

Q & A Technically Speaking

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Eukaryotic Expression: Transfection and Reporter Vectors

Promega Corporation offers a variety of reagents for the stable and transient transfection of cells, and a number of reporter vectors designed for investigating the *in vivo* expression of promoter and enhancer sequences. Convenient assay systems have been developed for the analysis of reporter gene expression.

Q What are the TransFast™, Tfx™ and Transfectam® Reagents and how do they work?

TransFast™ Transfection Reagent^(a) is comprised of the synthetic cationic lipid, N,N [bis (2-hydroxyethyl)-N-methyl-N-[2,3-di (tetradecanoyloxy)propyl] ammonium iodide and the neutral lipid L-dioleoyl phosphatidylethanolamine (DOPE). The TransFast™ Reagent is supplied as a dried lipid film that forms multilamellar vesicles upon hydration with water. The reagent neutralizes the negative charge of nucleic acids, allowing closer association of the liposome:DNA complex with the negatively charged cell membrane. Entry of the liposome:DNA complex into the cell may occur by the processes of endocytosis, or fusion with the plasma membrane via the lipid moieties of the liposome. Once inside the cell, the complexes often become trapped in endosomes and lysosomes. Endosomal disruption is facilitated by DOPE, which allows the complexes to escape into the cytoplasm. It is not known precisely how the liposome:DNA complex gains entry to the nucleus.

For a detailed description please refer to the [TransFast™ Transfection Reagent Technical Bulletin #TB260](#).

The **Tfx™ Reagents^(b)** are a mixture of a synthetic, cationic lipid molecule (N,N,N',N'-tetramethyl-N,N'-bis(2-hydroxyethyl)-2,3-di (oleoyloxy)-1,4-butane-diammonium iodide) and the neutral lipid L-dioleoyl phosphatidylethanolamine (DOPE). The Tfx™-10, Tfx™-20 and Tfx™-50 Reagents all contain the same concentration of the cationic lipid component, but differ in the molar ratios of DOPE. The Tfx™ Transfection Reagents are suitable for stable and transient transfection of various cell lines, although each reagent exhibits differing transfection efficiencies depending on the cell type of interest.

The cationic lipid component of the Tfx™ Reagents consists of a positively charged head group attached to a lipid backbone by an ester linkage. The positively charged headgroups associate with the negatively charged nucleic acids, resulting in the formation of multilamellar vesicles. These vesicles presumably facilitate the transfer of nucleic acids into cells by interaction of the lipid backbone with the cell membrane.

For a detailed description please refer to the [Tfx™-10, Tfx™-20 and Tfx™-50 Reagents Technical Bulletin #TB216](#).

Transfectam® Reagent^(c) is composed of dioctadecylamidoglycyl spermine (DOGS), a synthetic cationic lipopolyamine molecule. The spermine group is covalently attached through a peptide bond to the lipid moiety. The strong positive charge contributed by the spermine headgroup gives the molecule a high affinity for DNA (10^5 – 10^6 M⁻¹), coating the DNA with a cationic lipid layer that facilitates binding to the cell membrane. This reagent is recommended for stable and transient transfections of a variety of cell types.

For a detailed description please refer to the [Transfectam® Reagent Technical Bulletin #TB116](#).

Q How do I decide which transfection reagent to use for a particular cell type?

To help you decide which transfection reagent to use for the cell type of interest, please refer to the transfection assistant program available on the Promega web site at: www.promega.com/transfectionasst/. After the cell line of choice and the type of transfection to be performed (stable or transient) are entered, the program will display a list of transfection reagents and conditions that have been demonstrated to work with the cell type of interest. This information is based on experiments from various sources, including both published citations and data generated at Promega.





How do Promega's pGL3 Luciferase Reporter Vectors differ from the pGL2 Vectors?

The pGL3 Vectors^(d,e) were created by improving the pGL2 Vectors^(d). The improvements included modifications to both the luciferase gene and the vector backbone.

Luciferase gene (*luc* to *luc+*) modifications:

1. Removal of a peroxisome targeting sequence.
2. Deletion of the consensus binding sequences for AP1, AP2, SP1, TGT-3 and LF-A1 genetic regulatory proteins.
3. Improvement of codon usage for mammalian and plant cells.
4. Removal of potential glycosylation sites.

For additional information please refer to reference 1 and to Table 1 in the [pSP-luc+ Vector Technical Bulletin #TB208](#).

Table 1. Stability, Molecular Weights, Substrates and Sensitivities of Promega's Reporter Enzymes.					
Reporter Enzyme	mRNA Half-Life	Protein Half-Life	Molecular Weight	Substrate	Sensitivity
Firefly Luciferase	6 hours (4)	3 hours (4,5) (mammalian)	61kDa monomer (6)	beetle luciferin (4,9)	1fg (10 ²⁰ moles)
<i>Renilla</i> Luciferase	unknown	5.3 hours at 40°C (5)	34/35kDa monomer (5,7,8)	coelenterazine (5,8,11)	10fg (3 x 10 ¹⁹ moles)
Beta-Galactosidase	unknown	20 hours	465kDa tetramer (9) (116kDa each)	ONPG (5,9)	10 ⁴ 10 ⁵ molecules
CAT	6 hours (4)	50 hours (4)	80kDa tetramer (10) (20kDa each)	chloramphenicol (9)	510 x 10 ⁷ molecules

Vector modifications:

1. The SV40 early poly(A) signal was replaced with the SV40 late poly(A) signal to increase the efficiency of transcription termination and polyadenylation of the luciferase transcripts.
2. A synthetic poly(A) and transcriptional pause site were placed upstream of the multiple cloning site to terminate spurious transcription, which may initiate within the vector backbone.
3. The small T intron was removed to prevent reduced reporter gene expression due to cryptic RNA splicing.
4. A Kozak consensus sequence was inserted to increase the efficiency of translation initiation of the luciferase gene.
5. A unique *Nco* I site, for the construction of N-terminal fusions with *luc+*, was added to the 5'-end of the *luc+* gene.
6. A unique *Xba* I site was created immediately downstream of the *luc+* gene for subcloning purposes.
7. The *Sma* I site was moved to an internal position in the multiple cloning site so that blunt-end inserts can be cleaved by restriction enzymes on either side.

For a detailed description please refer to the [pGL3 Luciferase Reporter Vectors Technical Manual #TM033](#).



How does the relative activity of *Renilla* luciferase differ from that of firefly luciferase? What conditions are recommended for cotransfection of the two reporter vectors?

Firefly luciferase acts on beetle luciferin in the presence of ATP, magnesium and oxygen. *Renilla* luciferase, obtained from the sea

pansy *Renilla reniformis*, acts on coelenterazine in the presence of oxygen. Although their respective light outputs are similar, firefly luciferase has a molecular weight of 61kDa, and *Renilla* luciferase has a molecular weight of 36kDa. Therefore, when assaying equal mass amounts of the two enzymes, one is actually measuring 69% more molecules of *Renilla* luciferase. It has been empirically determined that, on a per mole basis, firefly luciferase provides approximately 53% greater luminescence intensity than *Renilla* luciferase when assayed using the Dual-Luciferase™ Reporter Assay (f.g) chemistry. Please refer to reference 2 for additional information.

Trans effects between promoters on cotransfected plasmids can potentially affect reporter gene expression (3). This is of particular concern when either the control or the experimental reporter vector contain very strong promoter/enhancer elements. The TK, SV40 and CMV promoters in the pRL Vectors^(h) are expressed at different levels in most mammalian cell types. The HSV-thymidine kinase promoter of pRL-TK is relatively weak and is most useful in providing neutral constitutive expression of the *Renilla* luciferase control reporter. The SV40 early and CMV immediate early promoter/enhancer regions provide high-level transcription and may be less suitable for coreporter applications involving experimental vectors with robust regulatory elements. In addition, the SV40 promoter and the CMV promoter contain transcription factor recognition sequences for SP1, AP1 and AP2. If the effects of such transcription factors on the experimental promoter are under investigation, the use of the SV40 and CMV control reporters is not recommended.

Transfection ratios of experimental vector to *Renilla* luciferase vector of 10:1, 50:1, 200:1 or greater are recommended to ensure independent genetic expression between the experimental and control reporter genes, to diminish or suppress the *trans* effects between promoter elements, and to control the level of expression of the cotransfected vectors.

For a detailed description please refer to the [Dual-Luciferase™ Reporter Assay System Technical Manual #TM040](#).



What is the stability, molecular weight, substrate and sensitivity of each of Promega's Reporter Enzymes?

Stability, molecular weight, substrate and sensitivity data for Promega's firefly luciferase, *Renilla* luciferase, beta-galactosidase and chloramphenicol acetyltransferase (CAT) enzymes are listed in [Table 1](#).



How do the pCAT[®]3 Reporter Vectors differ from the pCAT[®] Reporter Vectors?

Nine major modifications were made to the pCAT[®] Vectors to generate the pCAT[®]3 Vectors. These include:

1. The SV40 small T antigen intron was replaced with a chimeric intron 5' of the CAT gene to enhance expression and decrease cryptic splicing.
2. The poly(A) signal for CAT was changed from the early to the late SV40 poly(A) signal for more efficient transcription termination and polyadenylation.
3. A synthetic poly(A) and transcriptional pause site was inserted 5' of the CAT gene and multiple cloning sites to terminate spurious transcription upstream from the CAT gene.
4. A Kozak consensus sequence was created at the 5'-end of the CAT gene to optimize translation efficiency.
5. The multiple cloning sites were changed for compatibility with the pGL3 Vectors.
6. A fl *ori* was included in the vector backbone for generation of single-stranded DNA.
7. A unique *Nco* I site was created in the Kozak sequence to facilitate subcloning.
8. A unique *Xba* I site was created just downstream of the CAT gene.
9. The *Eco*R I site was removed from within the CAT gene, resulting in the amino acid change Phe⁷³ to Leu⁷³.

These modifications act to enhance CAT expression in transfected cells and increase cloning flexibility.

For a detailed description please refer to the [pCAT[®]3 Reporter Vectors Technical Manual #TM036](#).



What is the Beta-Galactosidase Assay System?

The Beta-Galactosidase Assay System is a colorimetric assay system that detects beta-galactosidase enzyme activity in cells transfected with a vector expressing this enzyme (e.g., the pSV-Beta-Galactosidase Control Vector). The pSV-Beta-Galactosidase Control Vector is designed to be used as an internal control for transfection efficiencies when cotransfected into mammalian cells. Equal volumes of cell extract and Assay 2X Buffer, which contains the substrate ONPG (o-nitrophenyl-beta-D-galactopyranoside), are mixed and incubated. The beta-galactosidase enzyme hydrolyzes the colorless substrate to o-nitrophenol, which is yellow. The reaction is inhibited with sodium carbonate, and the optical density is measured spectrophotometrically or with an ELISA plate reader at 420nm.



What reporter assays and protein assays are compatible with the Reporter Lysis Buffer (RLB), Passive Lysis Buffer (PLB) and Cell Culture Lysis Reagent (CCLR)?

A listing of reporter assays and protein assays that are compatible with each of Promega's lysis reagents and buffers is provided in [Table 2](#). Note that the BSA standard curve generated for protein quantitation should always contain the same final volume as the sample. Volumes can be adjusted using the same lysis buffer as the sample.

Table 2. Reporter and Protein Assays Compatible with Cell Culture Lysis Reagent, Reporter Lysis Buffer and Passive Lysis Buffer.		
Lysis Buffer	Compatible Reporter Assay	Protein Assay
Cell Culture Lysis Reagent (CCLR)	Luciferase only	First dilute the cell lysate 1:2 with water and use with BioRad #500-0116 DC protein assay or Pierce BCA protein assay #23225.
Reporter Lysis Buffer (RLB)	Luciferase Beta-Galactosidase CAT	Dilute the cell lysate 1:2 with water and use with BioRad DC protein assay #500-0116 (large-scale only) or Pierce BCA protein assay #23225. Alternatively, use 2-8µl of undiluted lysate with the Bradford assay.
Passive Lysis Buffer (PLB)	Luciferase Beta-Galactosidase CAT Dual-Luciferase™ Reagent	Pierce Coomassie® Plus protein assay (micro-scale). Use 100µl of the cell lysate with 1ml of the protein assay reagent.

REFERENCES

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7. Matthews, J.C. *et al.* (1977) *Biochemistry* **16**, 85.
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10. Shaw, V.W. (1975) *Meth. Enzymol.* **43**, 737.
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Ordering Information

Product	Size	Cat.#
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TransFast™ Transfection Reagent	1.2mg	E2431
Tfx™ Reagents Transfection Trio	5.4mg	E2400
Tfx™-10 Reagent	9.3mg	E2381
Tfx™-20 Reagent	4.8mg	E2391
Tfx™-50 Reagent	2.1mg	E1811
Transfectam® Reagent	1mg	E1231
	0.5mg	E1232
pGL3-Control DNA	20µg	E1741
pGL3-Enhancer DNA	20µg	E1771
pGL3-Promoter DNA	20µg	E1761
pGL3-Basic DNA	20µg	E1751
pRL-TK Vector	20µg	E2241
pRL-CMV Vector ⁽ⁱ⁾	20µg	E2261
pRL-null Vector	20µg	E2271
pRL-SV40 Vector	20µg	E2231
pCAT®3-Control Vector	20µg	E1851
pCAT®3-Enhancer Vector	20µg	E1881
pCAT®3-Promoter Vector	20µg	E1861
pCAT®3-Basic Vector	20µg	E1871
pSV-Beta-Galactosidase Control Vector	20µg	E1081
Luciferase Assay System ^(f)		E1500
Dual-Luciferase™ Reporter Assay System		E1910
CAT Enzyme Assay System with Reporter Lysis Buffer		E1000
Beta-Galactosidase Enzyme Assay with Reporter Lysis Buffer		E2000
Reporter Lysis Buffer, 5X	30ml	E3971
Passive Lysis Buffer, 5X	30ml	E1941
Luciferase Cell Culture Lysis Reagent	30ml	E1531

^(a)The cationic lipid component of the TransFast™ Transfection Reagent is covered by U.S. Pat. No. 5,824,812 and pending foreign patents.

^(b)The cationic lipid component of the Tfx™ Reagents is covered by U.S. Pat. Nos. 5,527,928, 5,744,625 and pending foreign patents.

^(c)Transfectam is a registered trademark of BioSeptra, Inc. The Transfectam® product was developed by J.P. Behr and J.P. Loeffler (under license from CNRS-ULP Strasbourg). Transfectam® Reagent is covered by U.S. Pat. No. 5,171,678.

^(d)The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

^(e)U.S. Pat. No. 5,670,356 has been issued to Promega Corporation for a modified luciferase technology.

^(f)U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289, and Australian Pat. No. 649289, have been issued to Promega Corporation for a firefly luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay.

^(g)U.S. Pat. No. 5,744,320 has been issued to Promega Corporation for quenching reagents and assays for enzyme-mediated luminescence.

^(h)The cDNA encoding luciferase from *Renilla reniformis* is covered by U.S. Pat. No. 5,292,658 assigned to the University of Georgia Research Foundation, Inc., and sublicensed from SeaLite Sciences, Inc., Norcross, GA. The pRL family of *Renilla* luciferase cDNA vectors is for research use only. Commercial manufacture

would require a license from SeaLite Sciences, Inc.

⁽ⁱ⁾Covered under U.S. Pat. No. 5,168,062 assigned to the University of Iowa Research Foundation.

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