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Production of Myxoma Virus Gateway Entry and Expression Libraries and Validation of Viral Protein Expression

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Abstract

Invitrogen's Gateway technology is a recombination-based cloning method that allows for rapid transfer of numerous open reading frames (ORFs) into multiple plasmid vectors, making it useful for diverse high-throughput applications. Gateway technology has been utilized to create an ORF library for Myxoma virus (MYXV), a member of the *Poxviridae* family of DNA viruses. MYXV is the prototype virus for the genus *Leporipoxvirus*, and is pathogenic only in European rabbits. MYXV replicates exclusively in the host cell cytoplasm, and its genome encodes 171 ORFs. A number of these ORFs encode proteins that interfere with or modulate host defense mechanisms, particularly the inflammatory responses. Furthermore, MYXV is able to productively infect a variety of human cancer cell lines and is being developed as an oncolytic virus for treating human cancers. MYXV is therefore an excellent model for studying poxvirus biology, pathogenesis, and host tropism, and a good candidate for ORFeome development.

Keywords

Myxoma virus; Gateway recombination cloning; entry clone; expression clone; protein expression

INTRODUCTION

Myxoma virus (MYXV) is a member of the *Poxviridae* family, a family of large double-stranded DNA viruses, most possessing from 150 to 300 viral genes. It is rabbit-specific, and prototype for the genus *Leporipoxvirus*; the virus is harmless to all other species tested, including humans. MYXV replicates exclusively in the cytoplasm of the host cell, and its genome encodes 171 open reading frames, 12 of which are duplicated in the terminal inverted repeats (TIRs) (Cameron et al., 1999).

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Internet Resources

<http://www.poxvirus.org/index.asp>

Web site for the Poxvirus Bioinformatics Resource Center, which provides resources and data for the study of poxviruses.

<http://www.idtdna.com>

The Web site for Integrated DNA technologies has an oligo analyzer under SciTools that is useful in designing primers. It calculates melting temperature, G-C content, etc.

<http://www.orfeomecollaboration.org/html/index.shtml>

Web site for The ORFeome Collaboration, a source of sequence-validated human and mouse ORFs in Gateway entry vectors.

<http://www.invitrogen.com/gateway>

The Invitrogen Web site is an excellent source for complete information about Gateway cloning technology.

To date, over 100 poxvirus genomes, including MYXV, have been completely sequenced (www.poxvirus.org/viruses.asp). Technologies are emerging to utilize this genomic information, and construction of cloned open reading frame (ORF) libraries, or ORFeomes, provides a link between the available genome sequences and downstream “omics” technologies (Rual et al., 2004a). The large poxvirus genomes, however, dictate the need for a less cumbersome cloning strategy than conventional restriction endonuclease/ligase methods allow. Invitrogen’s Gateway recombination cloning system provides an efficient method to generate ORFeome libraries (Fig. 14A.2.1), relying on the conservative site-specific recombination mechanism of bacteriophage lambda (Landy, 1989) and eliminating the need for complicated restriction enzyme digests and ligation protocols. This technology allows for rapid and directional transfer of a large number of ORFs into vectors suitable for protein expression and functional analysis (Hartley et al., 2000; Walhout et al., 2000). The Gateway system is therefore a valuable tool for sequentially cloning individual ORFs from large genomes, like MYXV, into vector libraries useful in a variety of applications for studying the viral proteins on a more global scale.

A number of MYXV genes encode proteins that can interfere with or modulate host anti-viral defense mechanisms, such as the host inflammatory response, signifying the potential utility of some as anti-inflammatory or anti-immune therapy reagents in clinical settings (Lucas and McFadden, 2004). Furthermore, MYXV is able to selectively infect a variety of human cancer cell lines (Sypula et al., 2004), and is being developed as an oncolytic virus for treating a number of human cancers. MYXV is also an excellent model for studying poxvirus biology, pathogenesis, and host tropism, making ORFeome library production using the Gateway system a useful tool for studying more global proteomic aspects of the virus.

The following protocols describe construction of a MYXV ORFeome library in Gateway entry and expression vectors (Table 14A.2.1), and verification of GST-tagged viral protein expression. Basic Protocol 1 addresses two-step PCR amplification of target viral genes with recombination sites appended. Basic Protocol 2 describes the production of Gateway entry plasmid clones using the BP recombination reaction, and Basic Protocol 3 outlines generation of Gateway expression plasmid clones using the LR recombination reaction. Basic Protocol 4 describes the steps for verifying GST-fusion viral protein expression in reticulocyte lysates.

PCR AMPLIFICATION OF TARGET GENES

In this protocol, primers that contain a partial *attB* recombination sequence flanking gene-specific sequence are used to amplify the gene of interest from MYXV genomic DNA template in a first round of PCR. A second round of PCR using a set of universal primers is then performed using the first PCR product as template; this reaction introduces the remainder of the *attB* recombination sequence needed to produce a PCR product that can be used in the Gateway BP reaction to create an entry clone. This two-step scheme (Fig. 14A.2.2) is utilized to circumvent the potential for errors and reduce cost when synthesizing the long primers necessary for amplifying the entire recombination sequence in a single PCR reaction.

The general scheme for designing gene-specific primers and the sequence for the universal primers are shown in Figure 14A.2.3. When designing the gene-specific primers, start with 15 to 20 nucleotides of either 5' or 3' gene sequence and add bases until a melting temperature in the 55°C to 68°C range is achieved. Integrated DNA Technologies (<http://www.idtdna.com>) offers a free online oligo analyzer that calculates melting temperature and G-C content. It is important to create primers that have melting

temperatures that are very similar, not just within a set of forward and reverse primers for a single ORF, but for all primers for all ORFs, whenever possible. This makes it possible to simultaneously run multiple PCR reactions to amplify a number of ORFs across the MYXV genome.

Once an optimum number of gene-specific nucleotides for the primers have been achieved, the recombination sequence can be added, resulting in primers that are in-frame. The gene-specific portion of the 5' *attB1* (forward) primer must begin with ATG, preceded by the Kozak sequence (for improved protein expression in mammalian cell systems) and the recombination sequence. The gene-specific part of the 3' *attB2* (reverse) primer starts at the end of the stop codon. However, the sequence of the stop codon is changed to encode leucine to allow for creation of a C-terminal fusion protein from the chosen expression vector plasmid, since the MYXV expression library described here is cloned into a recipient expression vector with a C-terminal tag (pANT7-cGST). Selected first- and second-round PCR products are shown in Figure 14A.2.4.

Materials

Sterile ultra-pure pyrogen-free water
10 × *Pfu* Ultra amplification buffer (provided with Agilent *Pfu* Ultra High-Fidelity DNA polymerase)
10 mM 4dNTPs (see recipe)
100 ng/μl genomic DNA
2.5 units/μl *Pfu* Ultra High-Fidelity DNA Polymerase (Agilent Technologies, cat. no. 600382)
50 pmol/μl gene-specific forward primer
50 pmol/μl gene-specific reverse primer
6× agarose gel loading buffer (Fisher, cat. no. BP633-5)
1% Agarose-TAE gel (see recipe)
SYBR Safe DNA gel Stain (Invitrogen, cat. no. S33102)
1-kb DNA ladder (NEB, cat. no. N3232L) or other similar size marker
50 pmol/μl universal forward primer
50 pmol/μl universal reverse primer
Thin-walled 0.2-ml PCR tubes
Thermal cycler
0.5-ml microcentrifuge tubes
Horizontal gel apparatus
Power supply
Gel-doc system

Perform first-round PCR

- 1 Prepare a master mix for 50-μl PCR reactions. Determine the number of reactions and include two to three extras to have enough volume. Multiply the

volume for each component by that number, and set up as follows for each 50- μ l reaction:

- 40 μ l sterile ultra-pure pyrogen-free water
- 5 μ l 10 $\times$ *Pfu* Ultra amplification buffer (1 $\times$ final)
- 1 μ l 10 mM 4dNTPs (0.2 mM final)
- 1 μ l genomic DNA template (100 ng)
- 1 μ l *Pfu* DNA polymerase (2.5 U).

Pfu Ultra amplification buffer at a 1 $\times$ concentration contains Mg^{2+} at 2 mM.

- 2 Transfer 48 μ l of the master mix into each 0.2-ml PCR tube.
- 3 Add 1 μ l (50 pmol) each of the forward and reverse gene-specific primers to each reaction.
- 4 Mix well, and perform PCR as follows:

First step:	2 min	94°C	(initial denaturation)
35 cycles:	30 sec	94°C	(denaturation)
	30 sec	55° to 68°C	(annealing)
	1 to 6 min	72°C	(extension)
Final step:	10 min	72°C	(final extension).

Annealing temperature depends on the primers' melting temperatures, which should be similar for all primers. Anneal at a temperature that is close to the melting temperature. Extension time should be a minimum of 1 min; as a general rule, extend 1 min per kb of nucleotides to be amplified.

An additional PCR step, holding at 4°C, may be added after the final extension step if it is necessary to leave samples in the PCR machine for an extended period after all steps are complete.

- 5 To verify that a PCR fragment of the correct size has been amplified, remove 5 μ l of each product and transfer into a 0.5-ml microcentrifuge tube, and add 2 to 3 μ l of 6 $\times$ agarose gel loading buffer. Run the product on a 1% agarose-TAE gel containing SYBR Safe DNA gel stain at a 1:6,000-10,000 dilution. Run 2 μ l of a 1-kb DNA ladder in one lane to determine PCR products' size.
- 6 Visualize the bands on a gel doc system to determine PCR size and whether or not multiple bands are present. If there are multiple bands, refer to the Support Protocol for purifying the proper band prior to performing second-round PCR.

Perform second-round PCR

- 7 Prepare a master mix for 50- μ l PCR reactions. Determine the number of reactions and include two to three extras to have enough volume. Multiply the volume for each component by that number, and set up as follows for each 50- μ l reaction:
 - 40 μ l sterile ultra-pure pyrogen-free water
 - 5 μ l 10 $\times$ *Pfu* Ultra amplification buffer (1 $\times$ final)
 - 1 μ l 10 mM 4dNTPs (0.2 mM final)

1 µl universal forward primer (50 pmol)

1 µl universal reverse primer (50 pmol)

1 µl *Pfu* DNA polymerase (2.5 U).

- 8 Transfer 49 µl of the master mix into each 0.2-ml PCR tube.
- 9 Add 1 to 2 µl of the first-round PCR product to each reaction.
- 10 Mix well, and perform PCR as follows:

First step:	2 min	94°C	(initial denaturation)
35 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	1 to 6 min	72°C	(extension)
Final step:	10 min	72°C	(final extension).

Extension time should be a minimum of 1 min; as a general rule, extend 1 min per kb of nucleotides to be amplified.

An additional PCR step, holding at 4°C, may be added after the final extension step if it is necessary to leave samples in the PCR machine for an extended period after all steps are complete.

- 11 To verify that a PCR fragment of the correct size has been amplified, transfer 5 µl of each product into a 0.5-ml microcentrifuge tube, and add 2 to 3 µl of 6× agarose gel loading buffer. Run the product on a 1% agarose-TAE gel containing SYBR Safe DNA gel stain at a 1:6,000 to 10,000 dilution. Run 2 µl of a 1-kb DNA ladder in one lane to determine PCR products' size.
- 12 Visualize the bands on a gel doc system to determine PCR size and whether or not multiple bands are present. If there are multiple bands, refer to the Support Protocol for purifying the proper band prior to performing the BP reaction in Basic Protocol 2.

PCR products may be stored up to 6 months at −20°C

- 13 Proceed to PCR product purification, if necessary (see Support Protocol), or proceed to Basic Protocol 2 to create an entry clone.

GEL PURIFY PCR PRODUCT

Occasionally a PCR reaction will generate additional PCR fragments that are smaller than the expected size product. To avoid incorporating these sequences into a vector through recombination, the correct size product can be purified from the PCR reaction by agarose gel electrophoresis and isolating the band that corresponds to the appropriate PCR product using a Qiagen Qiaquick Gel Extraction kit.

NOTE: All steps are performed at room temperature and all solutions in the kit are stored at room temperature.

CAUTION: When visualizing PCR products on a gel using a UV light box, wear a face shield for protection against UV radiation.

Materials

1% Agarose-TAE gel (see recipe)

SYBR Safe DNA gel stain

6× agarose gel loading buffer
PCR sample(s) from Basic Protocol 1
1-kb DNA ladder
QIAquickGel Extraction kit (Qiagen cat. no. 28706) containing:

Buffer QG

Buffer PE

QIAquick spin columns

2-ml collection tubes

3 M sodium acetate, pH 5.0 (see recipe)

Isopropanol

Sterile ultra-pure pyrogen-free water (pH 7.0 to 8.5)

Horizontal mini-gel apparatus

Power supply

2-ml microcentrifuge tubes

Balance

Low-frequency UV light box

UV face shield

Scalpel with no. 11 or similar blade

Heat block or water bath set at 50°C

Vortex mixer

Microcentrifuge

Additional reagents and equipment for determining the concentration of the purified PCR product (Gallagher and Desjardins, 2006)

- 1 Pour a 1% agarose-TAE gel containing SYBR Safe DNA gel stain at a 1:6,000 to 10,000 dilution. Add 1/6 volume of 6× agarose gel loading buffer to the PCR sample and load into two to three adjacent wells, depending on the comb used. Run the gel.

Also run a DNA size marker (e.g., 1-kb DNA ladder), but to avoid contaminating PCR samples, skip a well between the marker and samples. If multiple PCR products are being purified, skip a well between the different samples.

While the gel is running, weigh a 2-ml microcentrifuge tube for each sample.

- 2 After the bands have separated, visualize the PCR products on a UV light box on the low frequency setting. Cut out the desired band using a scalpel with a clean blade, and place it in a 2-ml microcentrifuge tube. Re-weigh the tube that now contains the gel slice and subtract the weight of the empty tube to determine the weight of the band.

Minimize the amount of time the PCR product is exposed to UV light to prevent DNA damage.

Keep the size of the slice as small as possible by excising extra agarose.

- 3 Add 3 gel volumes of Buffer QG (e.g., 300 μ l for every 100 mg of gel) from the QIAquick Gel Extraction kit to the tube containing the gel slice. Incubate for 10 min at 50°C, vortexing periodically to assist in dissolving the agarose, which must be completely solubilized.
- 4 After the gel is dissolved, the color of the solution should be yellow. If it is orange or purple, add 10 μ l of 3 M sodium acetate, pH 5.0.

Although the kit recommends adding sodium acetate if the color warrants it, the authors routinely add 10 μ l to ensure that the pH is within the appropriate range.
- 5 Add 1 gel volume of isopropanol (e.g., 100 μ l for every 100 mg of gel) and mix.
- 6 Place a QIAquick spin column in a 2-ml collection tube provided with the kit. Apply the sample to the column and centrifuge 1 min at 13,000 rpm, room temperature, in a microcentrifuge to bind DNA. If the sample volume is greater than the column reservoir (800 μ l maximum), discard the flow-through and reload the same column with more sample and repeat the centrifugation.

Do not exceed 400 mg of gel volume per QIAquick column. Divide samples that are larger than this into two or more columns.
- 7 Discard the flow-through and place the column back in the same collection tube. Wash the column by applying 750 μ l of Buffer PE to the column and centrifuging 1 min at 13,000 rpm, room temperature, in a microcentrifuge.
- 8 Discard the flow-through and centrifuge for an additional 1 min at 13,000 rpm, room temperature, to remove all traces of ethanol remaining from the Buffer PE.
- 9 Place the QIAquick column in a fresh sterile microcentrifuge tube and elute the DNA by adding 30 μ l of sterile ultra-pure, pyrogen-free water (pH 7.0 to 8.5) directly to the center of the column membrane. Let the column stand for 1 min at room temperature, then centrifuge for 1 min at 13,000 rpm, room temperature. Discard the column after centrifugation.
- 10 Confirm recovery, size, and purity of the purified PCR product by running 5 μ l of the PCR sample containing 1/6 volume of 6 $\times$ agarose gel loading buffer on a 1% agarose-TAE gel containing SYBR Safe DNA gel stain at a 1:6,000 to 10,000 dilution. Run a DNA size marker in an adjacent lane.
- 11 Determine the concentration of the purified PCR product (Gallagher and Desjardins, 2006).

BP CLONASE REACTION TO CREATE ENTRY CLONES

An entry clone library is created by BP reactions in which the individual *attB*-PCR fragments from the genes of interest (created in Basic Protocol 1) are transferred into an appropriate entry vector containing *attP* sequence, hence the term BP recombination reaction (see Fig. 14A.2.1). In the protocol described here, the PCR fragments are transferred into the *attP* sequence-containing pDONR222 entry vector (Invitrogen), or in some cases, pDONR221. Chemically competent One Shot Mach1 cells (Invitrogen) are then transformed with a portion of the BP reaction, the cells are plated, and colonies are PCR-screened to identify a positive clone. Positive clones are then sequence-verified with M13 forward and reverse primers.

NOTE: pDONR221 entry vector, also from Invitrogen, was used in constructing some of the early entry clones in the MYXV entry clone library. However, pDONR222 was used in the majority of cases; it differs from pDONR221 only in that it has three BsrG1 restriction sites. Both pDONR221 and pDONR222 contain M13 forward and reverse priming sites for sequencing entry clones with M13 forward and reverse primers.

NOTE: Although the authors use only One Shot Mach1 competent cells in the transformation following the BP recombination reaction, other *E. coli* strains may be used. However, One Shot Mach1 competent cells have the added benefit of mutations that aid in PCR product applications, minimizing nonspecific recombination, and promoting faster doubling time.

Materials

Sterile ultra-pure, pyrogen-free water
150 ng/μl pDONR222 entry vector (Invitrogen)
10 to 20 ng/μl second-round PCR product (see Basic Protocol 1)
Gateway BP Clonase II enzyme mix (Invitrogen, cat. no. 11789-100)
2 μg/μl proteinase K (provided with BP Clonase II)
One Shot Mach1 T1 Phage-Resistant Chemically Competent *E. coli* (Invitrogen, cat. no. C8620-03 or other *E. coli* competent cell, e.g., Seidman et al., 1997)
SOC medium (see recipe)
LB agar plates supplemented with 50 μg/ml kanamycin (see recipe)
5 U/μl *Taq* DNA polymerase (NEB, cat. no. M0273X)
10 × standard *Taq* reaction buffer (provided with *Taq* DNA polymerase)
50 mM MgCl₂
10 mM 4dNTPs (see recipe)
50 pmol/μl Universal forward primer
50 pmol/μl Universal reverse primer
6× agarose gel loading buffer
1% agarose-TAE gel (see recipe)
SYBR Safe DNA gel stain
1-kb DNA ladder
LB medium supplemented with 50 μg/ml kanamycin (see recipe)
100% glycerol, sterile
Plasmid DNA isolation kit (e.g., Qiagen)
M13 forward and reverse primers (Invitrogen, cat. nos. N520-02 and N530-02, respectively)
0.5- and 1.5-ml sterile microcentrifuge tubes
37°C shaking incubator
Ice bath

42°C water bath
37°C bacterial incubator
0.2-ml thin-wall PCR tubes
Thermal cycler

Prepare the BP reaction

- 1 Prepare reactions in 0.5-ml microcentrifuge tubes as follows (per reaction):
 - 2 µl sterile ultra-pure, pyrogen-free water (for a 10 µl final volume)
 - 1 µl (150 ng) entry vector (e.g., pDONR222, Invitrogen)
 - 5 µl PCR product (50 to 100 ng)
 - 2 µl BP Clonase II enzyme mix.

For multiple samples, a master mix can be prepared containing all components except the PCR product, if the PCR products' concentrations are all adjusted to the same concentration (10 to 20 ng/µl). In this case, make enough master mix for the number of samples plus two or three extras to compensate for loss through pipetting. Add 5 µl of the master mix to each tube, followed by the addition of 5 µl of the PCR product. Alternatively, the authors generally estimate the concentration of each PCR product by visualizing on a 1% agarose-TAE gel and comparing to a size/molecular weight standard (e.g., NEB 1-kb ladder, cat. no. N3232L), and add 0.5 to 1 µl of the PCR product to each BP reaction. The volume of water added is adjusted accordingly.

Include a negative control in which no PCR product is added to the reaction. Use sterile ultra-pure, pyrogen-free water in place of the PCR product.

Although Invitrogen's instructions call for a 10-µl reaction, it is possible to cut the reaction volume in half by reducing all components proportionately. If preparing a library, this reduction in reagent use can provide substantial savings.

- 2 Mix gently; incubate 1 to 2 hr at room temperature, or 25°C.

Although Invitrogen recommends incubation at room temperature, Park and LaBaer (2006) report that incubation at 25°C results in a more robust reaction.
- 3 Add 2 µl of 2 µg/µl proteinase K and incubate 10 min at 37°C.

Transform One Shot Mach1 T1 phage-resistant chemically competent *E. coli*

- 4 Thaw One Shot Mach1 T1 phage-resistant chemically competent *E. coli* on ice, then add 10 µl of the competent cells to a chilled 1.5-ml microcentrifuge tube.

If using competent cells prepared in-lab, use 25 µl for each transformation reaction. If using One Shot Mach1 competent *E. coli* purchased from Invitrogen, the authors have found that 10 µl of these cells is sufficient.

- 5 Add 2.5 µl of the BP reaction mix to the competent cells, mix gently, and incubate on ice for 30 min.
- 6 Heat shock at 42°C for 30 sec and then quickly chill on ice for 2 min.
- 7 Add 250 µl SOC medium to the tube and incubate for 1 hr at 37°C in a shaking incubator.
- 8 Plate 100 µl of transformation mix on an LB plate containing 50 µg/ml kanamycin.
- 9 Incubate the plate overnight at 37°C.

PCR screen for transformants

- 10 Prepare a PCR master mix for the number of colonies to be screened plus enough for two to four extra samples, multiplying the volume of each component by that number. The master mix contains the following components per 20-µl reaction:
 - 16.5 µl sterile ultra-pure, pyrogen-free water
 - 2 µl 10× *Taq* polymerase standard reaction buffer (1× final)
 - 0.6 µl 50 mM MgCl₂ (1.5 mM final)
 - 0.4 µl 10 mM 4dNTPs (0.2 mM final)
 - 0.2 µl universal primer 1 (10 pmol)
 - 0.2 µl universal primer 2 (10 pmol)
 - 0.1 µl *Taq* DNA polymerase (0.5 U).
- 11 Transfer 20 µl of the PCR master mix into 0.2-ml thin-wall PCR tubes.
- 12 Pick two to four colonies from each plate with a sterile pipet tip and prepare a replica LB plate containing 50 µg/ml kanamycin by touching each tip to a labeled spot on the plate. Place the same tip containing residual bacteria into a PCR tube containing PCR reaction mix, and pipet up and down several times to release the bacteria into the PCR mix.

Place the replica plate in a 37°C incubator. Replica colonies will be ready to pick and inoculate an overnight culture in 6 to 8 hr.
- 13 Use the following amplification cycles to perform PCR:

First step:	10 min	94°C	(to break up bacterial cells/initial denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	1 to 6 min	72°C	(extension)
Final step:	10 min	72°C	(final extension).

An additional PCR step, holding at 4°C, may be added after the final extension step if it is necessary to leave samples in the PCR machine for an extended period after all steps are complete.
- 14 Add 4 µl of 6× gel loading buffer to each PCR sample and run 10 µl on a 1% agarose-TAE gel containing SYBR Safe DNA gel stain at a 1:6,000 to 1:10,000 dilution. Run 2 µl of a 1-kb DNA ladder in one lane to determine the size of the PCR products.

Identify, prep, and sequence positive clones

- 15 Once a PCR product of the correct size is identified, pick the corresponding colony from the replica plate once it has grown (6 to 8 hr is usually enough, or overnight) and inoculate 5 ml LB containing 50 µg/ml kanamycin.
- 16 Incubate overnight at 37°C in a shaking incubator.
- 17 Remove a small volume (400 to 500 µl) of the culture and mix well with an equal volume of sterile 100% glycerol and store at –80°C.
Stored at –80°C, glycerol stocks should remain viable for 1 to 2 years.
- 18 Isolate plasmid DNA from the remaining culture using a commercially available miniprep kit (e.g., Qiagen).
- 19 Sequence the entry clone using M13 forward and reverse primers.
These primers will cover the ORF and the recombination (*attP*) regions, which is critical to identifying a good clone. Align the sequence against a reference sequence using BLAST (<http://www.ncbi.nlm.nih.gov/blast>) or other sequence alignment tool.
For larger ORFs for which complete sequence cannot be obtained with M13 forward and reverse primers, internal gene-specific primers must be designed for sequencing internal portions of the gene.
Because the recombination cloning process is not infallible, some PCR-screened colonies will not produce the correct-sized PCR product, or, occasionally, any product at all; in this case, repeat PCR screening of additional colonies. When more than one correct colony is identified by PCR screening, additional correct ones may be cultured for prepping and sequencing in case one has mutations. Alternatively, save the replica plate, and if only a single colony is prepped and found to have mutations upon sequencing, go back to the replica plate and prep a second colony that was found to be positive in the original PCR screening. The colonies on the replica plate will remain viable for a week or more, or can be transferred to a fresh plate to re-culture. Park and LaBaer (2006) generally pick four clones per gene to sequence, but identify and choose only a single fully sequenced and correct isolate as their representative clone.
- 20 Once a clone has been identified as having the correct sequence compared to a reference sequence, archive the clone by documenting the clone identifier (such as colony number) and maintaining a viable glycerol stock, and use it to transfer the ORF into an expression vector.

LR CLONASE REACTION TO CREATE EXPRESSION CLONES

The LR reaction is performed to generate an expression clone by recombination between the *attL*-containing entry clone and the *attR*-containing Gateway expression vector (as shown in Fig. 14A.2.1). ORFs cloned into pANT7-cGST [Harvard Institute of Proteinomics (HIP)], the expression vector used here, are under the T7 promoter for use in the TNT-Coupled Reticulocyte Lysate System (Promega) for protein expression verification; however, there are many expression vectors commercially available for a variety of applications. In choosing an expression vector, it is critical to use one that has a different antibiotic selection marker than the entry clone (which is kanamycin resistant); e.g., pANT7-cGST is ampicillin resistant.

Materials

Sterile ultra-pure, pyrogen-free water

150 ng/μl entry clone

150 ng/μl pANT7-cGST entry vector (HIP)

Gateway LR Clonase II Enzyme mix (Invitrogen, cat. no. 11791-100)

2 μg/μl proteinase K (provided with LR Clonase II)

E. coli competent cells (e.g., DH5α; Invitrogen, cat. no. 18258-012, or DH5α competent cells prepared in-lab)

SOC medium (see recipe)

LB-agar plates supplemented with 100 μg/ml ampicillin (see recipe)

5 U/μl *Taq* DNA polymerase

10 × standard *Taq* reaction buffer

50 mM MgCl₂

10 mM 4dNTPs (see recipe)

50 pmol/μl pANT7 forward screening primer (5' GTAAAACGACGGCCAG 3')

50 pmol/μl pANT7 reverse screening primer (5' GCCCGGATATAGTTCCTCCTTTCA 3')

6 × agarose gel loading buffer

1% agarose-TAE gel (see recipe)

1× TAE (see recipe)

SYBR Safe DNA gel Stain

1-kb DNA ladder

LB medium supplemented with 100 μg/ml ampicillin (see recipe)

100% glycerol, sterile

Plasmid DNA isolation kit (e.g., Qiagen)

0.5-ml sterile microcentrifuge tubes

25 °C incubator, optional

37°C shaking incubator

Ice bath

42 °C water bath

37°C bacterial incubator

Pipet tips, sterile

0.2 ml thin-wall PCR tubes

Thermal cycler

Additional reagents and equipment for growing *E. coli* DH5α competent cells (e.g., Seidman et al., 1997)

Prepare the LR reaction

- 1 Prepare reactions in 0.5-ml microcentrifuge tubes as follows (per reaction):

6 μ l sterile ultra-pure, pyrogen-free water to 10 μ l final volume

1 μ l (150 ng) entry clone

1 μ l (150 ng) pANT7-cGST expression vector

2 μ l LR Clonase II enzyme mix.

For multiple samples, a master mix can be prepared containing all components except the entry clone. Make enough master mix for the number of samples plus two or three extras to compensate for loss through pipetting. Add 9 μ l of the master mix to each tube, followed by the addition of 1 μ l of the entry clone.

Include a negative control in which no entry clone is added to the reaction. Use 1 μ l sterile ultra-pure, pyrogen-free water in place of the entry clone.

Although Invitrogen's instructions call for a 10- μ l reaction, it is possible to cut the reaction volume in half by reducing all components proportionately. If preparing a library, this reduction in reagent use can provide substantial savings.

- 2 Mix gently; incubate 1 to 2 hr at room temperature, or 25°C.

Although Invitrogen suggests incubation at room temperature, Park and LaBaer (2006) recommend incubation at a controlled 25°C.

- 3 Add 2 μ l of 2 μ g/ μ l proteinase K and incubate 10 min at 37°C.

Transform *E. coli* DH5 α Competent Cells

- 4 Thaw DH5 α competent cells on ice, and add 25 μ l of the competent cells to a chilled 1.5-ml microcentrifuge tube.

Use 10 μ l of competent DH5 α cells if using those purchased from Invitrogen.

- 5 Add 2 μ l of the LR reaction to the competent cells, mix gently, and incubate on ice for 30 min.

- 6 Heat shock at 42°C for 30 sec, and then quickly chill on ice for 2 min.

- 7 Add 250 μ l SOC medium to the tube and incubate for 1 hr at 37°C in a shaking incubator.

- 8 Plate 100 μ l of the transformation mix on an LB plate containing 100 μ g/ml ampicillin.

- 9 Incubate the plate overnight at 37°C.

PCR screen for transformants

- 10 Make a PCR master mix for the number of colonies to be screened plus enough for two to four extra samples, multiplying the volume of each component by that number. The master mix contains the following components per 20- μ l reaction:

16.5 μ l sterile ultra-pure, pyrogen-free water

2 μ l 10 $\times$ *Taq* polymerase standard reaction buffer (1 $\times$ final)

0.6 µl 50 mM MgCl₂ (1.5 mM final)
 0.4 µl 4dNTPs (0.2 mM final)
 0.2 µl pANT7 forward screening primer (10 pmol)
 0.2 µl pANT7 reverse screening primer (10 pmol)
 0.1 µl *Taq* DNA polymerase (0.5 U).

- 11 Transfer 20 µl of the PCR master mix into 0.2-ml thin-wall PCR tubes.
- 12 Pick two to four colonies from each plate with a sterile pipet tip and prepare a replica LB plate containing 100 µg/ml ampicillin by touching each tip to a labeled spot on the plate. Place the same tip containing residual bacteria into a PCR tube containing PCR reaction mix, and pipet up and down several times to release the bacteria into the PCR mix.

Place the replica plate in a 37°C incubator. Replica colonies will be ready to pick and inoculate an overnight culture in 6 to 8 hr.

- 13 Use the following amplification cycles to perform PCR:

First step:	10 min	94°C	(to break up bacterial cells/initial denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	50°C	(annealing)
	1 to 6 min	72°C	(extension)
Final step:	10 min	72°C	(final extension).

An additional PCR step, holding at 4°C, may be added after the final extension step if it is necessary to leave samples in the PCR machine for an extended period after all steps are complete.

- 14 Add 4 µl of 6× gel loading buffer to each PCR sample and run 10 µl on a 1% agarose-TAE gel containing SYBR Safe DNA gel stain at a 1:6,000 to 10,000 dilution. Run 2 µl of a 1-kb DNA ladder in one lane to determine the size of the PCR products.

Identify and prep positive clones

- 15 Once a PCR product of the correct size is identified, pick the corresponding colony from the replica plate once it has grown (6 to 8 hours is usually enough, or overnight) and inoculate 5 ml LB containing 100 µg/ml ampicillin.
- 16 Incubate overnight at 37°C in a shaking incubator.
- 17 Remove a small volume (400 to 500 µl) of the culture and mix well with an equal volume of sterile 100% glycerol and store at −80°C.

Stored at −80°C, glycerol stocks should remain viable for 1 to 2 years.
- 18 Isolate plasmid DNA from the remaining culture using a commercially available DNA prep kit (e.g., Qiagen).

There is no need to sequence the expression clone. Although nucleotide changes or deletions may be introduced by PCR, thus necessitating sequencing of the entry clone, transferring the ORF from the entry clone into the expression vector is straightforward. The expression clone is confirmed by protein expression, described in Basic Protocol 4.

VERIFY PROTEIN EXPRESSION BY TNT AND IMMUNOBLOT ANALYSIS

Once an expression plasmid clone has been created, it is important to confirm that the correct size protein is expressed. In the MYXV ORFeome library described here, the viral proteins are expressed as C-terminal GST-fusions, and can be quickly and easily verified using the TNT-Coupled Reticulocyte Lysate System (Promega). This kit requires minimal set up time and a 90 min reaction. A portion of that reaction can then be analyzed directly by SDS-PAGE, immunoblotting, and ECL to verify protein expression (Figs. 14A.2.5 and 14A.2.6).

CAUTION: Acrylamide (a neurotoxin) and other gel components for SDS-PAGE are highly toxic—use extreme caution when working with these chemicals. Wear appropriate PPE, such as laboratory coat and gloves, and dispose of all pipets and other waste according to your institution's hazardous waste management guidelines.

Materials

Sterile ultra-pure pyrogen-free water

TNT Coupled Reticulocyte Lysate System (Promega, cat. no. L4610) containing:

- TNT rabbit reticulocyte lysate
- TNT reaction buffer
- Amino acids minus methionine
- Amino acids minus leucine
- TNT T7 RNA polymerase

RNasin ribonuclease inhibitor (Promega, cat. no. N2511)

Expression clone plasmid prep at a concentration of 500 ng/μl (see Basic Protocol 3)

SDS-PAGE gel (8 to 12% acrylamide, depending on size of proteins; e.g., Gallagher, 2006, or can be purchased from Thermo Scientific, Invitrogen, or BioRad)

2× protein gel loading buffer (Fisher, cat. no. BP637-5)

2-mercaptoethanol

SDS-PAGE running buffer (see recipe)

SeeBlue Plus2 Pre-Stained Standard (Invitrogen, cat. no. LC5925)

Transfer buffer (see recipe)

Methanol

TBS-Tween (TBS-T; see recipe)

Blocking buffer (see recipe)

Primary antibody against tag (rabbit anti-GST polyclonal antibody, Fisher Scientific, cat. no. RB-9410-P)

Secondary HRP-tagged antibody (goat anti-rabbit-HRP: Jackson Immuno Research Labs, cat. no. 111-035-144 or Santa Cruz Biotechnology, cat. no. SC-2004)

Immobilon Western Chemiluminescent HRP substrate (Millipore, cat. no. WBKLS0500)

1000-, 200-, and 10-μl pipet tips, sterile

0.5- and 1.5-ml microcentrifuge tubes, sterile

Incubator or water bath set at 30°C

Water bath or heat block set at 90°C

Vertical Mini-gel apparatus (e.g., BioRad Mini-PROTEAN Tetra Cell, cat. no. 165-8004)

Power supply (e.g., BioRad, cat. no. 164-5052, which can supply constant voltage, current, or power; not all power supplies are compatible with the semi-dry blotter used here)

Several small containers for washing blots (e.g., 4-in. × 6-in. Rubbermaid container with lid, or pipet tip box lids)

Shaker platform (one at room temperature and one at 4°C)

Semi-dry blotter (e.g., Owl Separation Systems model HEP-1, Fisher Scientific)

Whatman 3MM paper (Fisher, cat. no. 05-714-5) cut to gel size

Hybond PVDF membrane (GE Healthcare Bio Sciences, cat. no. RPN303F) cut to gel size

Forceps

Seal-a-meal bags or other heat-sealable bags (Fisher, cat. no. 01-812-25A)

Seal-a-meal or other heat sealer apparatus

Vortex mixer

Sheet protector or Saran wrap

Light-proof cassette or folder for x-ray film

X-ray film

X-ray film developer

NOTE: Promega's TNT Coupled Reticulocyte Lysate System is a cell-free system that utilizes rabbit reticulocyte lysate in a one-step coupled transcription/translation reaction for protein expression.

NOTE: The DNA template used in the TNT reactions must be ethanol-, calcium-, salt-, and RNase-free. DNA prepped using Qiagen's QIAprep Spin Miniprep kit, which the authors use, meets these standards. Promega recommends its Wizard Plus Minipreps DNA Purification, but indicates that standard alkaline lysate preps according to the Sambrook and Russell (2001) method will also work.

NOTE: The package insert with this kit outlines the set up for 50 µl reactions; however, it is sufficient to set up reactions of 12.5 µl, and recommended, as the kits are costly. Each component of the reaction is reduced proportionately when assembling a reaction.

NOTE: The TNT kit is stored at -80°C, and upon removal from the freezer, the RNA polymerase should be placed on ice immediately. The reticulocyte lysate should be thawed rapidly by hand, and then transferred to ice. Keep all components of the kit on ice at all times.

NOTE: Wear gloves to reduce the risk of contaminating reactions with RNases, and sterilize all tips and tubes used in setting up the reactions.

NOTE: TNT kits can be purchased with T7, SP6, or T3 RNA polymerase. The MYXV ORF expression library reported here is cloned into the pANT7-cGST expression vector, which has a T7 promoter. Use only the T7 RNA polymerase provided with the kit.

Prepare the TNT reaction

- 1 Set up a master mix for multiple 12.5 μ l TNT reactions. Multiply individual components times the number of reactions plus extras to have enough for all reactions. Per reaction, add the following into a sterile 1.5-ml microcentrifuge tube and mix gently:
 - 4.5 μ l sterile ultra-pure, pyrogen-free water (to 12.5 μ l final volume)
 - 6.25 μ l TNT rabbit reticulocyte lysate
 - 0.5 μ l TNT reaction buffer
 - 0.25 μ l RNasin ribonuclease inhibitor
 - 0.125 μ l amino acids minus methionine
 - 0.125 μ l amino acids minus leucine
 - 0.25 μ l TNT T7 RNA polymerase.
- 2 Transfer 12 μ l of the mastermix into sterile 0.5-ml microcentrifuge tubes on ice.
- 3 Add 250 ng (0.5 μ l) of expression plasmid DNA to each tube.
- 4 Incubate 90 min at 30°C.
 - Samples may be stored ~1 to 2 weeks at -20°C, or analyzed immediately.

Immunoblot to analyze the expressed protein

- 5 Pour an SDS-PAGE mini gel(s).
 - Acrylamide-Bis percentage depends on the predicted size of the expressed proteins.
- 6 In a sterile microcentrifuge tube, place 3 to 4 μ l of each TNT reaction, and add an equal volume of 2 $\times$ protein gel loading buffer. Mix gently. Alternatively, 3 μ l of TNT reaction may be added to 12 to 14 μ l of 1 $\times$ protein gel loading buffer.
 - The protein gel loading buffer listed in the reagents for this protocol requires that 2-mercaptoethanol be added so that the final concentration of 2-mercaptoethanol, when added to the sample, is 1% (v/v).
- 7 Heat the samples for 2 min at 90°C, and then cool to room temperature. Briefly centrifuge the tubes 5 to 10 sec at 13,000 rpm, room temperature, in a microcentrifuge to collect any liquid that may accumulate in the cap due to condensation.
- 8 Load the gel, including one lane containing 15 μ l SeeBlue Plus2 pre-stained standard, and run.
- 9 Disassemble the apparatus when the run is complete, and remove the gel carefully into a small container containing ~25 ml transfer buffer. Wash for 10 min at room temperature on a gently rotating shaker platform.
- 10 Set up the semi-dry blotter apparatus with these components in the following sequence, wetting each as described below:

Two pieces of 3MM paper cut to gel size (bottom layer)

Gel

PVDF membrane cut to gel size

Two pieces of 3MM paper cut to gel size (top layer)

When placing the various components for the blot onto the apparatus, set up a small container of transfer buffer to wet each component. Wearing gloves and using forceps, dip the first piece of 3MM paper to fully wet it, and place it on the surface of the blotting apparatus, starting by setting one edge of the wet paper onto the surface, and rolling it down slowly and evenly to prevent air bubbles from gathering under the paper. A small tube or other item can be used to gently roll across the wet paper and squeeze out any remaining bubbles. Wet the second piece of 3MM paper and layer it on top of the first as before, again, removing any air bubbles.

Carefully remove the gel from the container of transfer buffer and place it on top of the two pieces of 3MM paper. If it is a higher percentage gel (10% to 12%), it can be picked up at either end with gloved hands, and placed on the 3MM paper lowering the center of the gel first, and then gently rolling out any air bubbles. If, however, the gel is too fragile to lift out of the transfer buffer by hand, the second piece of 3MM paper, rather than placing directly onto the first, can be slipped under the gel while still in the container of transfer buffer, and both the gel and second 3MM piece can be placed onto the first piece of 3MM paper as a single unit.

The PVDF membrane must first be wetted in methanol. It comes from the manufacturer sandwiched between two pieces of paper, and because it is important to avoid touching the membrane, handle it with forceps. Peel back the corners of the paper, and grasp the membrane on its edge with forceps. Free the membrane from the paper, and then dip it in a small container containing 15 to 20 ml methanol to wet all surfaces. Rinse off the methanol by dipping the membrane in transfer buffer. Using forceps, place the membrane on top of the gel, and roll out any air bubbles as before.

To prevent the PVDF membrane from drying, quickly wet another piece of 3MM paper and layer on top of the PVDF membrane, and layer a second piece of wetted 3MM paper on top of that, rolling out air bubbles at each step.

- 11 Place the lid of the blotting apparatus securely onto the base using care not to disturb the blot layers, and tighten gently.
- 12 Transfer at 10 to 12 V (200 to 300 mAmps) for 45 min at room temperature.

This setting and time work well for transferring proteins from ~30 to 100 kDa; adjustments in gel percentage and transfer time may need to be made for smaller or larger proteins.
- 13 Disassemble the blotting apparatus and carefully pull apart the layers of the blot with forceps.

Mark the side of the PVDF membrane that is touching the gel to designate the protein side. A small mark can be made on a corner with a Sharpie.

- 14 Place the PVDF membrane in a small container of TBS-T and wash 15 min at room temperature on a gently rotating shaker platform.
- 15 Pour off the TBS-T and block the membrane by adding ~20 to 25 ml of blocking buffer. Shake gently for 1 hr at room temperature on a shaker platform.

The remainder of the blocking buffer may be stored overnight at 4°C.

- 16 Pour off the blocking buffer, and rinse the blot briefly with TBS-T. Pour off the rinse, and wash three times, each time with ~25 ml TBS-T, shaking gently at room temperature for 5 min per wash.
- 17 Dilute anti-GST antibody 1:1,000 in 3 to 5 ml blocking buffer.

Fisher's rabbit-anti-GST antibody is sold at a concentration of 200 µg/ml. The manufacturer recommends using the antibody at a final concentration of 1 µg/ml (1:200 dilution); however, even at a dilution of 1:1,000, a strong signal is achieved, as the GST-tagged MYXV ORFs generally express well.

- 18 Using forceps, carefully place the PVDF membrane in a small Seal-a-meal bag, and pipet the anti-GST antibody over the surface of the protein side of the membrane.
- 19 Remove air bubbles and seal the pouch. Incubate overnight at 4°C with gentle rotation to maintain a wash of antibody across the surface of the membrane.
- 20 Remove the membrane from the pouch and wash three times in TBS-T as in step 16.
- 21 Dilute the secondary antibody (goat anti-rabbit-HRP) 1:10,000 in 20 ml blocking buffer remaining from step 15 above.

The goat anti-rabbit-HRP antibody from Jackson Immuno Research Labs is sold at a concentration of 0.8 mg/ml. The manufacturer recommends a dilution of 1:5,000 to 1:100,000 for immunoblotting applications. The antibody from Santa Cruz is sold at 0.4 mg/ml, and the manufacturer recommends a dilution of 1:500 to 1:10,000.

- 22 Pour off the third TBS-T wash from the membrane and add the diluted secondary antibody. Incubate 1 hr at room temperature with gentle shaking.
- 23 Pour off the antibody solution and wash the membrane three times as in step 16.
- 24 Remove the membrane from the wash, drain off excess liquid, and place in a clean container, protein side up.

Proceed immediately to ECL. Do not allow the blot to dry.

ECL to visualize expressed protein

- 25 Pipet an equal volume of the two Immobilon Western Chemiluminescent HRP substrate kit solutions (peroxide solution and Luminol reagent) into a tube and vortex briefly to mix.

A total of 750 µl to 1 ml of each solution for a final volume of 1.5 to 2 ml is sufficient to completely cover the surface of the membrane for a

single mini gel. Mix these solutions just before use; although they are light sensitive, there is no need to do this step in the dark as long as the solutions are used immediately after mixing.

- 26** Pipet the mixed HRP substrate solutions onto the surface of the membrane.

The entire surface of the membrane should be continuously covered by the solution.

- 27** Incubate 5 min at room temperature.

Pipet any solution that may spill off of the membrane back onto its surface as necessary.

This step does not need to be done in the dark.

- 28** Pick up the membrane with forceps, and drain excess liquid. Place the blot in a sheet protector or wrap in Saran wrap, smoothing out all wrinkles.

- 29** Immediately place the covered membrane in a light-proof cassette or folder, and in a dark room, place a piece of X-ray film over the membrane.

A very strong signal is typically generated with the anti-GST antibody used here. It is usually only necessary to briefly touch the film to the membrane to obtain a suitable exposure (e.g., 1 to 2 sec). However, the HRP substrate will gradually diminish in signal after 5 to 10 min, so it is important to obtain good exposures as quickly as possible.

- 30** Develop the film and obtain longer (or shorter) exposures as necessary.

Generally, four major bands from the SeeBlue Plus2 pre-stained marker will be detectable on the film, allowing for positioning it on the film and determining the size of the sample proteins and verifying that they are correct.

REAGENTS AND SOLUTIONS

Use ultrapure, pyrogen-free water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.

Agarose-TAE gel, 1%

Per 100 ml:

1 g agarose (Fisher, cat. no. BP160-500)

100 ml TAE (see recipe)

Melt agarose in TAE in a microwave oven until all agarose particles are completely dissolved. When the gel is still liquid but comfortable to the touch, add SYBR Safe DNA gel stain at a 1:6,000 to 10,000 dilution, mix well, and pour into the gel tray. Insert a comb and allow the gel to solidify before loading and running. SYBR Safe is a safe substitute for ethidium bromide, but it is light-sensitive and will lose its effectiveness with prolonged exposure to light. The gel should be used shortly after pouring.

Ampicillin, 100 mg/ml

Dissolve 10 g ampicillin sodium salt (Fisher, cat. no. BP1760-25) in 100 ml ultra-pure, pyrogen-free water, filter-sterilize using a 0.2- μ m filter, and divide into 1-ml aliquots or other convenient volume. Store indefinitely at -20°C .

Blocking buffer (5% w/v nonfat dry milk in TBS-T)

Per 100 ml:

5 g Carnation instant nonfat dry milk

TBS-T (see recipe) to 100 ml

May be stored overnight at 4°C

4dNTPs, 10 mM

Per 1 ml:

100 µl of 100 mM dATP (10 mM final)

100 µl of 100 mM dCTP (10 mM final)

100 µl of 100 mM dGTP (10 mM final)

100 µl of 100 mM dTTP (10 mM final)

600 µl sterile ultra-pure, pyrogen-free water

Combine ingredients, mix well, and store indefinitely at −20°C in small aliquots to avoid repeated freezing and thawing

NOTE: This recipe utilizes dNTPs sold in separate vials as a set (each at 100 mM) by Fisher Scientific (cat. no. AB-0315). Combine as above to make a 4dNTP solution containing each dNTP at a concentration of 10 mM.

Kanamycin, 25 mg/ml

Dissolve 2.5 g kanamycin (Fisher, cat. no. BP906-5) in 100 ml ultra-pure, pyrogen-free water, filter-sterilize using a 0.2-µm filter, and divide into 1-ml aliquots or other convenient volume. Store indefinitely at −20°C.

LB-agar plates plus ampicillin or kanamycin

Prepare 1 liter LB medium (see recipe) in a 2-liter flask, and before autoclaving, add 15 g Bacto agar (BD, cat. no. 214010; 1.5% w/v final) and a large stir bar to the flask. Cap or cover the flask with aluminum foil anchored with tape, and autoclave for 20 min. Remove from the autoclave and immediately place the flask on a stir plate and stir at a moderate speed (without introducing bubbles) for a few minutes until agar particles are completely mixed. Place the flask in a preheated 50°C water bath to allow LB-agar to cool prior to adding antibiotics (agar will solidify at temperatures below 50°C; antibiotics are not stable at higher temperatures). When the temperatures of the LB-agar and water bath have equilibrated, add ampicillin or kanamycin as follows, gently swirling (avoiding bubbles) to mix:

Ampicillin (to 100 µg/ml final concentration): 1 ml of 100 mg/ml stock (see recipe)

Kanamycin (to 50 µg/ml final concentration): 2 ml of 25 mg/ml stock (see recipe)

Immediately pour or pipet ~20 ml of LB-agar containing the appropriate antibiotic into 100-mm petri dishes (Fisher, cat. no. 08-757-12). If pouring, flame the lip of the flask in between pouring each plate, or if pipetting, use a 25-ml sterile pipet. After pouring, leave the plates

with the lids slightly ajar to prevent condensation from forming. This entire procedure can be done on the benchtop, but it is preferable to pour plates in a laminar flow hood or biosafety cabinet if available to reduce contamination of plates. After the plates have cooled to room temperature and condensation has evaporated, replace the lids on the plates and store at 4°C upside down in the bags in which the plates are packaged. Plates may be stored for several months at 4°C, but will eventually start to dry out and the effectiveness of the antibiotics will diminish.

LB medium

Per 1 liter:

10 g Bacto Tryptone (BD, cat. no. 211705)

10 g NaCl

5 g yeast extract (Fisher, cat. no. BP1422-500)

Mix the ingredients in 1 liter of ultra-pure pyrogen-free water. Autoclave for 20 min in a 2-liter flask, or in smaller convenient aliquots. Store indefinitely at room temperature.

SDS-PAGE running buffer (1×)

Dissolve 3.02 g Tris base (Fisher, cat. no. BP154-1; 25 mM final) and 14.4 g glycine (192 mM final) in 900 ml ultra-pure pyrogen-free water. Add 10 ml 10% (w/v) SDS (0.1% final), and adjust volume to 1 liter with ultra-pure pyrogen-free water. Autoclaving is not necessary, and buffer may be stored indefinitely at room temperature.

SOC medium

Dissolve 31 g SOC powder (MP Biomedicals, cat. no. 3031-012) in 1 liter ultra-pure, pyrogen-free water with stirring. Autoclave in 50- to 100-ml aliquots, and store indefinitely at room temperature.

Sodium acetate, pH 5.0, 3 M

Per 100 ml:

Dissolve 24.61 g sodium acetate anhydrous in 70 to 80 ml ultra-pure pyrogen-free water. Adjust pH to 5.0 with glacial acetic acid, adjust the volume to 100 ml with ultra-pure pyrogen-free water, and autoclave. Store indefinitely at room temperature.

TAE (Tris-Acetic acid-EDTA), 1×

Per 1 liter:

4.84 g Tris Base (40 mM final)

1.14 ml glacial acetic acid (0.114% v/v final)

2 ml 0.5 M EDTA, pH 8.0 (1 mM final)

Bring volume to 1 liter with ultra-pure, pyrogen-free water. Autoclaving is not necessary. Store indefinitely at room temperature.

TBS (Tris-buffered saline), 10×

Per liter:

80 g NaCl (1.37 M final)

2 g KCl (27 mM final)

30 g Tris base (248 mM final)

Dissolve in 800 ml ultra-pure, pyrogen-free water. Adjust pH to 7.4 with HCl; adjust volume to 1 liter. Store indefinitely at room temperature without autoclaving.

TBS-T (TBS-Tween-20)

Per liter:

100 ml 10× TBS (see recipe; 1× final)

Adjust volume to 1 liter with ultra-pure, pyrogen-free water, and add 1 ml Tween-20 (Fisher, cat. no. BP337-500; 0.1% final). Store indefinitely at 4°C without autoclaving.

Transfer buffer

Dissolve 3.02 g Tris base (25 mM final) and 14.4 g glycine (192 mM final) in 600 ml water. Add 200 ml methanol (20% v/v final), and then adjust the volume to 1 liter with remaining 200 ml ultra-pure pyrogen-free water. Store indefinitely at 4°C.

COMMENTARY

Background Information

Myxoma virus (MYXV) is a member of the *Poxviridae* family and prototype for the genus *Leporipoxvirus*; the virus is rabbit-specific and harmless to all other species tested, including humans. In European rabbits (*Oryctolagus cuniculus*), MYXV causes a lethal disease called myxomatosis; in two North American species, *Sylvilagus audubonni* and *Sylvilagus nuttalli*, it causes only minor localized lesions and not the lethal myxomatosis seen in European rabbits (Regnery, 1971; Silvers et al., 2010). MYXV is essentially nonpathogenic in its evolutionary hosts, members of the genus *Sylvilagus*, which include the South American tapeti and the North American brush rabbit (Kerr and McFadden, 2002; McFadden, 2005).

MYXV replicates exclusively in the cytoplasm of host cells, and its double-stranded DNA genome encodes 171 ORFs, 12 of which are duplicated in the TIRs (Cameron et al., 1999). Approximately 90 to 100 of these are presumably essential genes known to be conserved among all poxviruses. More than two dozen MYXV ORFs encode proteins that are known to have immunomodulatory, host range, anti-apoptotic, or virulence functions, and these genes are generally clustered towards the molecular ends of the genome (Barrett et al., 2001; Kerr and McFadden, 2002); a number of these host-interactive MYXV gene products have already been characterized (Stanford et al., 2007).

Among these MYXV proteins are several that are secreted and bind certain immune ligands; those that mimic cellular receptors are called viroreceptors, and include M001L (M-T1), M002L (M-T2), and M007L (M-T7). Virokines (e.g., M008.1L, or Serp-1, and M010L, or MGF) are also secreted, and imitate host immune effector molecules. Proteins such as M004L (M-T4), M005L (M-T5), and M011L are known as viromitigators, and can have host range functions or inhibit apoptosis (Kerr and McFadden, 2002). These characteristics suggest that a number of MYXV proteins may have potential use as therapeutics, and studies are underway to test the efficacy of several of these to treat various pro-inflammatory diseases. For example, Viron Therapeutics, is testing MYXV immunomodulatory protein M008.1L (Serp-1) in clinical trials for acute unstable coronary syndromes. Furthermore, Liu

et al. (2000) have shown that M-T7, a secreted glycoprotein that inhibits rabbit gamma interferon, can inhibit inflammatory responses in balloon angioplasty arterial injury in rabbits. The possibility for development of other MYXV immunomodulatory proteins as anti-inflammatory or anti-immune agents in a therapeutic setting is promising.

MYXV is also currently being developed as an oncolytic virus for treatment of human cancers. Although it is not pathogenic for humans, MYXV is able to productively infect a variety of human cancer cells in vitro and in vivo. For example, mice xenografted with human glioma tumors demonstrate increased long-term survival following treatment with MYXV (Lun et al., 2005).

Safety studies using MYXV as an oncolytic treatment look promising as well. Kim et al. (2009) showed that when normal and leukemic hematopoietic stem and progenitor cells (HSPCs) are treated with MYXV, only the leukemic cells, but not the normal human stem cells, are infected in vitro. Furthermore, when HSPCs from normal human bone marrow are treated ex vivo with MYXV and xeno-transplanted into immunodeficient mice that are first sublethally irradiated, the normal stem cells readily engraft, and at necropsy, there is no evidence for MYXV replication in any normal tissues.

Because of MYXV's large genome and its potential utility in a therapeutic setting, the availability of an ORFeome library in Gateway entry vectors is an important tool for studying global aspects of the viral protein repertoire. The technology has already been employed to generate ORFeomes for a number of other pathogens, including vaccinia virus, the vaccine strain used to eradicate smallpox, and the prototype orthopoxvirus (Zhang et al., 2009); *S. aureus*, the bacterium responsible for methicillin-resistant *Staphylococcus aureus* (MRSA) (Brandner et al., 2008); *Pseudomonas aeruginosa*, which causes a difficult to control infection in immunocompromised and cystic fibrosis patients (LaBaer et al., 2004); *Plasmodium falciparum*, the human malaria parasite (Aguiar et al., 2004); and for *E. coli* K-12, a laboratory strain (Rajagopala et al., 2010). Construction of a human ORFeome library is also underway (Brizuela et al., 2001; Rual et al., 2004b), and a collaboration formed in 2005, the ORFeome Collaboration, has as its goal to make available a sequenced ORF clone in Gateway vectors for the more than 18,000 human cDNAs (<http://www.orfeomecollaboration.org/html/index.shtml>).

The Gateway system facilitates cloning of large ORFeomes by eliminating the arduous and time-consuming process of conventional cloning strategies that rely on restriction enzymes and ligase reactions. It does so systematically and efficiently by utilizing bacteriophage lambda site-specific recombination; the technology has been described in detail by others (Landy, 1989; Walhout et al., 2000; Hartley et al., 2000; Park and LaBaer, 2006). Briefly, the process for creating a MYXV expression clone library as described here involves the following steps: (1) design gene-specific PCR primers for individual ORFs with partial *attB* sequence; (2) perform a first round of PCR using gene-specific primers and genomic DNA as template; (3) perform a second round of PCR using universal primers and the first-round PCR product as template; (4) create Gateway entry plasmid clones in which the termination codon has been mutated to a leucine to allow for later C-terminal tagging of the viral protein, using BP Clonase to facilitate recombination between the second-round PCR product containing the complete *attB* sequence and an entry vector with *attP* sequence; (5) transform bacterial cells and PCR-screen colonies to identify positive clones; (6) sequence individual clone(s); (7) create Gateway expression plasmid clones that have the genetic elements needed to express C-terminally tagged viral proteins, using LR Clonase to facilitate recombination between the *attL*-containing entry clone and *attR*-containing expression vector; (8) transform bacterial cells and PCR-screen colonies to identify positive clones; (9) verify tagged viral protein expression by TNT (Promega) and immunoblot analysis/ECL.

Complete instructions for using Gateway reagents and helpful guidelines can also be found on Invitrogen's Web site (<http://www.invitrogen.com/gateway>).

Individual genes in entry plasmid clones can also be easily shuttled to multiple expression plasmid vectors useful in a variety of applications (Walhout et al., 2000; Brizuela et al., 2001; Ramachandran et al., 2006), and the technology is well-suited to high-throughput methods. Expression vectors are available with various epitope tags (e.g., GST) and promoters, and proteins can be expressed in a variety of cell types and in numerous expression systems, including in vitro methods such as TNT, or in lentiviral, mammalian or bacterial systems (Park and LaBaer, 2006). Furthermore, a non-Gateway expression vector can easily be converted to a Gateway-compatible expression vector by ligating a conversion cassette into the expression vector of choice (for method, see Park and LaBaer, 2006), and allows for the inclusion of a variety of markers.

The versatility and efficiency of the Gateway recombination cloning system is therefore an invaluable tool for exploiting emerging proteomic techniques. For MYXV, the availability of a viral ORFeome library will certainly facilitate the study of viral-host interactions, virulence, and pathogenesis of the virus, and will be useful in developing its utility in therapeutic or clinical settings. Other applications will likely emerge as more Gateway-compatible expression libraries are developed.

Critical Parameters and Troubleshooting

PCR considerations

Primer design: Although it is preferable to design primers with a G-C content of ~50%, it is not always possible to achieve this because some ORFs may have a very high or very low G-C content at the 5' and/or 3' ends. Adjust the length of the gene-specific portion of the primer when designing the primers to attain G-C content as close to 50% as possible. Also, attempt to achieve similar melting temperatures for all primers.

PCR conditions: It is sometimes necessary to adjust PCR conditions in order to obtain a PCR product for some ORFs. Annealing temperature, cycle number, Mg^{2+} , and polymerase can all be adjusted to obtain a product for hard to amplify genes.

DNA polymerase: It is critical not only to obtain a positive PCR product for every ORF, but also to achieve high fidelity for every clone. Some polymerases are more successful than others in achieving a high success rate, both in terms of obtaining a PCR product, and in reducing or eliminating nucleotide changes. Although there are a number of high-fidelity DNA polymerases commercially available, the authors primarily use *Pfu* Ultra High-Fidelity DNA Polymerase (Agilent Technologies) in first- and second-round PCR reactions. It has been found to introduce fewer nucleotide changes during amplification than Platinum *Taq* High Fidelity (Invitrogen), which has also been tested. The authors have also used AccuPrime *Pfx* (Invitrogen) and found this polymerase to be useful when *Pfu* fails to produce a PCR product for a problematic ORF.

Creating entry clones

PCR product concentration and multiple bands: The amount and quality of PCR product added to a BP reaction can make a difference in successful generation of entry clones, especially for difficult-to-clone ORFs. Invitrogen recommends an equimolar amount of PCR product and entry vector [50 femtomoles (fmol) each] in the BP reaction. However, the authors have found that for ORFs that amplify well and for which it is possible to generate a "clean" PCR product that does not contain multiple bands, it is sometimes possible to simply add 0.5 to 1 μ l of a clean, abundant PCR product directly to the BP reaction without

quantifying or purifying. However, Invitrogen cautions against using more than 0.5 µg total of DNA in a 10-µl BP reaction. The PCR product should not contain multiple bands; if it does, adjustments can be made to the PCR conditions, or refer to the Support Protocol for gel-purifying PCR products.

Cloning large genes: Cloning larger genes into an entry vector can be problematic. Hartley et al. (2000) report that the efficiency of the recombination reaction decreases as the size of the ORF increases, but that this can be abrogated by using equimolar amounts of DNA (PCR product and entry vector), and by incubating for a longer period of time (overnight). MYXV ORF M134R, which at 6 kb is the largest MYXV ORF, presented such a problem; it was never successfully cloned in its entirety into an entry vector, and instead, was cloned in two parts, an upstream and downstream portion, both ~3 kb in size.

Storing BP reactions: Although Park and LaBaer (2006) reported success in storing BP reactions for up to 6 months at -20°C, in the authors' hands, efficiency was reduced when using frozen BP reactions in transformations.

Sequencing to verify entry clones: It is critical to completely sequence entry clones, since an occasional clone will have random mutations that can be a result of PCR amplification or mistakes in synthesis of the primers. Mutations that result from amplification may be eliminated or reduced by reducing the number of amplification cycles, or by using a different high-fidelity polymerase.

Furthermore, if the M13 forward and reverse sequencing primers do not cover 100% of a large ORF (generally those larger than ~1.4 to 1.5 kb), gene-specific primers should be designed and used to sequence the internal portion of these genes.

Creating expression clones—Although it is not necessary to sequence expression clones, it is important to verify that a protein of the expected size is actually expressed (see Basic Protocol 4).

Anticipated Results

PCR amplification—Using MYXV genomic DNA as template, a 99% success rate for amplifying the individual ORFs can be achieved. MYXV M134R was never successfully cloned as a full-length ORF; although a clean 6 kb first-round PCR product amplified, a second-round PCR product was never successfully generated. However, full-length PCR products can be produced for all other MYXV ORFs, and when multiple products are generated for a single reaction, the desired product can be isolated by gel purification for use as template in the second round of PCR amplification, or in the BP reaction.

Creating entry clones—The authors were able to achieve a 99% success rate in creating an entry clone library of full-length ORFs for MYXV. MYXV M134R entry clone was made in two parts; roughly 50% of the 6-kb ORF was PCR'd from the 5' or 3' end to create an upstream (5') entry clone, and a downstream (3') entry clone.

Mutations may also occur, depending on a variety of factors. Often it is enough to choose another colony to grow and sequence; it may be necessary, however, to start again with first round PCR, altering conditions or changing polymerase.

Creating expression clones—Greater than 95% success can be achieved in transferring ORFs from the entry to the expression vector, and re-sequencing is unnecessary. However, the efficiency of protein expression varies greatly. Some of the GST-tagged ORFs express at

very low (sometimes barely detectable) levels, a few appear to be smaller (or larger) than the expected size, and others generate GST-tagged proteins of multiple sizes. Approximately 4% to 5% of the expression clones do not appear to express at all in the test reticulocyte lysates.

Time Considerations

It generally takes ~8 days to complete these protocols (excluding sequencing), starting with generation of the first PCR product in Basic Protocol 1 and completing verification of protein expression in Basic Protocol 4. First- and second-round PCR products can be generated and purified in 1 day. Allow a little more than 2 days to complete the BP reaction, transformation of competent cells, PCR screening of transformants, and prepping entry clones. Time allotted for sequencing the positive clones varies, depending on sequencing facilities. Completing the LR reaction, transformation, PCR screening of transformants, and prepping expression clones takes a little more than 2 days, and verifying protein expression can be accomplished in 2 days.

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Figure 14A.2.1.

Overall strategy for Gateway recombination cloning of MYXV ORFs. Individual MYXV ORFs are PCR amplified using genomic viral DNA as a template in two PCR steps to generate a product with complete *attB* sequences flanking gene-specific sequence. This PCR product is incubated with a Gateway donor vector containing the *attP* sequence in a BP reaction to create an entry clone, which is then sequence verified. Note that the termination codon has been replaced with a leucine codon to allow for C-terminal tag fusions in subsequent Gateway expression vectors. The entry clone is incubated with pANT7-cGST expression vector in a LR reaction to generate an expression clone with the C-terminal GST tag; GST-tagged protein expression is verified using the TNT Coupled Reticulocyte Lysate System (Promega) and immunoblot analysis. Adapted from Park and LaBaer (2006) and Invitrogen (<http://www.invitrogen.com/gateway>).

Figure 14A.2.2.

Two-step PCR strategy. A first round of PCR is performed using MYXV genomic DNA as template and primers with gene specific and partial *att* sequence. A second round of PCR is then performed using the first-round PCR product as template, amplifying with universal *att* primers that generate a product containing the complete *attB* recombination sequence. The *att* sequence added to the gene-specific portion of the PCR product by successive rounds of PCR with these primers is indicated by the shaded areas. Adapted from Park and LaBaer (2006).

Figure 14A.2.3.

Primer sequence. For gene-specific primers, the partial *att* sequence is shown in capital letters. For the forward primer, Kozak sequence (CACC) precedes the ATG, which is followed by 15 to 30 nucleotides of viral gene sequence. In the reverse primer, the stop codon is replaced by the leucine anticodon (CAA) since all ORFs are ultimately cloned into a C-terminal GST expression vector (pANT7-cGST) to generate C-terminal fusion proteins. Adapted from Park and LaBaer (2006).

Figure 14A.2.4.

PCR amplification products prior to gel purification. **(A)** First-round PCR products for representative samples. **(B)** Second-round PCR products amplified from first-round PCR products shown in panel A. Although a full-length 6-kb product was obtained for M134A in a first round of PCR, this ORF did not amplify with universal primers for a second round of PCR. ORFs that did not amplify are indicated with an asterisk (*).

Figure 14A.2.5.

Protein expression for representative MYXV GST-tagged ORFs M001L-M066R. Those which did not express are indicated with an asterisk (*). Size markers are shown in kDa. Note that although GST-M047R and GST-M065R did not appear to express, protein expression was verified in a later immunoblot analysis. Furthermore, some expressed GST-fusion proteins migrate differently than what was predicted from the viral ORF.

Figure 14A.2.6.

Protein expression for representative MYXV GST-tagged ORFs M067L-M147R. Those which did not express are indicated with an asterisk *. Size markers are shown in kDa. Note that some expressed GST-fusion proteins migrate differently than what was predicted from the viral ORF.

Table 14A.2.1

Expression of Individual MYXV Open Reading Frames as GST-Fusion Proteins^a

MYXV ORF entry clone	Predicted size (kDa) of MYXV ORF	Observed size (kDa) of expressed MYXV ORF	MYXV ORF entry clone	Predicted size (kDa) of MYXV ORF	Observed size (kDa) of expressed MYXV ORF
M000.5L	33	33	M032R	87	87
M001L	54	54	M033R	55	55
M002L	61	61	M034L	136	136
M003.1L	42	Multiple bands	M035R	36	36
M003.2L	38	38	M036L	100	100
M004L	51	51	M037L	29	29
M004.1L	35	35	M038L	60	60
M005L	78	78	M039L	33	33
M006L	81	81	M040L	55	55
M007L	54	54	M041L	34	Multiple bands
M008L	81	81	M042L	68	55
M008.1L	66	66	M043L	72	72
M009L	81	81	M044R	100*	*
M010L	35*	*	M045L	90	90
M011L	44	44	M046L	38	38
M012L	42	Multiple bands	M047R	50	50
M013L	39	Multiple bands	M048L	39	39
M014L	82 ND	ND	M049R	73	73
M015L	61	61	M050R	32	40
M016L	34	34	M051R	44	44
M017L	34	Multiple bands	M052L	64	64
M018L	33	Multiple bands	M053R	54	54
M019L	49	49	M054R	62	62
M020L	74	74	M055R	52	52
M021L	94	94	M056R	36	Multiple bands
M022L	66	66	M057L	60	60
M023R	32	32	M058R	53	53
M024L	42	42	M059R	39	39

MYXV ORF entry clone	Predicted size (kDa) of MYXV ORF	Observed size (kDa) of expressed MYXV ORF	MYXV ORF entry clone	Predicted size (kDa) of MYXV ORF	Observed size (kDa) of expressed MYXV ORF
M025L	48	48	M060R	42	42
M026R	37	50	M061R	45	45
M027L	77	Multiple bands	M062R	43	43
M028L	106	106	M063R	49	60
M029L	38	Multiple bands	M064R	48	Multiple bands
M030L	50	Multiple bands	M065R	62	62
M031R	69	69	M066R	46	Multiple bands
M067L	40	40	M105L	36	Multiple bands
M068R	167 NV	NV	M106L	67	Multiple bands
M069L	45	45	M107L	47	Multiple bands
M070R	46	46	M108R	78	78
M071L	62	62	M109L	33	Multiple bands
M072L	112 NV	NV	M110L	38	38
M073R	47	60	M111R	73	73
M074R	60	60	M112R	43	43
M075R	41	41	M113R	68	68
M076R	120 ND	ND	M114R	152 ND	ND
M077L	41	41	M115L	46	60
M078R	52	52	M116L	41	41
M079R	49	49	M117L	59	59
M080R	112	112	M118L	34	Multiple bands
M081R	95	95	M119L	31	31
M082R	43	43	M120L	53	53
M083L	57	57	M121R	45	45
M084R	48	48	M122R	44	44
M085R	54	54	M123R	45	50
M086L	95	95	M124R	57	57
M087L	57	57	M125R	43	Multiple bands
M088L	86	86	M126R	55	55
M089L	42	42	M127L	74	74
M090L	50	50	M128L	56*	*

MYXV ORF entry clone	Predicted size (kDa) of MYXV ORF	Observed size (kDa) of expressed MYXV ORF	MYXV ORF entry clone	Predicted size (kDa) of MYXV ORF	Observed size (kDa) of expressed MYXV ORF
M091L	34 ND	ND	M129R	40	40
M092L	97	97	M130R	39	>50
M093L	43	Multiple bands	M131R	43	50
M094R	43	>50	M132L	45	50
M095L	66	66	M133R	87*	*
M096L	104	104	M134R	245 ND	ND
M097R	57	57	M135R	45	Multiple bands
M098L	34	34	M136R	45	45
M099L	124	124	M137R	58	58
M100R	60	60	M138L	57	57
M101L	43	50	M139R	46	Multiple bands
M102L	33	Multiple bands	M140R	86	86
M103L	36	Multiple bands	M141R	49	49
M104L	31	31	M142R	59	Multiple bands
M143R	51	51	M150R	80	80
M144R	58	58	M151R	62	62
M146R	37	Multiple bands	M152R	55	55
M147R	57	57	M153R	46	46
M148R	100	100	M154L	49	49
M149R	79	79	M156R	37	37

^a Each MYXV open reading frame (ORF) was deleted of its final termination codon and cloned into Gateway pDONR-221 or -222 entry plasmid. The viral cassettes were transferred into the Gateway pANT7-GST expression vector, and then expressed as C-terminal GST-fusion proteins in reticulocyte lysates. The viral proteins were identified by immunoblotting, using anti-GST antibody. An asterisk (*) indicates expression clones that did not express detectable GST-fusion protein. ND (not done) indicates an entry clone that could not be stably cassetted into the expression vector plasmid, and NV (not verified) specifies that no expression of any GST-fusion protein could be detected. "Multiple bands" indicate that GST-tagged proteins of multiple sizes were expressed. Protein sizes include the GST tag.