iCumate™ Inducible Lentiviral Expression System

Application Handbook

WELCOME

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Introduction

Inducible gene expression represents a fundamental technology that is irreplaceable to life sciences research.

Consequently, a sustained effort towards improving the current technology has provided a wide range of systems, employing inducers such as heavy-metals (8), steroid hormones (3), temperature (7), and antibiotics (i.e. tetracycline) for the manipulation of gene expression.

While many of these gene expression systems have been able to cater to specific, individual requirements, leaky gene expression, risk of undesirable pleiotropic effects caused by the inducer along with the associated experimental cost all present a range of outstanding issues that often limit their application.

Therefore, in a successful endeavor to address these underlying issues, **abm** has engineered the iCumate™ Inducible Lentiviral Expression System to effectively combine robust, yet easily tunable gene expression to meet the escalating, challenging demands for this core technology.

abm's iCumate™ Inducible Lentiviral Expression System utilizes a switchable interaction between the repressor (CymR), inducer (cumate) and operator (CuO) from the p-cym operon from *Pseudomonas putida* (2) that in turn regulates the strong CMV promoter.

In the 'OFF' state, the repressor (CymR) binds with high affinity to the operator sequence (CuO) that is strategically placed downstream of the CMV promoter (a strong viral promoter that is active in most mammalian cells) (Figure 1, p.2). Once bound to CuO, CymR blocks transcription through the CMV promoter, thus abrogating any downstream gene expression. Association of the inducer (cumate) with CymR renders it incapable of binding to CuO, thereby enabling transcription to proceed unhindered (Figure 1, p.2). The employment of a finely titratable inducer such as cumate, coupled to the strong read-out from the CMV promoter constitutes the core design for all iCumate™ inducible vectors available from **abm**.

An additional feature that makes **abm**'s iCumate™ Inducible Lentiviral Expression System highly desirable, is the non-toxic and cost effective nature of cumate as the inducer. Furthermore, induction with cumate does not lead to any significant background effect with respect to the host cells proteome expression profile. Lastly, unlike tetracycline that may exist in trace amounts in the fetal bovine serum (FBS) used for cell culture (thus causing leaky expression frequently associated with Tet-inducible systems), most FBS brands available on the market are completely free of cumate, making the iCumate™ Inducible Lentiviral Expression System the ideal choice for mediating non-leaky gene expression.

Figure 1a. Cumate switch 'ON'

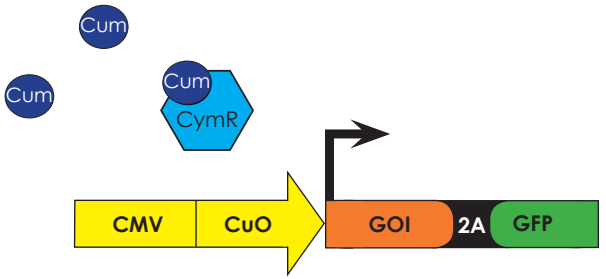

Figure 1b. Cumate switch 'OFF'

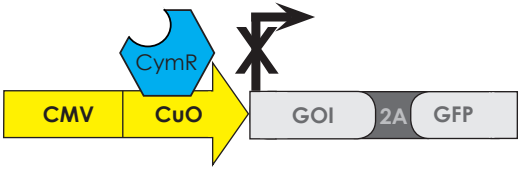

Figure 1. A schematic representation of the cumate 'switch'.
1a) The addition of cumate to the culture medium rapidly sequesters the bacterial repressor (CymR), preventing it from binding to the operator (CuO). This in turn enables transcription to proceed unhindered from the CMV promoter, inducing gene expression.
1b) Under cumate-free culture conditions, CymR binds to the operator sequence (CuO) placed downstream of the CMV promoter, therefore successfully and robustly blocking transcription.

Contents

1. Key Features

With an aim to utilize the full potential of one of the most robust and cost-effective gene expression systems on the market (based on the CMV promoter), **abm** has developed a comprehensive array of iCumate™ lentiviral vector kits and ready-to-use lentivirus particles for any human, mouse, and rat ORF, siRNA and miRNA for use with the iCumate™ expression system.

Highlights of the iCumate™ Inducible Lentiviral Expression System include:

- High transduction efficiency; based on a lentiviral vector format
- High level of induced gene expression; based on the strong CMV promoter
- Reliable, robust and finely titratable gene expression; particularly helpful for the expression of toxic proteins
- Easy reversal of induction; 'ON' and 'OFF' switch
- No basal/leaky gene expression
- Availability of any human, mouse, or rat ORF, siRNA and miRNA lentiviral vector (see pages 6-8)
- Flexibility of application, with All-in-One and dual vector formats

Since cumate is non-toxic to cells, it permits greater dynamic ranges of induction, allowing you to select the level of gene expression to further extremes (see figure 2), either through the addition of more cumate or higher MOI of your virus.

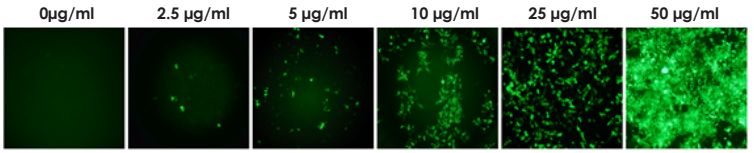

Figure 2. The dose-dependent control of GFP gene expression by cumate. The experiment was conducted with a stable clone of 293CymR-CMV-CuO-GFP cells, cultured in the absence of cumate as well as increasing concentrations of 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml and 50 µg/ml cumate. Cells were cultured in the presence or absence of cumate for 48 hours before GFP positive cells were assayed.

2. Components

2.1 Dual Vector Format

The dual vector format offers maximum flexibility with respect to the size of the insert, while ensuring the tightest regulation. This format provides the bacterial CymR repressor in one vector (Cat. No. iCu999 - figure 3a), with the gene of interest residing in a separate expression vector (for examples, see figures 3b and 3c). Similar to the All-in-One format, a drug selection marker and a reporter gene are also incorporated into the expression vector, all of which are available in a ready-to-use, viral particle format.

Figure 3a) Cat. No. iCu999

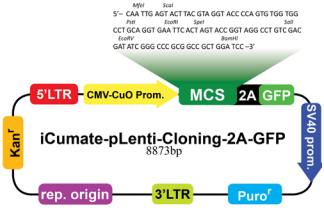

Figure 3b) Cat No. iCu003

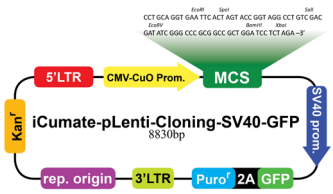

Figure 3c) Cat No. iCu007

Dual Vector	Cat. No
iCumate-pLenti-EF1α-CymR	iCu999
+	
iCumate-pLenti-Cloning	iCu001
iCumate-pLenti-Cloning-2A-GFP	iCu003
iCumate-pLenti-Cloning-2A-RFP	iCu005
iCumate-pLenti-Cloning-SV40-GFP	iCu007
iCumate-pLenti-Cloning-SV40-RFP	iCu009

2.2 All-in-One Format

The All-in-One format has the advantage of convenience, however leaky expression may present challenges in achieving tight regulation of gene expression. In addition, the inclusion of the CymR repressor, a selection marker, a reporter and the desired insert within the same vector, limits the insert size to 2.5 kb (see figure 3d). The All-in-One format is provided as a single plasmid vector or ready-to-use viral particles. A selection marker and reporter gene are included to facilitate stable cell line generation and effectively monitor the induction efficiency.

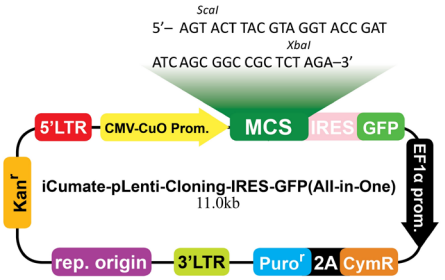

Figure 3d) Cat No. iCu013

All-in-One Vector	Cat No.
iCumate-pLenti-Cloning(All-in-One)	iCu011
iCumate-pLenti-Cloning-IRES-GFP(All-in-One)	iCu013
iCumate-pLenti-Cloning-IRES-RFP(All-in-One)	iCu015
iCumate-pLenti-siRNA-Cloning-GFP(All-in-One)	iCu017
iCumate-pLenti-miRNA-Cloning-GFP(All-in-One)	iCu019

2.3 Ready-to-Use, pre-made iCumate™ Lentiviral Vectors

The Ready-to-Use format provides you with iCumate™ inducible lentiviral vectors for any ORF, siRNA, or miRNA of human, mouse, and rat origin. Please visit our website www.abmGood.com/Lentivirus.html to determine the vector best suited to your needs prior to ordering. In addition, we will be more than happy to provide you with any custom-made vector you may require at an affordable cost and with the fastest turn-around time.

2.4 Control Vectors

A control should be included in all iCumate™ experiments. Our control vectors come available in a lentiviral vector format, with or without reporter gene expression to fulfil the requirements of any project. Please see the table below for a full listing of control vectors available :

Control Vector	Cat No.
iCumate-pLenti-Blank	iCu002
iCumate-pLenti-Blank-2A-GFP	iCu004
iCumate-pLenti-Blank-2A-RFP	iCu006
iCumate-pLenti-Blank-SV40-GFP	iCu008
iCumate-pLenti-Blank-SV40-RFP	iCu010
iCumate-pLenti-Blank(All-in-One)	iCu012
iCumate-pLenti-Blank-IRES-GFP(All-in-One)	iCu014
iCumate-pLenti-Blank-IRES-RFP(All-in-One)	iCu016
iCumate-pLenti-scramble siRNA-GFP(All-in-One)	iCu018
iCumate-pLenti-miRNA-Blank-GFP(All-in-One)	iCu020

3. Additional Materials Required

- 2nd Generation Packaging System Mix (Cat. No. LV003) OR 3rd Generation Packaging System Mix (Cat. No. LV053)
- ProAdhere 293T Cell Line (Cat. No. LV592)
- Lentifectin™ Transfection Reagent (Cat. No. G074)
- Speedy Lentivirus Purification (Cat. No. LV999)
- qPCR Lentivirus Titration (Titer) Kit (Cat. No. LV900)
- ViralPlus Transduction Enhancer (Cat. No. G698)
- Cumate Solution (30mg/ml), 500ul (Cat. No. CH065)
- Polybrene (Cat. No. G062)
- FBS (Cat. No. TM999)
- Cell culture medium: PriGrow III (TM003)

Protocols for the packaging of lentiviral particles can be found outlined in the following link: http://www.abmgood.com/His-Tag-Lentivirus-Expression-System.html

4. General Information on Lentivirus technology

4.1. Morphology

Lentiviral particles consist of an envelope and nucleocapsid, a nucleoid and matrix proteins. The enveloped virions assume a spherical to pleomorphic shape of 80-100 nm in diameter with its surface covered in dense inconspicuous spikes approximately 8 nm in length.

4.2. Physical Properties

Virions have a buoyant density of 1.13-1.18g/cm_3 in sucrose. They are sensitive to treatment with heat, detergents, and formaldehyde; however, their infectivity is not affected by irradiation.

4.3. How a Lentivirus Works

Once target cells are transduced with a recombinant lentivirus, the viral RNA is reverse-transcribed and actively imported into the nucleus (4) where it undergoes stable integration into the host genome (1). One to two days after the lentiviral genome is stably integrated into the host genome, you may begin to assay for the transient expression of your recombinant gene or use appropriate selection markers to generate a stable cell line for long-term, inducible gene expression studies.

4.4. VSV Glycoprotein Envelope

Most commercial vectors of retroviral origin are limited in gene delivery applications because of their restricted tropisms and generally low titers. For recombinant lentiviral vectors, these limitations are resolved by pseudotyping the vector with the G glycoprotein gene from Vesicular Stomatitis Virus Glycoprotein (VSV-G) envelope. The significant advantages associated with the use of VSV-G envelope include:

- Production of high titer lentiviral particles
- Increased viral particle stability
- Broader target cell ranges / tissue tropism
- Highly efficient transduction efficiency (9).

4.5. Packaging Limits

Recombinant lentiviral titers will decline with increasing insert size. The packaging limit for our lentiviral expression system is approximately 5.5 kb, above which, little to no virus will be produced.

5. Safety Guidelines

abm's iCumate™ Inducible Lentiviral Expression System employs third generation, self-inactivating, recombinant lentiviral vectors with minimal relation to the wild-type, Human HIV-1 Virus.

The lentiviral particles produced with this system are replication-incompetent and designed with a number of safety features to enhance their bio-safety. All lentiviral expression systems provided by **abm** include the following safety features:

- An enhancer deletion in the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral vector following transduction and integration into the target cell's genomic DNA
- Utilization of a RSV promoter upstream of 5'ΔLTR allows efficient Tet-independent production of viral RNA
- The number of lentiviral genes necessary for packaging, replication and transduction are limited to three (Gag/Pol/Rev). All three genes are encoded by separate plasmids that in turn lack packaging signals. These plasmids share no homology to the expression vector and therefore prevent the generation of a recombinant virus with replicative potential.

6. Infection Protocols

iCumate™ inducible expression can be achieved by using either a one-step or a two-step lentiviral transduction method. Wherever possible, the two-step method is preferred for achieving optimal results (i.e. tighter regulation and higher levels of induced expression).

The two-step transduction involves the initial generation of a CymR expressing (Cat. iCu999) stable cell line, followed by a second round of transduction with an inducible viral vector (see table on page 5) with a subsequent colony screening step. We have produced a 293-CymR stable cell line (Cat. T3016) which is also available for purchase; please see our website for more details.

The one step method involves co-transduction of the CymR repressor virus (Cat. iCu999) and the cumate inducible virus (see table on page 5) at the same time. Alternatively, inducible gene expression can be achieved by the transduction of target cells with an All-in-One iCumate™ expression lentivirus (see table on page 6 for All-in-One vectors to suit your project needs).

Note: In general, the one-step method is quick and relatively simple to execute. However, to achieve the best results (i.e. the tightest regulation and a high level of gene expression), the two-step procedure is recommended. For simplicity, the following protocol is given with the one-step protocol method, though either can apply.

1. Quantify virus titer. It is always useful to titer a viral preparation before proceeding with transduction, for the following reasons:

- To ensure viability of the viral stock.
- To determine the percentage of target cells that can be transduced with a pseudoviral stock.
- To control the number of copies of viral constructs delivered per target cell.
- To carefully determine the dosage of the selection marker by a standard killing curve assay.

2. On day one, plate target cells in a 24-well or 6-well plate at 30% density and incubate at 37°C with 5% CO_2 overnight.

3. On day two, co-transduce target cells with the CymR repressor virus along with an inducible expression virus at a ratio of 1:1. A broad range of 10, 20,

and 50 MOI for each virus in the presence of 0.8µg/ml polybrene and ViralPlus Transduction Enhancer (Cat. No. G698 - optional) should be tested to determine optimal results. Incubate cells at 37 °C with 5% CO_2 overnight. An additional well should be set up for selection control purposes as previously described.

4. On day three (24 hours later), add the selection drug (Puromycin) at an appropriate dosage as determined by a killing curve plus cumate at 30µg/ml. Alternatively, add cumate after a stable cell line is generated if the inducible gene is toxic to the host cells.
This is the critical step for successful clone selection in any stable cell line project. Please pay very detailed attention to the plating cell density, as this will determine the dosage of your selection drug. Ensure that the target cells are selected at the same density as those plated in your drug killing curve assay. It is highly recommended to have a selection control (non-tranduced cells - also at the same cell density) to monitor the selection process effectively, i.e. selection is complete when all cells in the control are dead.

5. Monitor the selection process by reporter gene expression.

6. Once selection is complete, a stable cell line can be established by performing a screen for clones or cell sorting with FACS.

7. Once a good, stable cell line is acheived, you will be able to perform experimental assays for inducible ORF expression or assay for specific gene knockdown following induced siRNA or miRNA expression.

8. The cumate induction can be turned OFF at any time. Simply remove and change the culture medium with fresh media excluding cumate. The induced expression will fade within 24-72 hours.

9. Similarly, the cumate induced expression can be turned back ON to test for repeated expression of your gene of interest. Simply add cumate back to the culture medium at any point to re-induce expression.

7. Troubleshooting

7.1. No or low reporter gene expression

a. Confirm the virus titer by a titer assay to ensure the viability of your

recombinant virus before transformation
b. Increase virus MOI for higher transduction efficiency
c. Optimize the ratio of the inducible expression and CymR repressor viruses. Use equal MOIs of both the expression and transactivator viruses, as excess CymR virus will result in the induction becoming weak or undetectable.

7.2. Cell Toxicity

a. Ensure that polybrene is used at an optimal concentration
b. Ensure that cumate is used at an optimal concentration
c. If the induced gene is toxic to target cells, titrate the cumate to induce appropriate levels of target gene expression.

8. The iCumate™ Technology

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Country	Type	Number	Date
Canada	Application	2,446,110	5/1/2002
United States	Patent	7,745,592	5/1/2002
United States Continution	Application	12/769,048	5/1/2002
Belgium	Patent	1385946	5/1/2002
Switzerland	Patent	1385946	5/1/2002
Germany	Patent	1385946	5/1/2002
France	Patent	1385946	5/1/2002
Britain	Patent	1385946	5/1/2002
Ireland	Patent	1385946	5/1/2002
Italy	Patent	1385946	5/1/2002

9. References

Further reading on the use of Lentiviral Vectors and methods for induced expression systems can be found here;

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