3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

***In Vitro* Mammalian Optimized Split Fluorescent Protein "Fold 'n' Glow"™ Solubility Assay Kits**

Catalog Number
APPA005 – *In Vitro* Mammalian Split GFP "Fold 'n' Glow" Solubility Assay Kit (Green)
APPA010 – *In Vitro* Mammalian Split CFP "Fold 'n' Glow" Solubility Assay Kit (Cyan)
APPA011 – *In Vitro* Mammalian Split YFP "Fold 'n' Glow" Solubility Assay Kit (Yellow)
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

The "Fold 'n' Glow"™ Protein Solubility Assay kit allows a test protein to be expressed as an N-terminal fusion with a fluorescent protein [i.e., Green Fluorescent Protein (GFP), Cyan Fluorescent Protein (CFP), and Yellow Fluorescent Protein (YFP)]. This allows the detection of protein properly folded in a given sample as the folding reporter gives a signal directly proportional to the amount of correctly folded protein.[1] The kit can be used for the detection and quantification of any protein by tagging and detecting either soluble or insoluble proteins.

Fluorescent protein (GFP, CFP, or YFP) fusions and split protein tags are widely used for the analysis of protein. These large tags can perturb protein solubility, misfold, and alter the processing of the protein. The split fluorescent protein technology used in the "Fold 'n' Glow" assay overcomes these problems. The protein tag is a genetically encoded, split fluorescent protein technology, engineered with small, soluble, self-associating fragments. Thus, it is a simple split fluorescent protein system that doesn't change fusion protein solubility, or require chemical ligation, fused interacting partners, co-expression, or co-refolding. Furthermore, while fluorogenic biarsenical FlaSH or ReASH substrates also overcome these limitations, they also require many other conditions not necessary when using the split fluorescent protein technology. The fluorescent protein system is a simple and easy tagging and detection system.[2] These kits may be used to quantify the expression level of the tagged protein, to determine the solubility of a protein, or to determine the solubility of a protein's domain.

The kit is a protein tagging and detection method that uses split fluorescent protein technology in a fluorescent complementation assay. The protein to be quantified is fused to a small fluorescent protein fragment (contained in the S11 plasmid) via a flexible linker. Expressed separately, neither the fusion protein of interest nor the fluorescent protein detector (Universal Detection Reagent S1-10) is fluorescent. When mixed, the properly folded fusion protein and detector spontaneously associate, completing the fluorophore. Misfolding or aggregation of the fusion protein makes the fluorescent protein tag inaccessible and prevents complementation, thus preventing fluorescence. Therefore, misfolded or aggregated proteins are not included in the quantification of the protein of interest.

Components

The kit contains sufficient reagents for one 96 well plate (96 tests).

Reagent	Amount
Mammalian S11 Single Plasmid (APPA006)	1 vial
Positive Control (APPA003)	1 vial
Universal Detection Reagent (S1-10): Green (APPA001) or Cyan (APPA008) or Yellow (APPA009)	20 mL

Universal Detection Reagent (S1-10) – Complementary fluorescent protein fragment. Supplied ready to use.

Positive Control (APPA003) – Positive control fluorescent protein fusion protein. Supplied ready to use at 5 μM.

Mammalian S11 Single Plasmid (APPA006) – Kanamycin (Kan^R) and Neomycin (Neo^R) resistance with *Bam*H I and *Nde* I restriction sites. Supplied at a concentration of 100 ng/mL. For additional restriction sites and plasmid information, see Appendix A.

Materials required, but not provided:

- GC5™ competent cells (Cat. No. G3169)
- Kanamycin
- Neomycin
- Eukaryotic transfection system
- IPTG (Ispropyl β-D-thiogalactopyranoside)
- LB growth media and plates
- *Bam*H I and *Nde* I restriction enzymes
- Ligation materials
- Metal affinity column
- TNG Buffer (50 mM Tris, pH 7.4, 0.1 M NaCl, and 10% glycerol)
- Bovine serum albumin (BSA)
- Plasmid isolation reagents
- 96 well plate
- Incubator
- Centrifuge
- Sonicator
- Microplate fluorescence reader
- Vortex mixer
- Water bath

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The components of this kit remain active for approximately 6 months when stored at –20 °C.

Depending on the particular usage requirements, it may be appropriate to re-aliquot reagents to smaller working volumes to avoid repeated freeze-thaw cycles or repeated pipetting from the same vial.

Procedures

A. Preparation of insert DNA
   1. Perform plasmid prep and/or PCR.
   2. Use *Nde* I (5′) and *Bam*H I (3′) restriction sites to perform a restriction digest that generates overhangs on the DNA insert.
      Optional: Purify digest fragment from agarose gel.

B. Preparation of S11 vector
   Appendix A contains additional plasmid information.
   1. To ensure a renewable source of plasmid DNA, transform the plasmid vector provided in this kit in an *E. coli* host strain. It is recommended bacterial frozen stocks be prepared for all transformed plasmids using standard molecular biology techniques.
   2. Purify plasmid DNA for cloning using a plasmid purification kit or other plasmid isolation technique.
   3. Perform restriction enzyme digest of the S11 vector using *Nde* I and *Bam*H I to prepare the plasmid for inserting DNA.
      Optional: Dephosphorylate the digest to decrease the non-recombinant background. Use molecular biology grade calf intestinal or shrimp alkaline phosphatase according to the manufacturer's directions.
   4. Perform ligation reaction according to manufacturer's instructions.
   5. Store vector at –20 °C until ready to use.

C. Clone DNA insert as an N-terminal fusion into S11 vector
   Appendix B contains additional S11 cloning information.
   1. Ligate the DNA insert with the digested S11 using standard DNA ligation protocol and manufacturer's protocol resulting in S11 fusion plasmid.
   2. Transform the S11 fusion plasmid in an expression host for high yields of quality plasmid. Use standard methods on the screening host used.
   3. Identify the positive clones using standard methods.
      Note: IPTG/X-gal screening is effective in the first 24 hours post plating as the T7 promoter is highly active and absorbs resources from the *LacZ* gene.
   4. Perform plasmid DNA purification, sequence to verify reading frame, or use *in vitro* transcription/translation.

D. Transfection of Eukaryotic System
Transfect the eukaryotic system of choice using the manufacturer's recommended protocol.

E. *In vitro* Complementation assay
1. Prepare 96 well plate(s).
   a. Block a 96 well flat bottom microplate for 10 minutes with 0.5% (w/v) bovine serum albumin in TNG buffer.
   b. Prepare 7 serial dilutions of Positive Control (SRS11, 5 μM) with TNG buffer (22 μL:22 μL) down to 39 nM, or lower depending on instrument sensitivity.
   c. Add 20 μL of the Positive Control (APPA003, 5 μM) and each serial dilution to the appropriate wells of the first two columns of a 96 well plate (see Appendix C).
   d. Add 20 μL aliquots of the protein fusion(s) prepared in Procedure, part C to the remaining wells in the same 96 well plate.
   e. Prepare a negative control of 0.5% BSA in TNG buffer and add 20 μL to the plate.
   f. Perform complementation by adding 180 μL aliquot of Universal Detection Reagent (S1-10, i.e., GFP, CFP, or YFP) to all the wells, except the blank.
2. Monitor the fluorescence kinetics with a microplate fluorescence reader or fluorimeter at 3 minute intervals for 15 hours. Use the excitation and emission wavelengths in Table 1 for monitoring fluorescence.

Table 1.
Fluorescence wavelengths

Fluorescent protein	$\lambda_{excitation}$	$\lambda_{emission}$
GFP	488 nm	525 nm
CFP	488 nm	500 nm
YFP	488 nm	550 nm

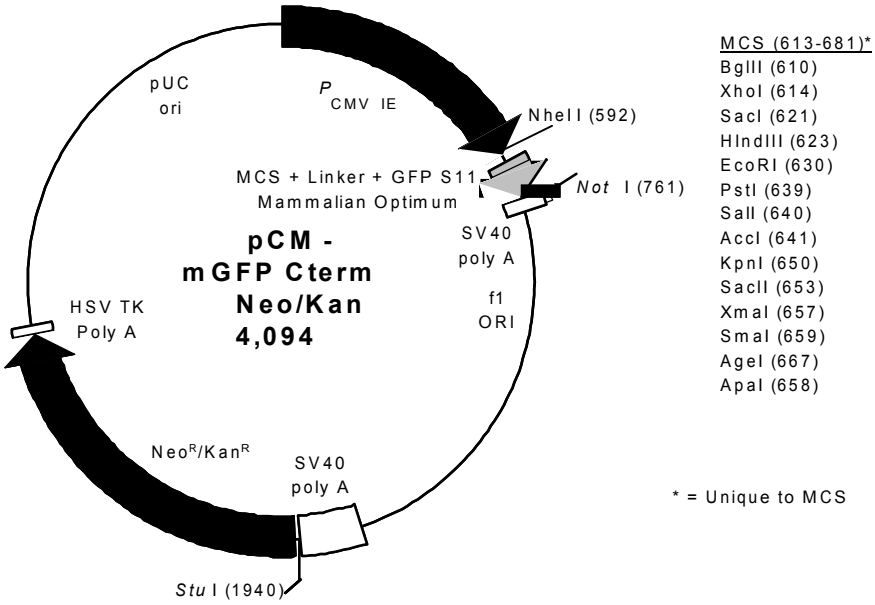
Note: Emission wavelengths can be ±25 nm

Data Analysis and Results
Subtract the blank fluorescence values from the final fluorescence values of the sample(s) and the positive control. Estimate protein concentration by comparing fluorescence on the standard curve.

References

1. Waldo, G.S. et al., Rapid protein-folding assay using green fluorescent protein. Nature Biotechnology, **17**, 691-695 (1999).
2. Cabantous, S. et al., Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. Nature Biotechnology, **23**, 102-107 (2004).
3. Cabantous, S. et al., New molecular reporters for rapid folding assays. PLoS ONE, **3**(6), e2387. Doi:10.1371/ (2008).

Appendix A
pCMV-mGFP Cterm S11 Neo Kan Vector Information



Feature Map of pCMV-mGFP Cterm S11 Neo Kan – Unique restriction sites shown flanking key modules. The *Not* I site follows the mGFP S11 stop codon. Genes are cloned with initiator codons just after *Nhe* I in the multiple cloning site (MCS) and in-frame with the downstream linker and GFP S11 module. The structure of expressed proteins of interest (POI) is POI-linker-GFP S11.

Begin MCS

561 **********GCTAGC**GCTACCGGACT**CAGAT**CTCGAG**CTC**AAGCTT**CGAATTC**TGCAGT 640
561 **********CGATCGCGATGGCCTGAGTCTAGAGCTCGAGTTCGAAGCTTAAGACGTCA 640
NheI BglII EcoRI
XhoI HindIII

Linker **GFP S11**

641 CGAC**GGTACC**GCGGGCCCG**GGATCC**ACCGGTCGCCACCATGGGCGACGGCGGCAGCGGCGGCGGCAGC**********
641 GCTGCCATGGCGCCCGGGCCCTAGGTGGCCAGCGGTGGTACCCGCTGCCGCCGTCGCCGCCGCCGTCG********** 720
KpnI BamHI

GFP S11 cont.'d **STOP (TAA)**

721 **********TAAAGCGGCCGCGAC**TCTAGA**TCATAATCAGCCATACCACATTT 800
721 **********ATTTCGCCGGCGCTGAGATCTAGTATTAGTCGGTATGGTGTAAA 800
XbaI

Plasmid Features and Locations

- Human cytomegalovirus (CMV) immediate early promoter: 1–589
  - Enhancer region: 59–465; TATA box: 554–560
  - Transcription start point: 538
  - C→G mutation to remove *Sac* I site: 569
- Multiple cloning site: 613-681
  - Linker GDGGSGGGS: 682-708
- GFP S11 mouse codon-optimized (GFP amino acids 215-230)
  - GFP S11: 709-756
  - Stop codon: 757-759
- SV40 early mRNA polyadenylation signal
  - Polyadenylation signals: 918-918 & 942-947
  - mRNA 3′ ends: 951 & 963
  - f1 single-strand DNA origin: 1010-1465
  - (Packages noncoding strand of POI-linker-GFP S11)
- Ampicillin resistance (β-lactamase) promoter
  - –35 region: 1527-1532; –10 region: 1550-1555
  - Transcription start point: 1562
- SV40 origin of replication: 1806-1941
- SV40 early promoter
  - Enhancer (72-bp tandem repeats): 1639-1710 & 1711-1782
  - 21-bp repeats: 1786-1806, 1807-1827 & 1829-1849
  - Early promoter element: 1862-1868
  - Major transcription start points: 1858, 1896, 1902 & 1907
- Kanamycin/Neomycin resistance gene
  - Neomycin phosphotransferase coding sequences:
  - Start codon (ATG): 1990-1992; stop codon: 2781-2784
- Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal
  - Polyadenylation signals: 3020-2025 & 3033-3038
- pUC plasmid replication origin: 3369-401

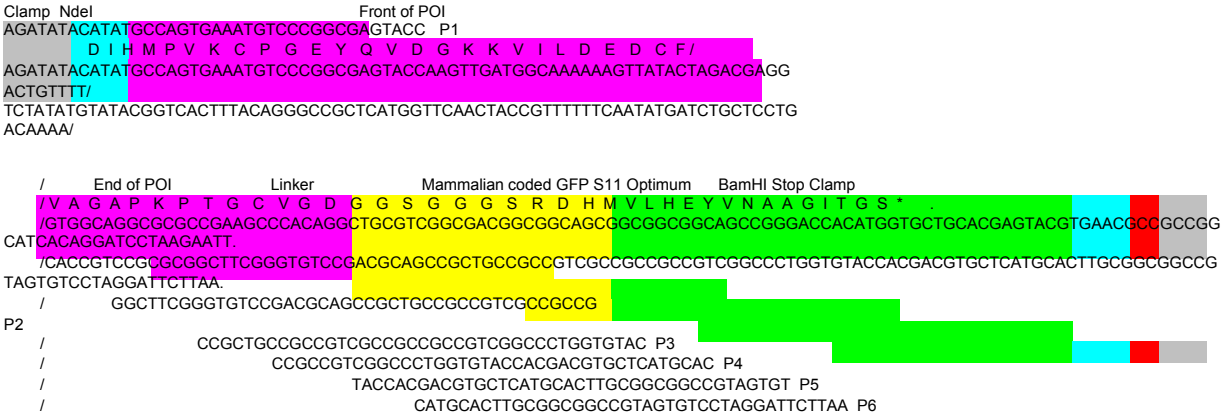
Appendix B
Alternate cloning techniques

Using PCR to Extend GFP S11 tag with mammalian codon usage onto target proteins of interest.

pCMV-mGFP Cterm S11 Neo Kan vector encodes a mammalian-codon optimized version of the 16 amino-acid engineered split GFP S11 detector fragment for tagging proteins with a C-terminal strand 11 of GFP "GFP S11". These GFP S11-tagged proteins can be detected using the so-called GFP 1-10 "detector" fragment, a highly engineered variant of strands 1-10 of green fluorescent protein. pCMV-mGFP Cterm S11 Neo Kan vector contains a the Neo Kan selection marker and so can be co-transfected with pCMV-mGFP 1-10 Hyg Amp Vector and co-transformants can be selected using neomycin and hygromycin in eukaryotes. It is also possible to add GFP S11 mammalian coding sequence for user-specific constructs and plasmids via PCR assembly and restriction cassette cloning, as described in the following examples.

Example 1.

Attaching GFP S11 to C-terminus of gene of interest (GOI) via flexible linker, and adding 5′ *Nde* I site and 3′ *Bam*H I site to assembled module.



Considerations:

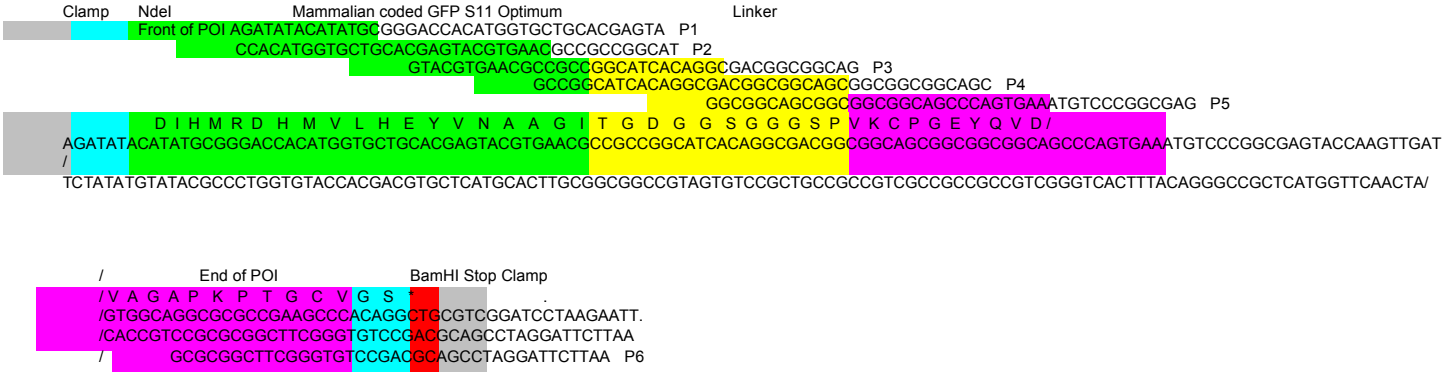
- Terminal primers that contain restriction sites also 5′ extensions or "clamps" to facilitate restriction enzyme binding to cognate restriction site. Such extensions are required by many restriction enzymes for efficient cutting.
- In this example, five sequential PCRs can be performed to obtain the final full-length product. A first PCR is performed using the primers P1 and P2, and the template protein of interest. The product is cleaned and used as a template for a second PCR with primers P1 and P3, and the product is cleaned and used as the template for a PCR using primers P1 and P4, etc. After the first PCR, fewer cycles (ca. 15 extensions) can be performed using a larger amount of template.
- Alternatively, a first PCR can be performed using a mixture of primers P1, P2, P3, P4, P5, P6 in the ratio 5:1:1:1:1:5 (i.e., the outside primers P1 and P6 are used in ca. 5-fold molar excess compared to each interior primer P2-p5. The product is cleaned and then used as a template for a PCR with primers P1 and P6, to ensure that the product is fully extended.
- Primers are generally ordered for synthesis in the 5′-3′ orientation. Be sure to order reverse complements for the lower primers P2-P6.

AGATATACATATGCCAGTGAAATGTCCCGGCGAGTACC	P1 as ordered
GCCGCCGCTGCCGCCGTCGCCGACGCAGCCTGTGGGCTTCGG	P2 as ordered
CATGTGGTCCCGGCTGCCGCCGCCGCTGCCGCCGTCGCC	P3 as ordered
CACGTACTCGTGCAGCACCATGTGGTCCCGGCTGCCGCC	P4 as ordered
TGTGATGCCGGCGGCGTTCACGTACTCGTGCAGCACCAT	P5 as ordered
AATTCTTAGGATCCTGTGATGCCGGCGGCGTTCACGTAC	P6 as ordered

- If several different proteins of interest (POI) and restriction sites are to be constructed with linker-GFP S11 tags, the same linker and GFP S11 coding primers can be used throughout as long as they do not containing POI or restriction site sequences. For example, P3, P4, P5 can be ordered once and used for many different cloning experiments.

Example 2.

Attaching GFP S11 to N-terminus of gene of interest via flexible linker, and adding 5′ *Nde* I site and 3′ *Bam*H I site to assembled module.



Considerations:

- Terminal primers that contain restriction sites also have 5′ clamp extensions to facilitate restriction enzyme binding to cognate restriction site. Such extensions are required by many restriction enzymes for efficient cutting.
- In this example, five sequential PCRs can be performed to obtain the final full-length product. For example, a first PCR is performed using the primers P5 and P6, and the template protein of interest. The product is cleaned and used as a template for a second PCR with primers P4 and P6, and the product is cleaned and used as the template for a PCR using primers P3 and P6, etc. After the first PCR, fewer cycles (ca. 15 extensions) can be performed using a larger amount of template.
- Alternatively, a first PCR can be performed using a mixture of primers P1, P2, P3, P4, P5, P6 in the ratio 5:1:1:1:1:5 (i.e., the outside primers P1 and P6 are used in ca. 5-fold molar excess compared to each interior primer P2-P5. The product is cleaned and then used as the template for a PCR with primers P1 and P6, to ensure that the product is fully extended.
- Primers are generally ordered for synthesis in the 5′-3′ orientation. Be sure to order reverse complements for lower primer P6.

AGATATACATATGCGGGACCACATGGTGCTGCACGAGTA	P1 as ordered
CCACATGGTGCTGCACGAGTACGTGAACGCCGCCGGCAT	P2 as ordered
GTACGTGAACGCCGCCGGCATCACAGGCGACGGCGGCAG	P3 as ordered
GCCGGCATCACAGGCGACGGCGGCAGCGGCGGCGGCAGC	P4 as ordered
GGCGGCAGCGGCGGCGGCAGCCCAGTGAAATGTCCCGGCGAG	P5 as ordered
AATTCTTAGGATCCGACGCAGCCTGTGGGCTTCGGCGCG	P6 as ordered

- If several different proteins of interest (POI) and restriction sites are to be constructed with linker-GFP S11 tags, the same linker and GFP S11 coding primers can be used throughout as long as they do not containing POI or restriction site sequences. For example, P2, P3, P4 can be ordered once and used for many different cloning experiments.

Appendix C
Suggested 96 well plate configuration

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Neat positive control**	**Neat positive control**	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
B			Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
C			Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
D			Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
E			Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
F			Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
G			Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	**Blank**	**Blank**
H	**.39 nM positive control**	**.39 nM positive control**	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	**BSA negative control**	**BSA negative control**