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Monitoring Protein–Protein Interactions Using Split Synthetic Renilla Luciferase Protein-Fragment-Assisted Complementation

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Abstract

In this study we developed an inducible synthetic renilla luciferase protein-fragment-assisted complementation-based bioluminescence assay to quantitatively measure real time protein–protein interactions in mammalian cells. We identified suitable sites to generate fragments of N and C portions of the protein that yield significant recovered activity through complementation. We validate complementation-based activation of split synthetic renilla luciferase protein driven by the interaction of two strongly interacting proteins, MyoD and Id, in five different cell lines utilizing transient transfection studies. The expression level of the system was also modulated by tumor necrosis factor α through NF κ B-promoter/enhancer elements used to drive expression of the N portion of synthetic renilla luciferase reporter gene. This new system should help in studying protein–protein interactions and when used with other split reporters (e.g., split firefly luciferase) should help to monitor different components of an intracellular network.

Protein–protein interactions regulate different cellular processes, such as transcription, translation, cell division, signal transduction, and oncogenic transformation. To modulate many cellular events, it is essential to know about a variety of proteins and the types of interactions that can occur among them. To understand biological interactions between proteins in cells, several techniques have been developed and studied.¹ The yeast two-hybrid system² uses a “bait” protein connected to a DNA binding domain and is used to find a “prey” protein that is connected to a transcription activation domain. When the bait and prey proteins interact, they bring together the DNA binding and transcription-activating domains, which transactivate the expression of a reporter gene of choice. The limitation of the two-hybrid system is that the set of detectable protein interactions must occur in the nucleus to transactivate the reporter gene.³ To overcome this limitation, several techniques have been developed for studying protein–protein interactions, including the split-ubiquitin system,⁴ Sos recruitment system,^{5–7} β -galactosidase complementation,¹ G-protein fusion system,^{8,9} and fluorescence resonance energy-transfer (FRET) system. FRET is a useful technique to

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study the real time interactions between proteins, but the availability of fluorescent labels and autofluorescence can sometimes be limiting.^{9,10} The protein-fragment-assisted complementation of dihydrofolate reductase and β -lactamase has also been used for studying protein–protein interactions in bacteria and mammalian cells,^{10,11} but the sensitivity and complex assay procedures can be limiting.

The applications of various bioluminescence optical reporter genes have been well studied in both prokaryotic and eukaryotic cells and in small living animals. The use of optical reporters allows for high sensitivity with a relatively simple assay. In our previous studies, we reported an inducible yeast two-hybrid system with firefly luciferase¹² and a split firefly luciferase complementation system¹³ to study protein–protein interactions in cell lines and noninvasively in small living animals using a cooled charge coupled device (CCD) camera. An intein-mediated reconstitution of split firefly luciferase has also been reported previously in cell culture⁹ and in cells implanted in mice.¹³ Inteins are protein domains that perform a cis splicing reaction to excise themselves posttranslationally from nascent polypeptide chains, forming a new peptide bond between the exteins.¹⁴ These approaches can be used to study protein–protein interactions not only in cell culture but also in intact living small animals. Recently, we also reported imaging both intact firefly luciferase and intact renilla luciferase reporter gene expression in living mice while utilizing D-luciferin and coelenterazine substrates, respectively, without any cross-reactivity.¹⁵ To monitor different intracellular protein networks, it will be essential to have a multireporting system for use with both intact cells and living animals. For this, it is important to generate different optical split reporter proteins with substrate specificity. The enzymes of firefly luciferase and renilla luciferase react with different substrates with no cross-reactivity. The firefly luciferase emission spectrum is in the range 575–610 nm, whereas that of renilla luciferase is 440–550 nm. Therefore, in the current study, we used synthetic renilla luciferase (*hrLuc*), a second optical bioluminescence reporter gene for developing a protein-fragment-assisted complementation system.

The general features required for designing a protein-fragment-assisted complementation assay (PCA) (Figure 1), also referred to as split protein technology, are the need for a relatively small monomeric protein, well-established crystal structure, simple assay system, and generalizable applicability. Renilla luciferase (rluc), a monomeric 36-kDa, does not require ATP or posttranslational modification for its activity and also functions as a genetic reporter immediately following translation.¹⁶ The cDNA encoding renilla protein catalyzes coelenterate luciferin (coelenterazine) oxidation to produce light that luciferase was originally cloned from the marine organism *Renilla reniformis* (Sea pansy).¹⁷ This native renilla luciferase (*rluc*) gene sequence contains codons that are not frequently used in mammalian cells, which limits its expression efficiency in mammalian cells. The synthetic renilla luciferase is a systematically redesigned renilla luciferase gene with only codon changes for higher expression in mammalian cells (Promega, Technical manual no. 237 (1–3)). The protein encoded by both reporter genes is identical. The recovered activity from protein fragments of a reporter protein is anticipated to be lower than that of an intact reporter protein.¹³ This and the need to study different levels of interactions between proteins in the cellular network make it necessary to develop a highly sensitive reporter

system. Therefore, we used the gene sequence coding for synthetic renilla luciferase in this study to develop such a system. Although synthetic renilla luciferase, the modified form of renilla luciferase has many of the required components needed for developing a protein-fragment-assisted complementation assay, a crystal structure is lacking to identify potential sites to generate suitable fragments of the protein. Hence, in this study, for the first time, we validate a split synthetic renilla luciferase-based complementation system to study protein-protein interactions by selecting six different split sites. We selected the split sites to also allow future study of intein-mediated reconstitution of renilla luciferase. The intein-mediated splicing of split protein fragments requires the amino acid cysteine to be at the +1 position of the C part of the protein fragment to generate efficient reconstitution.^{3,9} Furthermore, the presence of more than one consecutive glycine molecule in a protein serves as a natural flexible linker. Considering those two factors, we selected six different split sites to generate fragments for the protein-fragment-assisted complementation strategy. Three of these sites were before cysteine molecules, one was before two consecutive glycine residues, one was at a convenient restriction enzyme site, and one was selected at random.

The complementation-based recovery of split protein activity was studied in five different cell lines. The system was studied with a constitutive CMV promoter and modulated by using TNF- α , an interleukin that controls NF κ B promoter/enhancer elements in cells. The signal measured from the complementing synthetic renilla luciferase (hrluc) fragments driven by a MyoD-Id protein-protein interaction shows significantly higher renilla luciferase activity than control studies that also include fragments without interacting proteins or with two noninteracting proteins (MyoD and p53). The split synthetic renilla luciferase strategy developed in the current work should be useful for studying protein-protein interactions when utilized alone or in combination with other split reporters, such as split firefly luciferase.

EXPERIMENTAL PROCEDURES

Chemicals, Enzymes, and Reagents

Restriction and modification enzymes and ligase were purchased from New England Biolabs (Beverly, MA). PCR amplification was used for generating the fragments of N and C portions of the synthetic renilla luciferase gene at each split point using the primers shown in Table 1 and the template plasmid phRL-CMV from Promega (Madison, WI). The cDNA of genes Id and MyoD were amplified from Promega's CheckMate Mammalian two-hybrid system kit containing vectors pBIND-Id and pACT-MyoD, respectively, by using the primers listed in Table 1. The NF κ B promoter/enhancer element was used from the vector pNF κ B-Luc of Stratagene (La Jolla, CA). Superfect transfection reagent, plasmid extraction kits, and DNA gel extraction kits were purchased from Qiagen (Valencia, CA). TNF- α and antibiotics for bacterial culture were purchased from Sigma (St. Louis, MO). CheckMate Mammalian two-hybrid kit was purchased from Promega. Coelenterazine was purchased from Biotium (Hayward, CA). Bacterial culture media were purchased from Difco (Franklin Lakes, NJ). Cell culture medium, FBS, penicillin, streptomycin, sodium bicarbonate, and all cell culture plates were purchased from GIBCO BRL (Frederick, MD).

Construction of Plasmids

The N and C portions of the synthetic renilla luciferase gene for each split point linked in-frame with the coding sequence for the interacting proteins were amplified from the plasmid templates using the corresponding primers indicated in Table 1. The primers were designed with convenient restriction enzyme sites for cloning. A linker sequence (GGGGS)₂ was added to the forward primer of Id and reverse primer of MyoD. The clones were constructed in a pcDNA 3.1(+) vector backbone. The clones confirmed by sequencing were used for the study (Figure 2C). The detailed methods of construction of the plasmids are available upon request.

Cell Culture

Human embryonic kidney cancer cells, 293T (ATCC-CRL-11268, Manassas, VA) were grown in MEM supplemented with 10% FBS and 1% penicillin/streptomycin solution. The N_{2a} cells (mouse neuroblastoma cells) were obtained from V. P. Mauro (Scripps Research Institute, La Jolla, CA) and COS-1 (monkey kidney cells) cells were grown in DMEM (high glucose) supplemented with 10% FBS and 1% penicillin/streptomycin. C6 rat glioma cells were maintained in glucose-deficient DMEM supplemented with 0.01% histidinol, 10% FBS, and 1% penicillin/streptomycin/glucose. U87 human malignant glioma cells purchased from ATCC (HTB-14) were grown in MEM supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.15% sodium bicarbonate, 1% penicillin/streptomycin, and 10% FBS.

Cell Transfection and Luciferase Assay

Transfections were performed in 80% confluent 24-h-old cultures of 293T, COS-1, N_{2a}, C6, and U87 cells. In 12-well plates, 100 ng/well of DNA for plasmids containing the N portion of synthetic renilla luciferase gene (*N-hrluc*) and 500-ng/well for plasmids containing the C portion of synthetic renilla luciferase gene (*C-hrluc*) were used for transfection. For transfection, different combinations of DNA were mixed with 75 μ L of serum-free medium and 6 μ L of Superfect and were kept at 25 °C for 10 min. The cells were washed twice with phosphate buffered saline (PBS) pH 7.0. The DNA/superfect complex was mixed with 400 μ L of complete medium and added to the cells. The cells were incubated at 37°C with 5% CO₂ for 3 h. After 3 h, the cells were washed twice with PBS, 1 mL of complete medium was added, and the cells were incubated at 37 °C with 5% CO₂. The cells were assayed for luciferase activity after 24 h. For comparison of different optical reporters, 100 ng/well of DNA from *fluc*, *hrluc*, *rluc*, and *N-hrluc* plasmids with coding sequences was used. The plasmids containing *N-hrluc* DNA concentration (100 ng/well) were considered for the comparison of different optical reporter genes. Because *C-hrluc* provides only the remaining part of the protein for activity recovery, even if it is expressed in greater quantity, there will not be any increase in the net hrluc activity due to its low background activity. Volumes of Superfect used were as recommended by the manufacturer. For cell induction, 0.05 μ g/mL TNF- α was added immediately after transfection and assayed 24 h later. The luminometer assay for renilla luciferase was performed by following a previously published protocol.¹⁵ In brief, the cells were lysed in 200 μ L of 1 $\times$ passive lysis buffer from Promega by placing them in a shaker for 15 min at 25 °C. The lysates were collected and centrifuged for 5 min at

10 000 rpm at 25 °C. The samples were assayed by mixing 20 μ L of cell supernatant (renilla luciferase enzyme), 1 μ L of the substrate coelenterazine (1 mg/mL), and 100 μ L of 0.05 M sodium phosphate buffer at pH 7.0, followed by photon counting in the luminometer (Turner Designs, model no. T 20/20, Sunnyvale, CA) for 10 s at 25 °C. Similarly, using Promega's luciferase assay kit, the assay for firefly luciferase was performed. The readings were normalized by measuring the concentration of proteins from the cell lysates by using the Bio-Rad (Hercules, CA) protein assay reagent and represented as relative light units (RLU) per microgram of protein per minute.

RESULTS AND DISCUSSION

Split Synthetic Renilla Luciferase Shows Complementation-Based Restoration of Enzyme Activity by the Fragments Generated from Two out of Six Chosen Split Sites

Complementation-based restoration of enzyme activity by two interacting proteins requires correctly folded portions of the proteins in close proximity. We selected six different sites to generate fragments of N and C portions of the synthetic renilla luciferase reporter protein to study the protein–protein interaction-assisted folding to form active enzyme (Figure 2A, B). We used the flexible linker (GGGS)₂ between the interacting proteins and the fragments of synthetic renilla luciferase to enhance proper folding (Figure 2C).¹⁸ The split site selected at nucleotide position 687–688, located after coding regions for two consecutive glycine molecules, shows efficient recovery of the hrluc activity through the interaction of the MyoD and Id proteins (Figure 3). The cells cotransfected with vector constructs carrying N and C portions of the synthetic renilla luciferase gene without interacting proteins or with noninteracting proteins (MyoD and p53) show significantly higher signal than mock-transfected cells but significantly lower than that obtained from MyoD and Id interaction. The split site at nucleotide position 669–670 shows complementation activity that is 20% less than the split site at nucleotide position 687–688 (data not shown). The other four sites studied did not lead to fragments that recovered significant activity relative to background. Construct *N-hrluc*–Id shows significantly higher ($P < 0.05$) activity than the *C-hrluc*–MyoD construct or studies employing mock transfection. The recovery of activity from the split sites at nucleotide positions 669–670 and 687–688 indicates that both of these split points are probably in a region of the hrluc protein that only partially affects the active site(s) of the protein molecule. The identification of other sites to generate protein fragments that might yield potentially better complementation-assisted activity necessitates the availability of a well-established crystal structure for the protein or the selection of additional split sites by permutation combination. There may be a possibility that future studies could reveal more optimal split sites, but the 687–688 split site should be useful for future studies because of its relatively low background and high complementation-based signals. The remainder of the studies were, therefore, performed only with the constructs made using the split site at nucleotide position 687–688.

The Signal Achieved Through Split Synthetic Renilla Luciferase Activity Is Significantly Higher than Split Firefly Luciferase after MyoD–Id Interaction

To compare the sensitivity of split synthetic renilla luciferase and split firefly luciferase with the interacting proteins MyoD and Id, transfection and cotransfection of different vector

constructs with and without interacting proteins and intact native renilla luciferase, synthetic renilla luciferase, and firefly luciferase were studied in 293T cells. The signal achieved through MyoD–Id interaction-mediated split synthetic renilla luciferase activity from the cells cotransfected with constructs is significantly more ($P < 0.01$) (a factor of ~2) than the cells cotransfected with vector constructs with N and C portions of firefly luciferase fragments with the same interacting proteins (Figure 4A). The signal achieved through MyoD–Id interaction-mediated split synthetic renilla luciferase activity from the cells cotransfected with constructs is $10 \pm 2\%$ of the cells transfected with intact synthetic renilla luciferase, 8 ± 1 times more than the activity seen from cells transfected with native renilla luciferase, and $90 \pm 5\%$ of the activity of cells transfected with intact firefly luciferase (Figure 4A). The N portion of the split protein encoded by 75% of the *hrluc* gene shows significant signal over mock-transfected cells ($P < 0.05$) (Figure 3). The activity obtained from the cells transfected with *C-hrluc* and *C-hrluc*–MyoD is not significantly different from mock-transfected cells (Figure 3). These results indicate that the split synthetic renilla luciferase system is more sensitive than the split firefly luciferase system under the conditions tested. These data also support the finding that the split synthetic renilla luciferase is as robust as the native firefly luciferase.

Split Synthetic Renilla Luciferase Shows Protein-Fragment-Assisted Recovery of Enzyme Activity by MyoD–Id Interactions in All Five Different Cell Lines Studied

Transfection and cotransfection of the N and C portions of *synthetic renilla luciferase* gene with and without interacting proteins were studied in 293T, C6, COS-1, N_{2a} and U87 cells. The cotransfection of N and C portions of synthetic renilla luciferase with the interacting proteins MyoD and Id shows significantly higher ($P < 0.01$) activity (15 ± 5 times) than the cells transfected with *N-hrluc*, *N-hrluc*–Id, and *N-hrluc* + *C-hrluc* in 293T cells (Figure 3). The ratio of recovered activity obtained in C6, U87, COS-1, and N_{2a} cells was similar to 293T cells. The magnitudes of the activity obtained through protein interactions from different cell lines studied are on the order of 293T (highest), N_{2a} ($60 \pm 5\%$ activity of 293T cells), COS-1 ($45 \pm 10\%$ activity of 293T cells), U87 ($30 \pm 5\%$ activity of 293T cells), and C6 ($20 \pm 10\%$ activity of 293T cells). The variations in the activity observed in different cell lines are likely due to different transfection efficiencies and different transcriptional/translational efficiencies. The efficiency of transfection and the level of transgene expression depends on various parameters, including the types of promoters used, types of cell lines used, types of vector backbone used for cloning the transgene, and also the types of proteins expressed.¹⁹

Split Synthetic Renilla Luciferase Activity Can Be Modulated by TNF- α by Controlling the Expression Level of One Fragment under NF κ B Promoter/Enhancer Elements

The protein–protein interaction-mediated split synthetic renilla luciferase activity can be modulated by controlling the level of expression of one of the two fragments generated for the study. The NF κ B promoter/enhancer element was used for modulating the level of expression of *N-hrluc*–Id. Transfection and cotransfection of 293T cells with *N-hrluc*–Id carrying NF κ B promoter/enhancer elements and *C-hrluc*–MyoD driven by the CMV promoter induced with TNF- α for a period of 24 h show a significant ($P < 0.01$) increase (30 ± 5 times) in their enzyme activity over the cells without TNF- α (Figure 4B). The cells

transfected with NF κ B–*N-hrluc*–Id with TNF- α show activity similar to *N-hrluc* under CMV promoter. The signal seen by the cells transfected with NF κ B–*N-hrluc*–Id without TNF- α and *C-hrluc* with and without TNF- α is not significantly different from the mock-transfected cells. These results verify the ability to modulate the signal by controlling levels of transcription of one of the two split reporters.

To date, several techniques have been developed for studying protein–protein interactions, and each has its own advantages and limitations. Many proteins identified for studying protein interactions have been identified by the formation of homotetramers by intracistronic complementation of mutants.¹⁰ Larger proteins may be sterically hindered during the complementation process.²⁰ Selections of irreversible mutants with no self-complementation properties are important for developing an intracistronic complementation system. The protein-fragment-assisted complementation assay uses the fragments of the protein that lack the self-complementation problem. Therefore, it is essential to use small monomeric reporter molecules that might avoid all of the above-mentioned obstacles to development of an ideal system to study protein–protein interactions for various applications. The synthetic renilla luciferase encoding a 36-kDa monomeric optical reporter protein is a suitable small protein identified for studying protein–protein interactions through a protein-fragment-assisted complementation strategy. The limitation associated with the use of renilla luciferase is its relatively rapid reaction kinetics requiring early time-point measurements.¹⁵ Because of its optical nature with signal amplifiable through an enzymatic process, it may prove to be a unique reporter system for studying protein–protein interactions in cells and small living animals.

This system can be further extended for studying protein–protein interactions using different protein partners with variable affinity to potentially obtain a significant signal from weaker interactions. The split synthetic renilla luciferase can also be tested with intein-mediated reconstitution approaches in further studies, and some of the current split sites have already been selected on the basis of this potential future application. The splicing-mediated split-protein approach generates reconstituted complete protein and is less dependent on the characteristics of the split sites, as long as the required components for the protein splicing mechanism are present.^{21–23} In contrast, the protein-fragment-assisted complementation approach described here maintains the portions of the protein in a nonligated state. Hence, it is important to identify the exact site to generate fragments that can fold properly without disturbing the active site.²⁴ Leucine-zipper-based complementation²⁵ and intein (VMA1)-mediated protein splicing³ have been studied in GFP (green fluorescent protein) and its variant EGFP (enhanced green fluorescent protein), respectively, using fragments generated by two different split positions. These studies support the fact that it is not necessarily one identical site that would work optimally for both complementation and intein-mediated splicing strategies. The splicing mediated split firefly luciferase has been reported with amino acid modifications at the split sites to compensate splicing machineries.^{3,9} As well, intracistronic complementation using β -galactosidase has been reported with the selection of two inactive mutants, one with a deletion of 30 amino acids and a second with the N-terminal 788 amino acids that restore enzyme activity when complementation occurs.^{1,20} A drawback associated with the use of mutants for studying protein complementation is the

formation of self-complemented molecules that produce reporter signal even in the absence of protein interactions. In this study, we succeeded in obtaining active split renilla luciferase fragment-assisted complementation through protein–protein interaction. The development of advanced systems to quantify optical reporter signals from both cell culture and living subjects may lead to many new applications to help understand fundamental intracellular processes as well as development of drugs to modulate them.

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Abbreviations

hrluc	synthetic renilla luciferase enzyme/protein
hrluc	synthetic renilla luciferase reporter gene
N-hrluc	N-terminal portion of synthetic renilla luciferase enzyme/protein
N-hrluc	N-terminal portion of synthetic renilla luciferase reporter gene
C-hrluc	C-terminal portion of synthetic luciferase enzyme/protein
C-hrluc	C-terminal portion of synthetic renilla luciferase gene
fluc	firefly luciferase enzyme/protein
split-fluc	N and C portions of firefly luciferase enzyme/protein
TNF-α	tumor necrosis factor α

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Figure 1.

Schematic diagram showing the protein-fragment-assisted complementation (PCA) strategy using split synthetic renilla luciferase (hrluc) to monitor protein–protein interactions. N-terminal portion of synthetic renilla luciferase is attached to protein X through the linker peptide (GGGGS)₂, and C-terminal portion of synthetic renilla luciferase is connected to protein Y through the linker (GGGGS)₂. Interaction of proteins X and Y recovers hrluc activity through protein complementation and produces light in the presence of the substrate coelenterazine and oxygen.

Figure 2.

A. Schematic diagram showing different split points with nucleotide positions in 5' to 3' direction. The right-directed arrow (→) indicates the forward priming position and the left-directed arrow (←) indicates the reverse priming positions. The positive signs at nucleotide positions 669–670 and 687–688 indicate the split points restored activity during complementation with interacting proteins MyoD and Id. B. Amino acid (311) sequence of synthetic renilla luciferase protein with bolded amino acids showing the split sites. C. Diagram showing the plasmid constructs (i, ii) made for each split site with interacting proteins MyoD and ID under the control of the CMV promoter with linker (GGGS)₂. (iii). The *N-hrluc*–Id construct with NFκB promoter/enhancer elements to modulate the system.

Figure 3.

Protein-protein interaction mediated fragment-assisted complementation of the split *hrluc* system in transiently transfected 293T cells. The signal from cells cotransfected with both *N-hrluc*-Id and *C-hrluc*-MyoD shows significant recovered activity as compared to cells transfected with *N-hrluc*-Id alone and also significant recovered activity as compared to all other plasmids shown. The signal from cells transfected with *C-hrluc*-MyoD is not significantly different from mock-transfected cells. The error bar is the SEM of six samples.

Figure 4.

A. Comparison of protein–protein interaction-mediated fragment-assisted complementation of split firefly luciferase, split synthetic renilla luciferase and native reporters of firefly luciferase, renilla luciferase, and synthetic renilla luciferase in transiently transfected 293T cells. The error bar is the SEM of six samples. B. The TNF- α -modulated protein–protein interaction-mediated fragment-assisted complementation of the split *hrluc* system in transiently transfected 293T cells. The error bar is the SEM of six samples.

Table 1

Nucleotide Sequence and the Positions of PCR Primers with Linkers Used for Constructing the Different Split Synthetic Renilla Luciferase Clones

primer name	primer sequence (5' → 3')	position
N-forward primer	ATAT GCTAG CCACCATGGCTTCCAAGGTGT	1–16
NP.1. reverse primer	ATAT GAATT CGCGAGCCCACCACTGAGGCC	69–50
NP.2. reverse primer	ATAT GAATT CTCTAGCCACGGGCTCGATGT	216–197
NP.3. reverse primer	ATAT GAATT CAGCCCCCAGTCGTGGCCCA	369–350
NP.4. reverse primer	ATAT GAATT CATCCTCCTCGATGTCAGGCC	486–467
NP.5. reverse primer	ATAT GAATT CGATCTCGCGAGGCCAGGA	669–652
NP.6. reverse primer	ATAT GAATT CGCCTCCCTTAACGAGAGG	687–670
C-reverse primer	ATAT CTCGAG TACTGCTCGTTCTTCAGCAC	933–913
CP.1. forward primer	ATAT GGATC CTGCAAGCAAATGAACGTGCTG	70–90
CP.2. forward primer	ATAT GGATC CTGCATCATCCCTGATCTGATC	217–237
CP.3. forward primer	ATAT GGATC CTGTCTGGCCTTTCACACTCC	370–390
CP.4. forward primer	ATAT GGATC CATCGCCCTGATCAAGAGCGAA	487–507
CP.5. forward primer	ATAT GGATC CCCTCTCGTTAAGGGAGGGCAA	670–689
CP.6. forward primer	ATAT GGATC CAAGCCCGACGTCGTCCAGATT	688–708
Id-forward primer with linker	ATAT GAATT CGGTGGCGGAGGGAGCGGTGGCGGAGGGAGCCATAAATTC	
Id-reverse primer	ATAT CTCGAG ATTAACCCTCACTAAAGG	
MyoD-forward primer	ATTAG GCTAG CCGGAGTGGCAGAAAGTTAAGACG	
MyoD-reverse primer with linker	ATAT GGATC CGCTGCCACCTCCGCCTGAACCGCCTCCACCAAGCACCTGATAAATCGCATTGGGGT	

^a The bold letters in each primer sequence are the regions of the restriction enzyme recognition site.