A COMPLEMENTATION ASSAY FOR IN VIVO PROTEIN STRUCTURE/FUNCTION ANALYSIS IN Physcomitrella patens (FUNARIACEAE)¹

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Abstract

Protein structure/function relationships can be investigated using a structural model to generate hypotheses and an in vitro reconstitution assay for functional analysis of protein variants. However, this approach is only available for some proteins. For example, elucidation of the structure/function relationships for bacterial cellulose synthases has progressed due to two recent technical advances—solving of the crystal structure of Rhodobacter sphaeroides (van Niel 1944) Imhoff et al. 1984 cellulose synthase (Morgan et al., 2013) and in vitro reconstitution of functional cellulose synthase from purified R. sphaeroides proteins (Omadjela et al., 2013). A computational model for the cytoplasmic region of a plant cellulose synthase (designated CESA) from Gossypium hirsutum L. (Sethaphong et al., 2013) has been used to postulate functions of the structural features that distinguish CESA from bacterial cellulose synthases (Slabaugh et al., 2014a). However, methods for routine in vitro assay of CESA activity are not available for functional testing (Guerriero et al., 2010).

In vivo complementation assays provide alternatives to in vitro assays for protein functional analysis. This approach was used effectively to test an engineered point mutation in an Arabidopsis thaliana (L.) Heynh. CESA that was predicted to impair protein function based on computational modeling (Slabaugh et al., 2014b). However, due to the time and space required for genetic transformation and backcrossing, A. thaliana is not ideal for screening large numbers of mutations. As in all mosses, the protonema and gametophores of Physcomitrella patens (Hedw.) Bruch & Schimp. are haploid. Thus, mutant phenotypes can be detected immediately without the need for backcrossing to produce homozygous diploid lines. This makes P. patens a faster alternative to A. thaliana for assays that involve genetic transformation (Cove, 2005; Cove et al., 2006).

Physcomitrella patens cell walls contain the same classes of polysaccharides as vascular plant cell walls, with some differences in side-chain structure (Roberts et al., 2012). Also, P. patens has rosette cellulose synthesis complexes (Roberts et al., 2012) and seven CESA genes (Roberts and Bushoven, 2007). However, consistent with the absence of lignified vascular tissue in mosses, the PpCESA genes are not orthologous to the CESA genes that synthesize primary and secondary cell walls in seed plants (Roberts and Bushoven, 2007). We have previously generated ppcesa5 knockout lines that are deficient in gametophore production. In these lines, the gametophore buds typically produce irregular clumps of tissue, but occasionally form aberrant gametophores consisting of a few small deformed leaves with irregular phyllotaxy (Goss et al., 2012). When transformed with a vector that drives constitutive expression of
the wild-type P. patens PpCESA5 protein, these lines produce normal gametophores (Goss et al., 2012). This provides a platform for developing a complementation assay to test CESAs proteins that have been modified by site-directed mutagenesis with the extent of rescue of the mutant phenotype providing a measure of protein function.

Here we describe a complementation assay in P. patens for analysis of structure/function relationships in plant CESAs proteins that is rapid and has modest space requirements. Although designed for analysis of CESAs proteins, this assay could be adapted to study structure/function relationships of other proteins where the knockout phenotype can be easily scored.

METHODS AND RESULTS

Vector construction and ppcesa5KO-2 transformation—Expression vectors carrying a wild-type (positive control) or a mutated (test) PpCESA5 coding sequence fused at the N-terminus to a triple hemagglutinin (3XHA) epitope tag were constructed using PCR fusion and MultiSite Gateway cloning (Atanassov et al., 2009). To create Gateway entry clones carrying point mutations, gene fragments were amplified from wild-type PpCESA5 cDNA clone pdp24095 (RIKEN BioResource Center, http://www.brc.rikencei.or.jp/lab/epd/Engr/) using primer pairs that added an att fl site at one end and introduced a mutation at the other (Appendix 1; Appendix 2, Fig. A1, A2). The fragments were fused in a single overlap extension reaction (Appendix 2) and cloned into pDONR 221 P5-P2 according to the manufacturer’s instructions (Life Technologies, Grand Island, New York, USA). CESAs entry clones were sequence verified using primers listed in Appendix 3. To create the 3XHA entry clone, two oligonucleotides (Appendix 1) were fused, cloned into pDONR 221 P1-P5r (Life Technologies), and sequence verified using primer M13 Forward (~20) (Life Technologies).

Each CESAs entry clone was transferred along with a S3XHA entry clone to the pTHAct1Gate destination vector using LR Clonase II Plus as described by the manufacturer (Life Technologies). The pTHAct1Gate vector contains an Act1 promoter from rice, which drives constitutive expression in P. patens, and sequences that target the vector to the P. patens 108 locus, which can be disrupted without producing a phenotype (Perroud and Quatrano, 2006).

Protoplasts prepared from mutant P. patens line ppcesa5KO-2 (Goss et al., 2012) were divided into five or six samples (6 × 10^6 protoplasts each) and each sample was transformed with a test (R453K, R453D, or R453G), positive wild-type CESAs5 expression, or empty pTHAct1 negative control vector (Table 1), following the detailed protocol of Roberts et al. (2011). Regenerated protoplasts were subjected to two rounds of selection (Roberts et al., 2011), which typically resulted in 10–40 vigorously growing, stably transformed lines for each vector (Appendix 2, Fig. A3). Results from an experiment (i.e., five to six transformations using the same batch of protoplasts) were excluded from the statistical analysis when fewer than 10 lines were obtained for the positive or negative control transformations. In cases where one or more test vectors from a transformation using the same batch of protoplasts were excluded from the evaluation phenotypes using primary colonies derived from independently generated lines for each vector were compared to the positive and negative controls.

Table 1. Expression vectors used to transform Physcomitrella patens.

| Name       | Expression cassette        | Comments                                      |
|------------|---------------------------|----------------------------------------------|
| CESAs      | Act1:3XHA-PpCESA5         | Positive control                             |
| Empty      | None                      | Negative control                             |
| R453K      | Act1:3XHA-ppcesa5-R453K   | Homologous to fra6*                          |
| R453G      | Act1:3XHA-ppcesa5-R453G   | Variant of R453K                             |
| R453D      | Act1:3XHA-ppcesa5-R453D   | Variant of R453K                             |

*Zhong et al., 2003.

To make the assay as rapid as possible, we evaluated phenotypes using primary colonies derived from independently transformed protoplasts and selected for stable antibiotic resistance. In contrast to tissue subcultured from wild-type and mutant lines, the positive and negative control transformations produced multiple colonies, each representing an independent stably transformed line, that could be directly and quantitatively compared to colonies derived from test transformations. Comparisons were based on the percentage of stably transformed lines that produced gametophores and on gametophore morphology. A dissecting microscope (Meiji EMZ-TR, Meiji Techno Co. Ltd., Tokyo, Japan) was used at 30× to count the number of lines with and without gametophores. Colonies and individual gametophores were photographed using a stereomicroscope (Leica M165 FC with Leica BFC310 FX camera, Leica Microsystems, Buffalo Grove, Illinois, USA) and individual leaves were photographed using a compound microscope (Olympus BX51, Olympus Corporation, Lake Success, New York, USA) with a Spot Flex camera (Diagnostic Instruments, Sterling Heights, Michigan, USA). The positive control CESAs5 vector complemented the ppcesa5KO-2 phenotype, with 78% ± 3.8% of lines (average for seven independent transformations: 138 stably transformed lines) producing gametophores that generally resembled wild-type gametophores (Fig. 1A). When ppcesa5KO-2 was transformed with the empty negative control vector, only 13% ± 4.9% of lines (average for seven independent transformations: 182 stably transformed lines) produced gametophores. These were distinctly abnormal with fewer than 10 small misshapen leaves and abnormal phyllotaxy (Fig. 1A) and resembled those that are produced at low frequency by the untransformed ppcesa5KO-2 line (Goss et al., 2012). Fewer than 10% of stable lines produced gametophores in a typical negative control transformation, and even when the percentage was higher the gametophores were never normal in appearance. To ensure consistency, colonies were scored for presence and absence of gametophores without considering gametophore morphology. Statistical analysis (see below) confirmed that this was adequate for distinguishing test vectors that complemented or failed to complement the mutant phenotype. However, deviations from expected morphologies were recorded, and this information was used to identify vectors that partially complemented the mutant phenotype (see below).

We tested the complementation assay with expression vectors carrying the PpCESA5 coding sequence with three different point mutations at R453 (Table 1). The R453K mutation is homologous to fra6, a recessive mutation in A. thaliana (AtCESA8, R362K) that results in decreased fiber wall thickness and cellulose content, but has a less severe phenotype compared to other atcesa8 mutant alleles (Zhong et al., 2003). Although AtCESA8 expression is correlated with secondary cell wall deposition and fra6 is defective in fiber development, the affected residue is conserved (Zhong et al., 2003). Thus, the phenotypes for homologous mutations in different CESAs genes would be expected to depend on the developmental function of the mutated CESAs. When the ppcesa5KO-2 line was transformed with the R453K expression vector, the percentage of stable lines producing gametophores was intermediate between the positive and negative control and gametophore morphology was similar to wild type (Fig. 1). When the basic polar R453 residue was instead replaced with an acidic polar D residue (R453D), all stable lines lacked gametophores (Fig. 1). Finally, replacement of R453 with a nonpolar G residue (R453G) yielded results similar to the positive control for both percentage of stable lines with gametophores and gametophore morphology (Fig. 1).

Statistics and experimental analysis—A two-tailed Fisher’s exact test of independence (Sokal and Rohlf, 1981) was used for statistical analysis of complementation. Statistics were calculated using the 2 × 2 contingency table at http://vassarstats.net/tab2x2.html and sample calculations were verified manually. We first confirmed that the numbers of colonies with and without gametophores were significantly different for the CESAs5 positive control and the empty negative control vectors (P < 0.0001, pooled results from seven experiments). Comparison of each test vector to the positive and negative controls using counts pooled from two or three transformations (Fig. 1B) showed that R453K and R453D differed significantly from the positive control (P < 0.0001), but not the empty negative control vector (P = 0.27 and P = 0.50, respectively). In contrast, R453G differed significantly from the negative control (P < 0.0001), but not the CESAs5 positive control vector (P = 0.14). Although R453K did not differ significantly from the negative control vector in the number of colonies with and without gametophores, the gametophores that were produced resembled those observed following transformation with the CESAs5 positive control vector (Fig. 1A), indicating that the encoded protein may be partially functional. The vectors tested fall into three classes (1) R453G fully complements, (2) R453D does not complement, and (3) R453K partially complements the ppcesa5KO-2 phenotype. Thus, this relatively rapid assay allowed us to screen mutations and tentatively identify those that generate functional, non-functional, and partially functional proteins. Because these three classes of mutations could be distinguished based on statistical comparison to positive and negative controls, we were able to streamline the assay by not genotyping the
stable transformatants. The failure of gametophore production in 12–39% of the stable lines from transformation with the positive control CESA5 vector can potentially be explained by (1) disruption of the promoter or coding sequence of the vector during integration, which would result in lack of transgene expression, or (2) disruption of another locus that is necessary for gametophore development due to mistargeting of the vector, which would explain lack of complementation when protein expression is detected (see below). This is supported by the observation that transformation of P. patens with a gene disruption library resulted in a high proportion of lines with gametophore defects (Egener et al., 2002). These mistargeting events are stochastic (Kamisugi et al., 2006), and statistical analysis confirms that they do not interfere with the ability to distinguish complementing from noncomplementing vectors and partially complementing vectors.

Protein determination—Before concluding that a specific mutation impairs the function of an encoded protein, it is necessary to rule out lack of expression of the full-length transgenic protein as an explanation for the failure of the vector to complement the mutant phenotype. This was tested by Western blot analysis of microsomal proteins isolated from arbitrarily selected stable lines representing a small proportion of lines tested for complementation (see Appendix 2 for protein extraction methods). Equal masses of protein extracted from 13 stable CESA5 positive control lines, or 12 stable lines transformed with a particular test vector along with one CESA5 positive control line, were separated by polyacrylamide gel electrophoresis (PAGE) and transferred to membranes for total protein determination (positive control), an empty vector control or vectors driving expression of PpCESA5 carrying engineered point mutations (R453K and R453G). Scale bars shown in the first column apply to all images in the row. B. pcesa5KO-2 complementation rates for vectors driving expression of PpCESA5 carrying engineered point mutations compared to positive and negative control vectors from the same experiment. Means for two (R453K and R453D) or three (R453G) experiments are shown with P values determined using a two-tailed Fisher’s exact test of independence.

Comparison to other methods—Complementation assays provide means to test hypotheses about the functions of specific proteins and protein modifications and are particularly useful when a robust in vitro assay is not available, as is the case for CESA proteins (Guerriero et al., 2010). Arabidopsis thaliana has been used previously for genetic analysis of engineered CESA variants (Wang et al., 2006; Chen et al., 2010; Bischoff et al., 2011; Slabaugh et al., 2014b). However, P. patens has the advantages that transformation, selection, and scoring can be completed in 33 d and less space is required to maintain cultures. In A. thaliana, production of transformants requires three months (Zhang et al., 2006), with additional time needed for backcrossing to obtain homozygous lines. Because the dominant phase of the P. patens life cycle is haploid, backcrossing in unnecessary.

In P. patens, an assay has been developed for using image analysis to screen for mutants that affect protoxenial growth and to test for transient complementation of those phenotypes (Bibeau and Vidali, 2014). In contrast to this highly sensitive assay for detecting protoxenial growth defects, the assay reported here is suitable for analysis of proteins involved in other aspects of P. patens development. It is common practice to use complementation analysis to verify knockout phenotypes in P. patens, and cross-species complementation has been used to test for functional conservation of vascular plant and P. patens proteins (e.g., Bravo-Garcia et al., 2009). Here we have described a systematic approach for quantitative analysis of complementation based on comparison to positive and negative controls that can be used for functional testing of heterologous genes, as well as engineered mutations.

An effective complementation assay is based on a mutant with a measurable phenotype that is easy to score. The absence of normal gametophores in the ppcesa5KO mutants (Goss et al., 2012) is readily observed with a dissecting
allowing classification of engineered proteins as either fully functional, partially functional, or nonfunctional, (3) engineering of genes through PCR fusion without introduction of restriction or recombination sites, (4) epitope-tagging to facilitate testing for expression of full-length transgenic protein through Western blotting, and (5) efficiency due to rapid development of *P. patens* and no requirements for backcrossing or genotyping. Thus, *P. patens* is well suited for rapid identification of mutations, which can be further investigated in other organisms, if desired.

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In summary, the strengths of this complementation assay include (1) a clear phenotype that can be easily scored, (2) inclusion of positive and negative controls and statistical analysis allowing classification of engineered proteins as either fully functional, partially functional, or nonfunctional, (3) engineering of genes through PCR fusion without introduction of restriction or recombination sites, (4) epitope-tagging to facilitate testing for expression of full-length transgenic protein through Western blotting, and (5) efficiency due to rapid development of *P. patens* and no requirements for backcrossing or genotyping. Thus, *P. patens* is well suited for rapid identification of mutations, which can be further investigated in other organisms, if desired.

**CONCLUSIONS**

In summary, the strengths of this complementation assay include (1) a clear phenotype that can be easily scored, (2) inclusion of positive and negative controls and statistical analysis allowing classification of engineered proteins as either fully functional, partially functional, or nonfunctional, (3) engineering of genes through PCR fusion without introduction of restriction or recombination sites, (4) epitope-tagging to facilitate testing for expression of full-length transgenic protein through Western blotting, and (5) efficiency due to rapid development of *P. patens* and no requirements for backcrossing or genotyping. Thus, *P. patens* is well suited for rapid identification of mutations, which can be further investigated in other organisms, if desired.
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APPENDIX 1. Primers used in vector construction. The att sites are shown in yellow, start codons are shown in green, stop codon is shown in red, and engineered mutations are shown in blue.

| Primer          | Sequence                                                                 |
|-----------------|--------------------------------------------------------------------------|
| CESA5-attB5     | 5' - GGGGACAACCTTTGTAATAACAAAAGTTGCCATGAGGCTAATGCGGCTTTAT-3'             |
| CESA5-attB2     | 5' - GGGGACCACTTTGTAATACAAAAGCTGGGTATACAAAGTTGCCATGAGGCTTTAT-3'          |
| R453G-SF1       | 5' - GTGAAGGAGCCGCGCTATGAAAGCGA-3'                                      |
| R453G-SR1       | 5' - TCGCTTCATAGCCGCGCTCCTTCAC-3'                                      |
| R453F-SF1       | 5' - GTGAAGGAGCCGCGAGTATGAAAGCGA-3'                                     |
| R453F-SR1       | 5' - TCGCTTCATAGCCGCGCTCCTTCAC-3'                                      |
| 3XHA-attB1      | 5' - GGGGACAACCTTTGTAATAACAAAAGTTGCCATGAGGCTAATGCGGCTTTAT-3'             |
| 3XHA-attB5      | 5' - GGGGACAACCTTTGTAATAACAAAAGTTGCCATGAGGCTAATGCGGCTTTAT-3'             |

http://www.bioone.org/loi/apps
**APPENDIX 2.** Benchtop protocol for the *Physcomitrella patens* complementation assay.

**Vector construction**

PCR fusion for Gateway: This protocol was modified from Atanassov et al. (2009).

1. Design attB5 and attB2 primers that include the att sites along with sequences from your gene of interest, including the start and stop codons, and SF1 and SR1 primers that include an engineered mutation with about 12 nucleotides upstream and downstream of the mutation (Appendix 1, Fig. A1).

2. Amplify fragments F1 and F2 (Fig. A1) in 50-μL reactions using Phusion DNA polymerase (M0530S, New England Biolabs, Ipswich, Massachusetts, USA) and recommended cycling conditions. We used a 25-s annealing step at 68°C, but this may need to be adjusted depending on the primers used.

![Fig. A1. A schematic of site-directed mutagenesis through PCR fusion.](image)

3. Run 1 μL of each PCR reaction on a gel with a calibrated ladder (Fig. A2) to verify fragment size and estimate DNA concentration.

4. If correct, proceed to PCR fusion with the following caveats:
   - Total DNA mass must not exceed 800 ng.
   - Add fragments in equimolar amounts. Example: F1 is ~2500 bp and F2 is ~1000 bp, thus 480 ng of F1 and 320 ng of F2 were used (Fig. A2).

![PCR Fusion mix:](image)

5. PEG purify the PCR fusion product using 30% PEG/MgCl₂ included in the Invitrogen MultiSite Gateway Pro (12537-102) or BP Clonase II (11789-020) kit according to the manufacturer’s instructions (Life Technologies, Grand Island, New York, USA) and resuspend the pellet in 7 μL of TE buffer.

**Expression vector construction:**

1. Clone the PCR fusion product (3.5 μL) into pDONR P5-P2 in a BP reaction using Invitrogen MultiSite Gateway Pro according to the manufacturer’s instructions (Life Technologies).

2. Transform competent *E. coli* with 2 μL of the BP reaction and incubate on the appropriate selective medium according to the manufacturer’s instructions (e.g., Invitrogen TOP10, Life Technologies).

3. Isolate plasmid DNA from four overnight cultures inoculated with single colonies using a commercial kit according to the manufacturer’s instructions (e.g., QIAprep Spin Miniprep Kit, QIAGEN, Venlo, The Netherlands).

4. Design and perform restriction digests to identify plasmids containing the expected insert. For the CESA5 entry clones, 2.5 units each of EcoRI-HF and EcoRV-HF were used in 20-μL reactions according to the manufacturer’s instructions (New England Biolabs).
Fig. A2. PCR products separated on a 1% agarose gel (100 V, 25 min). Concentrations of F1 and F2 were both estimated at ~240 ng/μL. However, F1 is ~2500 bp and F2 is ~1000 bp. Thus, 2 μL of PCR reaction F1 was combined with 1.33 μL of PCR reaction F2 for the PCR fusion reaction.

5. Sequence a plasmid with the expected restriction fragment pattern to verify the mutation and the absence of PCR errors. The primers used to sequence the CESA5 entry clones are listed in Appendix 3.

6. To create an expression vector, clone the sequence-verified plasmid along with a pDONR P1-P5r entry clone containing an epitope tag (available from the author) into pHAct1Gate (available from Pierre-Francois Perroud, Washington University, St. Louis, Missouri, USA) using LR Clonase II Plus as described by the manufacturer (Life Technologies).

7. Transform competent E. coli with the LR reaction and incubate on the appropriate selective medium as in step 2.

8. Isolate plasmid DNA from four overnight cultures inoculated with single colonies as in step 3.

9. Digest 1 μL of each plasmid prep with 2.5 units of SwaI in a 20-μL reaction according the manufacturer’s instructions (New England Biolabs) to screen for the expected restriction fragment pattern (2519-bp backbone plus the expression vector of approximately 10,000 bp).

10. Sequence a plasmid with the expected restriction fragment pattern to confirm proper integration of both entry clones into the destination vector. Primer P395 (Appendix 3) was used for expression vectors containing CESA5 entry clones.

Expression vector preparation:
1. Isolate at least 300 μg of plasmid DNA using a commercial midiprep kit (e.g., GenElute HP Plasmid Midiprep Kit [Sigma-Aldrich, St. Louis, Missouri, USA] or Nucleobond Xtra Midi [Machery-Nagel, Düren, Germany]) or another method.

2. Linearize 100 μg of plasmid DNA with 100 units of SwaI in a 200-μL reaction according the manufacturer’s instructions (New England Biolabs).

3. Ethanol precipitate DNA using sterile technique as described previously (Roberts et al., 2011).

Protoplast isolation and transformation
1. Prepare protoplasts from two plates according to the detailed protocol provided in Roberts et al. (2011) through step 3.3.11 using a P. patens line in which your gene of interest is knocked out. We have achieved higher regeneration rates when protoplasts were isolated separately from two plates and combined in the final wash step.

2. After resuspension in 3M medium (step 3.3.11), transfer 6 × 10^5 protoplasts to each of 5–6 tubes containing 50 μg of DNA (one positive control, one negative control, and up to four test vectors) and proceed with transformation and selection as described (Roberts et al., 2011).

Complementation assay
1. After the second round of selection, array stably transformed lines (Fig. A3) on plates containing BCDAT medium (Roberts et al., 2011).

2. Incubate at 25°C with fluorescent lights at a photon flux density of 85 μM m^-2 s^-1 for 7 d.

3. To score the cesA5KO phenotype, use a dissecting microscope at 30× to count the number of lines with gametophores. To test complementation of a different gene, a scoring method can be developed that is appropriate for the mutant phenotype.

4. If necessary, use a compound microscope to further analyze the morphology of specific tissues (protonemal, gametophores, etc.).

Statistics
In the case of the ppcesaR453 mutants, Fisher’s two-tailed exact test (Sokal and Rohlf, 1981), calculated using the 2 × 2 contingency table at http://vassarstats.net/tab2x2.html, was used to test the significance of test rescue rates vs. rescue rates of the positive and negative controls. However, another test may be more appropriate depending on how the phenotype is scored.

Microsomal protein extraction
This protocol was modified from Hutton et al. (1998).

1. Collect colonies for protein extraction when they are 0.75–1 cm in diameter (2–3 wk after they are arrayed on BCDAT, Fig. A3D).

   • Remove traces of agar and blot colonies firmly with tissue to remove surface water.

   • Place each colony in a preweighed 1.5-mL centrifuge tube on ice.
Reweigh tubes to determine tissue weight (expected range 8–12 mg).
Tissue may be stored at −80 °C prior to protein extraction.
Samples and reagents must remain on ice for the remainder of this protocol.

2. Add 300 μL of extraction buffer to each tube (50 mM HEPES [pH 7.6], 0.1 mM EDTA, 500 mM sucrose, 1 : 100 protease inhibitor cocktail for plant cell lysate, P9599 [Sigma-Aldrich]).
3. Homogenize tissue using a motor-driven pestle (Z359971, Sigma-Aldrich) until no visible clumps remain.
4. Centrifuge at 10,000 × g, 10 min, 4 °C.
5. Collect supernatant and centrifuge at 10,000 × g, 15 min, 4 °C.
6. Transfer supernatant to an ultracentrifuge tube and centrifuge at 107,400 × g, 30 min, 4 °C.
7. Remove supernatant and resuspend pellet in 32 μL of sample buffer (30 mM Tris [pH 6.8], 2% SDS, 0.00002% bromphenol blue, 10% glycerol).

Protein quantification and normalization

Determine the protein concentrations of microsomal extracts using the Pierce BCA Protein Assay kit (23225, Thermo Scientific Inc., Rockford, Illinois, USA) according to the manufacturer’s instructions with the following modifications:

- Centrifuge samples and standards for 30 s, 13,000 × g.
- Test 7-μL samples of microsomal extracts (in duplicate) and each standard (expect 500 μg/mL or higher for microsomal extracts).

Western blot

1. Normalize samples to the lowest concentration by adjusting loading volume or adding sample buffer to adjust concentration.
2. Add 10% β-mercaptoethanol to each sample.
3. Run samples (at least 5 μg, but ideally 7–10 μg, total protein) on an SDS-PAGE gel and transfer proteins to a PVDF membrane.
4. Stain membranes with Ponceau S to confirm consistent protein loading (Romero-Calvo et al., 2010). Additional washing with TBST (50 mM Tris [pH 7.5], 150 mM NaCl, 0.05% Tween 20) may be required to remove stain.
5. Probe membrane with an antibody that detects the transgenic protein. Our method follows, but other methods could be used.
   - Block membrane in 5% (w/v) milk protein in TBST, 80 rpm, room temperature (RT), 1 h.
   - Rinse membrane with TBST.
   - Incubate membrane with 1:1000 anti-HA (HA.11 Clone 16B120 [BioLegend, Dedham, Massachusetts, USA]) in 5% (w/v) blocking agent in 1× TBST + 0.02% sodium azide, 80 rpm, 4°C, 12–16 h.
   - Wash membrane in TBST, 80 rpm, 4 min. Repeat three times.
   - Incubate membrane in 1:4000 HRP-conjugated antismouse (A4416, Sigma-Aldrich), 80 rpm, RT, 1 h.
   - Wash membrane in TBST, 80 rpm, 4 min. Repeat three times.
   - Develop membrane using Pierce ECL Western Blotting Substrate kit (32106, Life Technologies) according to the manufacturer’s instructions.