Published in final edited form as:

Biotechniques. 2024 ; 76(7): 299–309. doi:10.1080/07366205.2024.2343609.

pJoseph2: a family of plasmids as positive controls for bacterial protein expression, transfections, and western blots

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Abstract

Epitope tagging represents a powerful strategy for expedited identification, isolation, and characterization of proteins in molecular biological studies, including protein–protein interactions. We aimed to improve the reproducibility of epitope-tagged protein expression and detection by developing a range of plasmids as positive controls. The pJoseph2 family of expression plasmids functions in diverse cellular environments and cell types to enable the evaluation of transfection efficiency and antibody staining for epitope detection. The expressed green fluorescent proteins harbor five unique epitope tags, and their efficient expression in *Escherichia coli*, *Drosophila* Schneider's line 2 cells, and human SKOV3 and HEK293T cells was demonstrated by fluorescence microscopy and western blotting. The pJoseph2 plasmids provide versatile and valuable positive controls for numerous experimental applications.

MULTIDISCIPLINARY ABSTRACT

Epitope tagging, a fundamental technique in molecular biology, involves attaching short amino acid sequences (epitope tags) to target proteins for their efficient identification and study. This technique has evolved since its inception, enabling diverse applications in protein research. Notably, CRISPR/Cas9 gene editing has enhanced epitope tagging by enabling the tagging of endogenous genes, expanding its versatility. However, reproducibility challenges exist, demanding positive controls for troubleshooting. The pJoseph2 family of plasmids was developed to address

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Author contributions

E Robinson: undertook conceptual design, performed experiments, and wrote the manuscript; E Barajas Alonso: performed experiments and edited the manuscript; JA Waters: performed experiments and edited the manuscript; C Bileckyj: undertook conceptual design, edited the manuscript, and obtained funding; CD House: edited the manuscript and obtained funding; CA Johnston: undertook conceptual design, edited the manuscript, and obtained funding; and RM Cripps: undertook conceptual design, edited the manuscript, and obtained funding.

Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, stock ownership or options and expert testimony.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

this need, providing robust positive controls for various epitope-based experiments, from bacterial expression to *Drosophila* and mammalian cell studies. This resource enhances the reliability and accuracy of epitope tagging, benefiting researchers across disciplines.

METHOD SUMMARY

This method introduces the pJoseph2 family of plasmids, a set of tools designed to improve the reproducibility and consistency of protein expression and epitope tagging experiments. Proteins expressed from these plasmids contain multiple epitope tags, providing researchers with a valuable resource for troubleshooting and validating epitope tagging experiments using bacterial, *Drosophila*, and mammalian cells.

Keywords

epitope tag; GFP; multitag; transfection; western blot

1. Background

Epitope tagging is a widely used technique in molecular biology that involves fusing a short amino acid sequence, called an epitope tag, to a target protein of interest. The epitope tag serves as a molecular handle, allowing for the expedited identification, isolation, and characterization of the tagged protein, in the absence of an effective antibody against the parent protein of interest. This approach was pioneered by Munro and Pelham [1], who fused a short sequence from the tachykinin peptide hormone Substance P to *Drosophila* HSP70, to follow the stability and localization of HSP70 deletion mutants expressed in tissue culture. Since that time, a large number of epitope tags, and their cognate monoclonal antibodies, have been developed (reviewed in Brizzard [2]).

Following the initial use of epitope tagging to track the expression of a single protein, the technology has been developed to offer several applications in protein research. For example, epitope tags can be used to study protein–protein interactions by introducing tags into one or multiple proteins and investigating their interactions through coimmunoprecipitation or pull-down assays (see, e.g., Mende et al. [3]). These approaches aid in the elucidation of cellular pathways and molecular mechanisms. Similarly, immunoprecipitation of a tagged protein expressed in living cells followed by MS is a powerful approach to identifying the components of protein complexes [4]. Also, immunoprecipitation of tagged transcription factors crosslinked to chromatin followed by high throughput sequencing is now a standard approach to identify transcription factor occupancy in the genome [5]. This approach was further simplified by using CRISPR/Cas9 genome editing to tag endogenous genes in living organisms [6].

However, despite its wide usage, achieving consistent and reproducible results with epitope tagging can be challenging. Factors such as variations in transfection efficiency, differences in antibody staining, and variability in protein expression levels can contribute to inconsistent experimental outcomes. These challenges are particularly exacerbated in situations where research groups perform these assays intermittently rather than routinely,

and where there is the potential for degradation of reagents. In these instances, accurately troubleshooting the causes of a failed experiment is important to obviate the unnecessary replacement of valuable molecular reagents.

To address these challenges and enhance reproducibility, we developed a series of plasmids, collectively known as the pJoseph2 family, as positive controls for epitope-based experiments. The green fluorescent protein (GFP) encoded by each plasmid is flanked by five additional epitope tags. It is crucial to note that the intention is not to link the multitag construct to another protein. Instead, our goal is to integrate these reagents alongside protein expression and epitope tagging experiments, ensuring that every component involved in protein expression and detection is functional. These plasmids can be used as positive controls for bacterial expression, transfection in *Drosophila* or mammalian cells, and western blotting, and will aid in troubleshooting experimental problems with any of these approaches. Altogether, these plasmids offer the potential to evaluate the efficiency of protein purification, assess transfection outcomes, and optimize cell sorting techniques.

2. Materials & methods

2.1. Ethics statement

All recombinant DNA approaches were carried out following authorization from the San Diego State University Institutional Biosafety Committee. No animal or human subjects were used for this research.

2.2. Epitope tag design & cloning

The pJoseph2 plasmids were constructed to incorporate five unique epitope tags adjacent to the GFP coding region. The basic coding sequence of Joseph2 was synthesized using GBlocks and inserted in a pUCIDT-AMP plasmid by Integrated DNA Technologies (IDT, IA, USA). This product was amplified using primers pJoseph2/pEXP1 F and pJoseph2/pEXP1 R for cloning into pEXP1 (Thermo Fisher Scientific, MA, USA), and primers pJoseph2/pAHW F and pJoseph2/pAHW R for cloning into pAHW (*Drosophila* Genomics Resource Center 1095; generated by the Murphy Laboratory, Carnegie Mellon University) and pMH-HA (Addgene no. 101767, An et al. [7]). Primer sequences used for polymersae chain reaction (PCR) are described in Table 1. Note that these primers include attB1 and attB2 sequences to facilitate Gateway cloning [8]. PCR products from the first reactions were inserted into pDONR221 (Thermo Fisher Scientific) using Gateway Technology in a BP Reaction. Sequence-verified clones were then used to create the final plasmids by LR Reactions into either pEXP1 (containing the 6xHis tag sequence), or pAHW and pMH-HA (containing the Hemagglutinin (HA) tag sequence). Final clones were sequence-verified before their use for expression. The Joseph2 family plasmids will be submitted to Addgene.

2.3. Protein purification from Escherichia coli

E. coli BL21 DE3 cells (Invitrogen, CA, USA) were transformed with pEXP1/Joseph2 and grown in LB broth plus ampicillin at 37°C overnight. This culture was diluted 1:10 into 50 ml of fresh LB plus ampicillin and grown to optical density at 600nm wavelength (OD600) of ~0.5 before induction with 100 μM isopropyl ß-D-1-thiogalactopyranoside

(IPTG) (Invitrogen) for 4 h. Cells were then harvested by centrifugation, and recombinant protein was isolated under denaturing conditions, to maximize the yield of protein, using the ProBond Purification System (Thermo Fisher Scientific) and Ni-NTA resin (Qiagen, Hilden, Germany). Purified protein was analyzed by SDS-PAGE for purity and the concentration was determined based on A_{280} using a NanoDrop device (Thermo Fisher Scientific).

2.4. Transfection

For expression in *Drosophila* cells, Schneider's line 2 (S2) cells were grown in Schneider's medium and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, MA, USA) at 25°C. S2 cells were plated on a 24-well plate at a concentration of 6×10^5 cells/well. After allowing cells to adhere for 1 h, the supplemented medium was removed and 300 μl of serum and antibiotic-free medium that contained 0.5 μg of pAHW/Joseph2 plasmid and 5 μl transfection reagent (FuGENE HD, Promega, WI, USA) was added to experimental wells. Control wells only received the nonsupplemented medium and transfection reagent. Transient transfections were incubated for 24–32 h before harvesting and conducted in triplicate.

For expression in mammalian cells, SKOV3 ovarian cancer cells (American Type Culture Collection, VA, USA) were seeded at 50% confluency using six-well plates in RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin and incubated at 37°C/5% CO_2 overnight. HEK293T cells were seeded at 50% confluency in six-well plates using Dulbecco's modified Eagle medium supplemented with 10% FBS and 1% penicillin/streptomycin and incubated at 37°C/5% CO_2 overnight. Both cell line wells were then transfected with 2 μg pMH-HA/Joseph2 using Lipofectamine 3000 (Thermo Fisher Scientific) prepared in Opti-MEM (Thermo Fisher Scientific) according to the manufacturer's instructions. Transfected cells were grown for 48 h prior to evaluation via microscopy and 72 h prior to flow cytometry and western blotting.

2.5. Flow cytometry

To quantitatively assess transfection efficiency in human cells, cells were collected using CellStripper, and GFP expression was measured via flow cytometry. Untransfected SKOV3 cells were used to establish negative gates and the frequency of GFP-expressing cells after pMH-HA/Joseph2 transfection was evaluated on a BD FACSMelody Cell Sorter (BD Biosciences, NJ, USA) in the San Diego State University Biology Flow Cytometry Core.

2.6. Immunofluorescence & confocal microscopy

To visualize transfection efficiency, transfected cells were grown on 13 mm diameter coverslips in tissue culture plates. Coverslips for S2 and SKOV3 cells were first coated with 0.2% gelatin in 1× phosphate-buffered saline (PBS). Then, 24–48 h following transfection, cells were fixed with 4% formaldehyde in 1× PBS for 8 min and washed twice with 1× PBS. Next, cells were blocked with 1% bovine serum Albumin in 1× PBS with 0.1% v/v Triton X-100 to minimize nonspecific binding. Fluorescent staining was performed by incubating the fixed cells with reagents as shown in Table 2. Nuclei were visualized using 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) (Thermo Fisher Scientific) at 5 μg/ml and F-actin was visualized using Alexa Fluor™ 568 Phalloidin (Thermo Fisher

Scientific). Finally, coverslips carrying cells were washed and mounted on glass slides. Confocal microscopy was performed using an Olympus FV3000 (MA, USA) instrument to visualize the expression and subcellular localization of the epitope-tagged proteins. The HEK293T cells were observed live under transmitted light microscopy.

2.7. Western blot analysis

Purified protein or cell lysates were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked using 1× PBS with 0.1% v/v Tween-20 and 5% w/v nonfat milk and then probed with primary antibodies specific to the epitope tags incorporated in the pJoseph2 plasmids (Table 2). Membranes were incubated with primary antibody overnight at 4°C with gentle agitation. After primary antibody incubation, the membranes were washed to remove unbound antibodies. For horseradish peroxidase (HRP)-conjugated primary antibodies, bound antibody was visualized immediately after washing. For nonlabeled primary antibodies, blots were incubated with a secondary antibody conjugated to either HRP or a fluorophore and washed before imaging.

For HRP-conjugated antibody detection, protein bands were visualized using an enhanced chemiluminescence reagent (Thermo Fisher Scientific) and captured using a Bio-Rad ChemiDoc MP imaging system (BioRad, CA, USA). For fluorescence-based detection, bound fluorescent secondary antibodies were directly imaged using the same instrument.

3. Results & discussion

3.1. Creation of expression plasmids

To create the pJoseph2 family of plasmids, we first designed and ordered a dsDNA fragment that we termed Joseph2, which could be used as a template to create all three plasmids. This template comprised the coding sequence for enhanced GFP (F64L S65T), which was flanked on the 5′ side by a sequence encoding 3xHA and 3xMYC tags and flanked on the 3′ side by a sequence encoding 3xFLAG, 3xV5, and 6xHis tags (Figure 1). Each component sequence was separated by sequences for a 5xGly-Ser linker, to allow for concurrent binding of antibodies to each component tag. An earlier version of this construct, termed Joseph, contained only one copy of each epitope but did not allow clear detection in western blots. Therefore, we modified the design to include three copies of each epitope as described here.

To create the pEXP1 version of the pJoseph2 plasmid, we used this dsDNA as a template for PCR using the primers Joseph2 pEXP1 F and Joseph2 pEXP1 R. These primers will amplify all the templates except for the 3′ 6xHis tag, because pEXP1 provides an N-terminal His tag as part of its design. The primers also contain attB sites, so that the product can be used for Gateway cloning, and the reverse primer contains a stop codon (Table 1). The PCR product was inserted into pDONR221 using BP clonase to create the entry plasmid pDONR/Joseph2EXP, and resulting clones were sequence-verified using M13 primer before proceeding to the next step. Next, pDONR/Joseph2EXP was used in an LR reaction with pEXP1 to create the destination plasmid pEXP1/Joseph2 (Figure 1) for *E. coli*

transformation. Correct and in-frame insertion of the Joseph2 sequences was confirmed by sequencing using T7 and T7 term primers.

A common destination plasmid was employed in the generation of pAHW/Joseph2 and pMH-HA/Joseph2 constructs. Initially, a PCR product generated using primers pJoseph2/pAHW F and pJoseph2/pAHW R (Table 1) was integrated into the pDONR221 vector using BP clonase enzyme, resulting in the formation of the entry plasmid pDONR/Joseph2AHW. Following this step, the resulting clones were subjected to sequence verification, utilizing the M13 primer, prior to advancing to the subsequent stages. Next, pDONR/Joseph2AHW underwent an LR reaction with pAHW, leading to the creation of the destination plasmid pAHW/Joseph2 (Figure 1), intended for transfection into *Drosophila* S2 cells. The accuracy and proper alignment of the Joseph2 sequences were affirmed through sequencing utilizing ACT primers (IDT).

Additionally, the pDONR/Joseph2AHW plasmid was employed in an LR reaction alongside pMH-HA, culminating in the production of the destination plasmid pMH-HA/Joseph2, for use in transfecting human SKOV3 and HEK293T cells. The correct and proper alignment of the Joseph2 sequences within this construct was also validated through sequencing, utilizing CMV-Forward and BGHR primers (IDT).

3.2. Expression of Joseph2 in E. coli

After confirming the sequences of pEXP1/Joseph2 we proceeded with protein expression in *E. coli BL21 DE3 E. coli* cells. Cells were grown to an (OD600 of 0.5–0.8, then we initiated transcription from the T7 promoter with 100 μM IPTG for 4 h. Coomassie Blue staining verified that a 4-h induction period yielded optimal protein expression, and visible GFP expression was evident in induced but not noninduced cells through the distinctive green coloration of the cell pellet (not shown). We next prepared cell lysates for protein purification under denaturing conditions, utilizing Ni-NTA agarose through affinity chromatography to purify the His-tagged proteins. Protein was routinely purified at a concentration of >100 μg/ml.

To validate protein expression, we ran 44.4 ng samples on an SDS-PAGE gel, transferred the proteins to the nitrocellulose membrane, and stained the membrane with Ponceau S to visualize all proteins. We observed the purification of a protein with an apparent mass of approximately 65 kDa (Figure 2A, lane 2), which is slightly larger than the predicted size of Joseph2 generated from this plasmid, which is 49.3 kDa (Figure 1). This variance in size could be attributed to the presence of urea during the denaturing protein purification process, a known factor that can influence the migration of certain proteins in SDS-PAGE, as demonstrated in previous studies [9]. However, even in bacterial lysates that lack urea, the protein appears at ~65 kDa (Figure 2, lanes 3 and 4), indicating that in this system the generated protein runs anomalously slowly. We hypothesize that some modification of the protein occurs when expressed in *E. coli* that affects its mobility, although this does not affect its use as a positive control protein (see remainder of this section below).

We next performed western blotting to determine if each epitope could be detected, employing six distinct tag-specific antibodies (Table 2). In all cases, we were able

to clearly detect Joseph2 without a significant background signal (Figure 2A, lanes 5–10). We additionally sought to demonstrate that multiple tags within Joseph2 could be simultaneously detected using different primary antibodies, and thus performed fluorescent western blotting using fluorescently labeled secondary antibodies with nonoverlapping spectra. In this approach, we simultaneously detected the purified protein using HA and MYC antibodies (Table 2).

In these fluorescent western blots, we first observed that even the denatured Joseph2 protein retained fluorescence in the green channel (Figure 2B, lane 3). Subsequently, we validated the expression of the target protein, demonstrating the simultaneous visualization of HA (far-red) and MYC (blue) tags (Figure 2B, lanes 4 and 5).

This approach, incorporating multiple fluorescent channels, strengthens our confidence in the accuracy and reliability of our protein expression results. However, it is important to note that Joseph2 purified under denaturing conditions, subjected to SDS-PAGE, and transferred to nitrocellulose membrane still retains significant fluorescence in the green channel. While this fluorescence can be a valuable positive control for detecting Joseph2 protein, this means that Joseph2 cannot be used to test the efficacy of antibodies that fluoresce in the GFP range.

3.3. Expression of Joseph2 in Drosophila S2 cells

We next determined if Joseph2 could be expressed and detected in *Drosophila* S2 cells, using the plasmid variant pAHW/Joseph2 (Figure 1). Transfected S2 cells served as our experimental sample, while nontransfected S2 cells were utilized as the negative control. At 24–32 h after transfection, cells were fixed and stained with DAPI and fluorescent Phalloidin. GFP, DAPI, and fluorescent Phalloidin were visualized using confocal microscopy. In the nontransfected cells, there was no GFP fluorescence detectable (Figure 3A), whereas in the transfected samples, GFP-positive cells were evident (Figure 3B). This indicated the successful expression and detection of Joseph2 in *Drosophila* S2 cells using the pAHW/Joseph2 plasmid variant.

To determine if the expressed Joseph2 protein could be detected in lysates of transfected cells, we separated lysates by SDS-PAGE and performed western blotting using the same antibodies used for the detection of protein expressed in *E. coli*. In all cases, we confirmed the presence of all six tags, evident by well-defined bands of the correct sizes (Figure 3C). Note that the Joseph2 protein in these experiments migrates at ~55 kDa. This is closer to its predicted MW, although it still seems to migrate more slowly than predicted.

3.4. Expression of Joseph2 in mammalian cells

Finally, we initiated the transfection of human SKOV3 cells, a human cancer cell line derived from ovarian adenocarcinoma, and HEK293T human embryonic kidney cells using the pMH-HA/Joseph2 plasmid. As before, we employed the transfected cells as our experimental, contrasting them with nontransfected cells as the negative control, in order to discern if GFP expression predominantly stemmed from the pMH-HA/Joseph2 plasmid. Similar to our approach with the transfected S2 cells, we proceeded with cell fixation and staining at 48 h post-transfection, imaged using confocal microscopy. Once

again, GFP expression was conspicuously observed in the transfected cells, but absent in the nontransfected cells (Figure 4A & B).

To quantitatively assess GFP expression, we performed flow cytometry of untransfected and transfected SKOV3 cells to determine if Joseph2-expressing cells could be detected and distinguished from nontransfected cells. The experimental cells showed GFP expression in 37.5% of cells, whereas the negative control cells exhibited a minimal 0.082% of cells detected in the GFP channel (Figure 4C). These results indicate that our plasmid achieves a transfection efficiency that corresponds with the optimal transfection efficiency for SKOV3 cells with Lipofectamine 3000, which the manufacturer reports to be optimized at approximately 40%.

Finally, we evaluated protein expression via western blot analysis of cell lysates from untransfected and transfected cells, using six tag-specific antibodies. For SKOV3 cells, we observed considerable background stain and poor signal intensity, which was surprising given the clear results from microscopy and flow cytometry. However, when we performed western blot analysis of transfected HEK293T cells with the pMH-HA/Joseph2 plasmid we consistently noted a band of approximately 55 kDa in size in the lysates of transfected HEK293T cells (Figure 4D).

Altogether in two different mammalian cells, the expression of the multitagged protein of interest was validated using three distinct analytical approaches. Consequently, these pMH-HA/Joseph2 plasmids can be used as a positive control in these, and presumably other, mammalian cells.

Overall, our study has presented the successful utilization of the pEXP1/Joseph 2, pAHW/Joseph2, and pMHHA/Joseph2 plasmids for validating the expression and detection of epitope-tagged proteins of interest. This design proves effective across model systems ranging from *E. coli* to *Drosophila* S2 cells, as well as mammalian SKOV3 and HEK293T cells. These plasmids will be made freely available for use by other groups. As researchers begin to utilize these plasmids in diverse experimental setups, it will be valuable to evaluate their performance across different cell lines, and organisms. In the development of the pJoseph2 plasmids, we purposefully designed these constructs with strategically spaced epitope tags adjacent to the GFP coding region. This deliberate arrangement aimed to minimize potential influences arising from the relative locations of the epitopes within the polypeptide. Based on this design, we anticipate that the relative order of the epitope tags is unlikely to exert a major impact on the performance of these plasmids. Moreover, while it is essential to evaluate the performance of antibodies when conducting epitope tagging experiments, we have not tested a wide array of different antibodies. Instead, our approach focuses on providing researchers with standardized tools for epitope detection to enhance experimental reproducibility without requiring extensive antibody assessment. Such analyses could provide insights into any potential limitations or biases of the pJoseph2 system, thus guiding its refinement and adaptation to varying research needs.

4. Conclusion

By integrating multiple epitope tags to facilitate successful protein expression across various cell types, including *E. coli*, *Drosophila*, and human cells, we harnessed a diverse set of analytical methods, leading to the successful detection of our protein of interest. This approach provides a reliable means of troubleshooting experimental issues, enhancing the reliability of results, and advancing our understanding of protein–protein interactions, cellular pathways, and gene regulation. Building upon the foundational work by Munro and Pelham [1], the pJoseph2 plasmids can be a valuable asset for researchers in the field.

5. Future perspective

We envision that these plasmids can be used in research laboratories to solve the challenges caused by intermittent use of molecular biological reagents, such as for protein expression and purification in both prokaryotic and eukaryotic cells, for fluorescence microscopy, and for western blotting with standard epitope tags. It might also be valuable to generate a next-generation version of these plasmids wherein a restriction or recombinase site can be introduced so that additional epitope tags can be inserted. The pJoseph2 plasmids might also be valuable in laboratory classes for students to learn the basics of protein expression and detection.

Financial disclosure

The authors declare no competing financial interests. This work was supported by grant nos. GM061738 and GM124498 to RM Cripps, NSF 2205405 to CA Johnston, and CA260281 to CD House. C Bileckyj is supported by a predoctoral fellowship from the American Heart Association. JA Waters was partially supported by the San Diego State University/University of California San Diego Moores Cancer Center Comprehensive Partnership (U54 CA132384 and U54 CA132379). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Data availability statement

The plasmids pEXP1/Joseph2, pAHW/Joseph2, and pMHHA/Joseph2 will be submitted to Addgene following the publication of this work.

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Papers of special note have been highlighted as: •• of considerable interest

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Article Highlights

Background

- Epitope tags are broadly used in molecular biology for the purification and analysis of expressed recombinant proteins.
- Reproducible use of tagged proteins relies upon effective protein expression, purification, and detection using western blotting.
- These multistep techniques can suffer from experimental error and degradation of reagents.
- Therefore, there is a need for reagents to confirm that biochemicals are functional and that experimental steps have been performed correctly.
- We designed a set of plasmids to meet this need, termed the pJoseph2 family.

Materials & methods

- Three plasmids were generated, for expression in *Escherichia coli*, *Drosophila* S2 cells, or mammalian cells.
- Each plasmid was designed to express green fluorescent protein (GFP) tagged with MYC, Hemagglutinin, V5, 6xHis, and FLAG epitopes.

Results & discussion

- Expression of the tagged GFP was successfully achieved in each cell type and confirmed through visual observation.
- All epitopes could be detected through western blotting of purified proteins or cell lysates.

Conclusion

- The pJoseph2 family of plasmids can be used as positive controls for bacterial expression and purification, cell transfection, and western blotting by detecting GFP or several commonly used epitope tags.

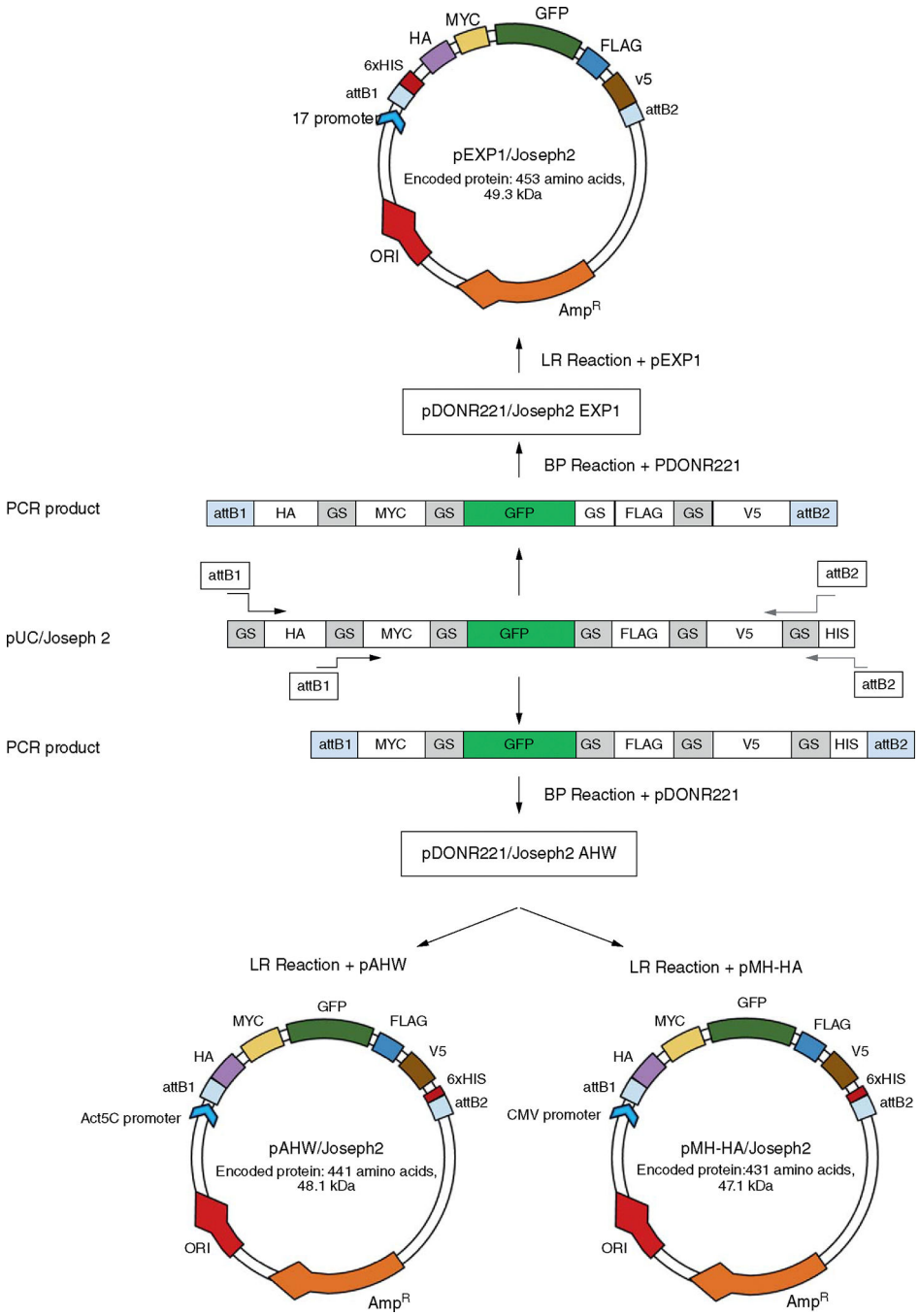

Figure 1.
Construction of pJoseph2 plasmids.

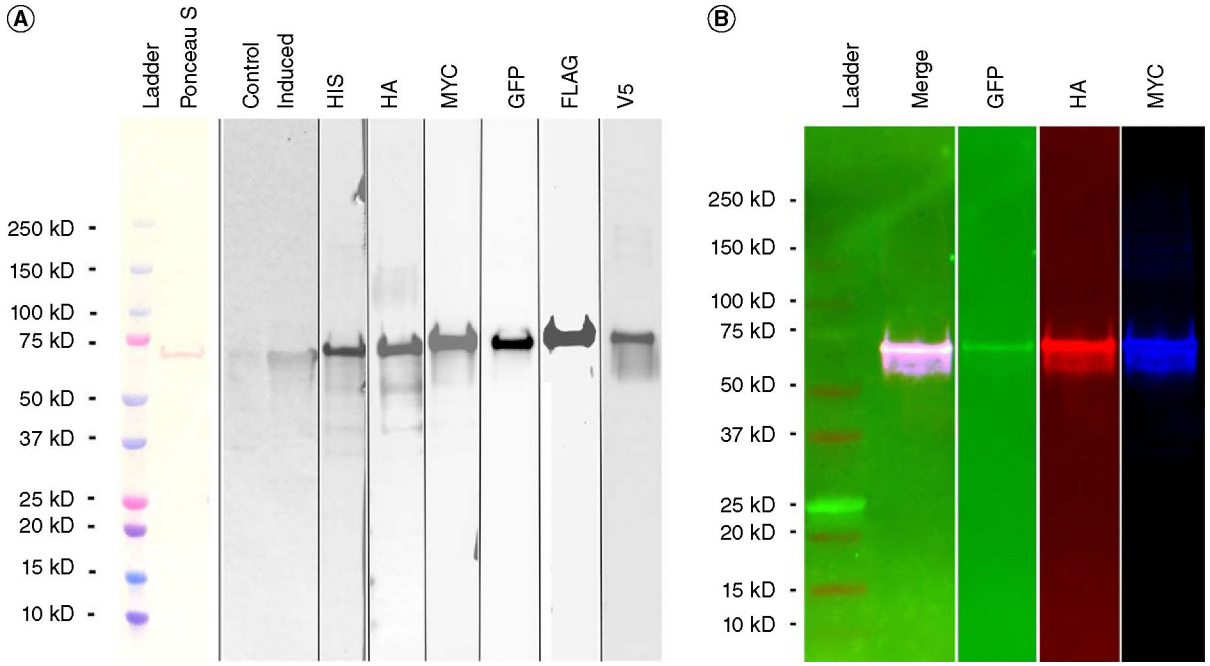

Figure 2.

Purification, expression, and detection of Joseph2 in *Escherichia coli*. **(A)** Joseph2 protein purified from *E. coli* transformed with the pEXP1/Joseph2 plasmid was run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and stained with Ponceau S alongside MW markers (lanes 1 and 2). Lanes 3 and 4: Joseph2 protein in uninduced (control) and induced *E. coli* lysates, detected with anti-His antibody. Lanes 5–10: duplicate nitrocellulose membrane strips were then reacted with antibodies recognizing the indicated epitopes. In all cases, a band of approximate apparent MW of ~65 kD was specifically recognized. **(B)** A similar nitrocellulose membrane strip was reacted with the indicated primary and fluorescently labeled secondary antibodies, and localized antibodies were detected alongside the endogenous green fluorescence of Joseph2.

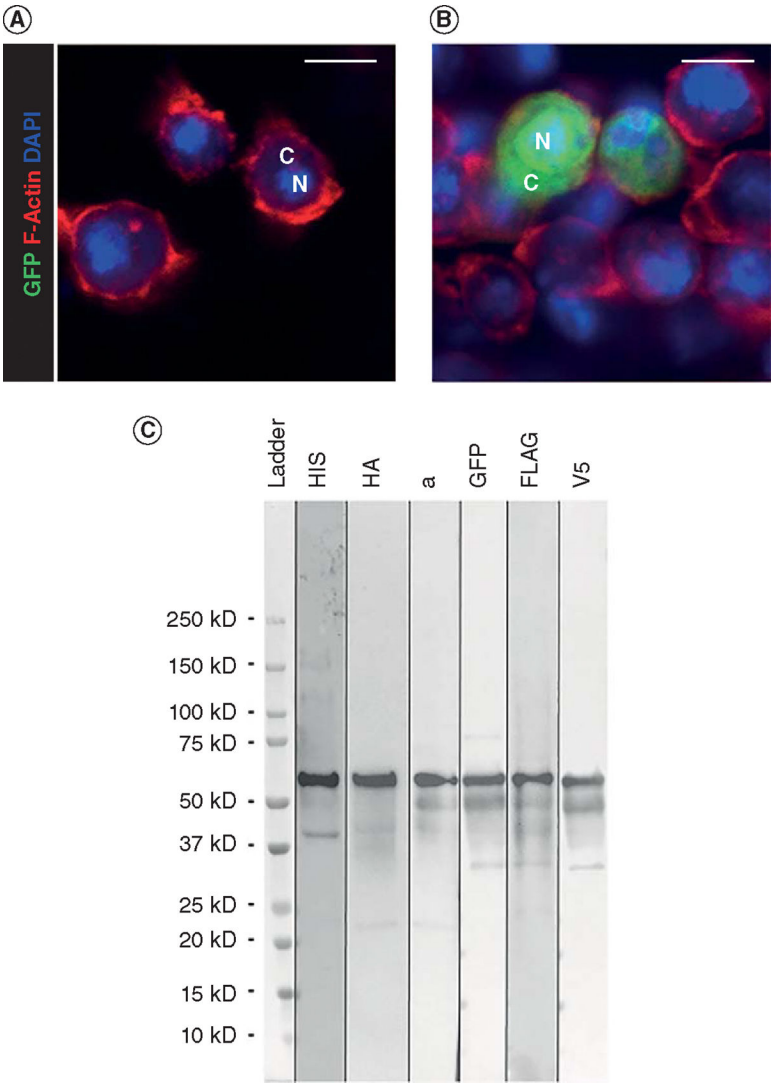

Figure 3.

Expression and detection of pJoseph2 using *Drosophila* S2 cells. S2 cells were either **(A)** untreated or **(B)** transfected with pAHW/Joseph2, fixed, and stained with fluorescently labeled Phalloidin (to detect F-actin, red) and DAPI (blue). These fluorophores, plus endogenous GFP signal, were detected by confocal microscopy. Bar 5 μm. **(C)** Lysates from S2 cells transfected with pAHW/Joseph2 were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with antibodies recognizing the indicated epitopes. In all cases, a band of approximate apparent MW of ~55kD was specifically recognized. C: Cytoplasm; DAPI: 4′,6′-Diamidino-2-phenylindole dihydrochloride; GFP: Green fluorescent protein; N: Nucleus; S2: Schneider's line 2 cells.

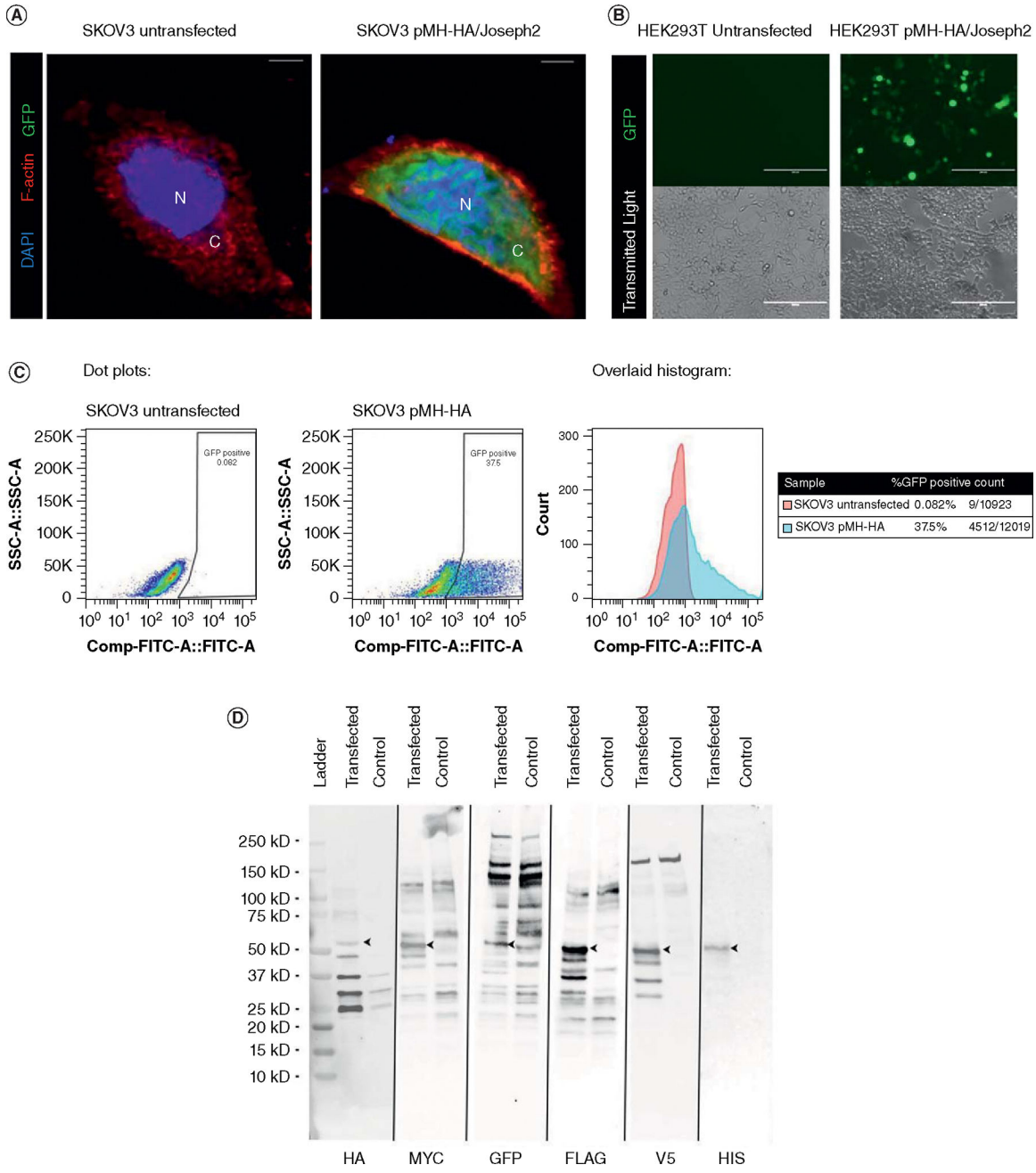

Figure 4.

Expression and detection of pJoseph using human SKOV3 cells. **(A)** SKOV3 cells were either untreated or transfected with pMH-HA/Joseph2, fixed, and stained with fluorescently labeled Phalloidin (to detect F-actin) and DAPI. These fluorophores, plus endogenous GFP signal, were detected by confocal microscopy. Bar 5 μm. **(B)** HEK293T cells either control or transfected with pMH-HA/Joseph2 were imaged live for endogenous GFP signal by transmitted light microscopy. Bar 200 μm. **(C)** Representative gating for flow cytometry assessing the population of GFP+ cells in SKOV3 nontransfected controls (left) or pMH-HA/Joseph2 transfected cells (right) with a histogram demonstrating the shift in

GFP+ cells (below). **(D)** Lysates from HEK293T cells transfected with pMH-HA/Joseph2 were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with antibodies recognizing the indicated epitopes. In all cases a band of approximate apparent MW of ~55 kD was specifically detected in transfected, but not nontransfected cells. C: Cytoplasm; DAPI: 4′,6′-Diamidino-2-phenylindole dihydrochloride; GFP: Green fluorescent protein; N: Nucleus.

Table 1.

Oligonucleotide sequences.

Name	Sequence
pEXPI F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGGCAGTGGAAGCGGTTGCTATCCCTACGATGTCCCTG
pEXPI R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACGAACCGCTACCCGTAGAATCGAGG
pAHW F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTCGGGCAGCGGAAGCGAGCAAAAGC
pAHW R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAATGGTGATGATGATGGTGGCTTCCAC
M13 F	GTAAAACGACGGCCAG
M13 R	CAGGAAACAGCTATGAC
T7	TAATACGACTCACTGTAGGG
T7 term	GCTAGTTGTTGCTCAGCGG
EGFP-C-F	CATGGTCCTGCTGGAGTTCGTG
EGFP-C-R	GTTCAGGGGGAGGTGTG
CMV	CGCAAATGGGCGGTAGGCGTG
BGHR	TAGAAGGCACAGTCGAGG

Table 2.

Antibody table.

Tags/antibodies	Name	Host animal	Dilution	Manufacturer	Catalog no.
His	6x-His Tag Monoclonal Antibody (HIS.H8) HRP	Mouse	1:5000	Invitrogen	MA1-21315-HRP
Hemagglutinin (HA)	HA-Tag Monoclonal Antibody HRP	Mouse	1:5000	Santa Cruz Biotechnology	sc-7392 HRP
MYC	Anti-Mec/c-Myc Antibody (9E10) HRP	Mouse	1:1000	Santa Cruz Biotechnology	sc-40 HRP
Gre en fluore scent protein	Ant i-GFP Ant ibody (B-2) HRP	Mouse	1:1000	Santa Cruz Biotechnology	sc-9996 HRP
FLAG	Ant i-OctA-Probe Antibody (H-5) HRP	Mouse	1:1000	Santa Cruz Biotechnology	sc-166355 HRP
V5	Anti-V5-Pro be Antibody (C-9) HRP	Mouse	1:1000	Santa Cruz Biotechnology	sc-271944 HRP
HA: fluorescent western blot primary antibody	HA Tag Monoclonal Antibody (2–2.2.14)	Mouse	1:5000	Invitrogen	26183-1MG
HA: fluorescent western blot secondary antibody	Goat anti-Mouse IgG (H+t) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 647	Goat	1:5000	Invitrogen	A32728
MeC: fluorescent western blot primary antibody	Myc-Tag (71D10) Rabbit mAb	Rabbit	1:5000	Cell Signaling Technology	2278S
MeC: fluorescent western blot secondary antibody	Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 790	Goat	1:5000	Invitrogen	A11369
F-actin	568 Phalloidin	–	1:400	Thermo Fisher Scientific	A12380
Nucleus	DAPI (4’, 6’ -diamidino-2-phenylindole dihydrochloride)	–	1:500	Thermo Fisher Scientific	D3571

HRP: Horseradish peroxidase.