 9 and 10 to incubate cells with secondary antibody (*Refer to Note* ³²). Incubate at room temperature in the dark for 45 minutes.
15. Repeat step 11 to carefully move the coverslip from the parafilm to the 24-well plate.
16. Wash three times with 1X PBS (5 min/wash) on a horizontal shaker.
17. Mount coverslips (face down) onto glass microscope slides using a drop of mounting medium containing DAPI. Incubate coverslips on slides for 5 minutes at room temperature. Use a kimwipe to wipe off and absorb any excess mounting medium.
18. Seal the edge of the coverslips with small drops of clear nail polish.
19. Examine slides under a confocal microscope to visualize antibody-tagged intracellular proteins (*Refer to Note* ³³).

²⁹Permeabilizing more than 10 minutes may lead to over permeabilization of the cells, which can result in fragile cells and broken nuclei.

³⁰Primary antibody dilution will vary according to the manufacturer's recommendations. The 50 μ L antibody solution can be stored at 4°C for reuse. If expression of the GFP-fusion protein is low, incubating with a primary anti-GFP antibody will accentuate and increase the fluorescence of the fusion protein.

³¹The cover slip is oriented in this manner so that the cells on the surface of the coverslip will be in complete contact with the primary antibody solution.

³²Secondary antibody dilution will vary according to the manufacturer's recommendations.

³³Store immunostained coverslips on glass slides at 4°C and protect it from light.

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Basic BLAST

Choose a BLAST program to run.

<u>nucleotide blast</u>	Search a nucleotide database using a nucleotide query <i>Algorithms: blastn, megablast, discontinuous megablast</i>
<u>protein blast</u>	Search protein database using a protein query <i>Algorithms: blastp, psi-blast, phi-blast, delta-blast</i>
<u>blastx</u>	Search protein database using a translated nucleotide query
<u>tblastn</u>	Search translated nucleotide database using a protein query
<u>tblastx</u>	Search translated nucleotide database using a translated nucleotide query

Fig. 1.

The protein and nucleotide BLAST programs available using the NCBI database.

Table 1

Volumes and Concentrations of PCR Reaction Components

<i>Components</i>	<i>Volume</i>	<i>Final Concentration</i>
Water	to 25 μ L	
10X Buffer	2.5 μ L	1X
25 mM MgCl₂	2.0 μ L	1.5 – 2 mM
10 mM dNTPs	0.5 μ L	200 μ M
10 uM Forward Primer	1.0 μ L	0.1 μ M – 1 μ M
10 uM Reverse Primer	1.0 μ L	0.1 μ M – 1 μ M
<i>Taq</i> DNA Polymerase^a	0.25 μ L	0.05 units/ μ L
cDNA template	Variable	less than 10 μ g
Total Volume	25 μ L	

^aUse a commercial high-fidelity *Taq* DNA Polymerase that is capable of amplifying PCR products up to 5-kb in size and has the ability to add single 3' adenine overhangs at the end of the PCR product.

Table 2

PCR cycling conditions

<i>Cycle step</i>	<i>Temperature</i>	<i>Duration</i>	<i>Cycle</i>
Initial Denaturation	95°C	1–5 minutes	1
Denaturation	95°C	30 sec – 1 minute	25 – 35
Annealing	55°C	30– 45 seconds	
Extension	72°C	1 minute per 1 kb	
Final Extension^b	72°C	15 – 30 minutes	1
Hold	4°C	∞	

^bThe long final extension time is critical to ensure that the PCR products are full length and completely adenylated for subsequent TA cloning.

Table 3

Volumes and Concentrations for 10 µL PCR reaction

<i>Components</i>	<i>Volume</i>	<i>Final Concentration</i>
10X Buffer	1.0 µL	1X
25 mM MgCl₂	1.0 µL	1.5 – 2 mM
10 mM dNTPs	0.2 µL	200 µM
<i>Taq</i> DNA Polymerase^a	0.2 µL	0.05 units/µL
DNA	7.6 µL	Up to 10 µL
Total Volume	10 µL	