Restriction-ligation-free (RLF) cloning: a high-throughput cloning method by *in vivo* homologous recombination of PCR products

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ABSTRACT. In this study, we optimized a restriction-ligation-free (RLF) method to save time and cost of constructing multiple plasmids with the same gene insert, and examined the efficacy of RLF on high-throughput multi-plasmid cloning. This method utilizes the precise DNA repair and recombination systems within *Escherichia coli*, which allows to bypass the *in vitro* restriction and ligation enzyme reactions commonly included in

routine cloning procedures. A homologous arm is linked to the 5'-end of the forward primer used to amplify both the target gene and vector. A different homologous arm is linked to the 5'-end of the reverse primer. Therefore, genes can be cloned into the vectors by homologous recombination after co-transformation of the amplified target gene and the linearized vector, which bear the same homologous arm on either end. More than twenty-four different plasmids were generated by this method, which uses two simple polymerase chain reaction steps. This method is highly efficient in cloning any gene of interest into any vector at any site without sequence constraints, as no restriction and ligation reactions are required.

INTRODUCTION

Gene cloning, an indispensable technique in molecular biology, is essential for the study of gene expression, regulation, and function. Numerous gene cloning methods, including sticky-end cloning (Scharf et al., 1986), blunt-end cloning (Costa et al., 1994), TA cloning (Holton and Graham, 1991; Marchuk et al., 1991), ligation independent cloning (LIC) (Aslanidis and de Jong, 1990; Shuldiner et al., 1990; Hsiao, 1993; Yang et al., 1993; Kaluz and Flint, 1994; Tillett and Neilan, 1999), and site-specific recombination systems (Hartley et al., 2000), have been developed over the years. These cloning approaches are widely used and have proven to be highly efficacious in most instances; however, they present several limitations because of the extensive enzymatic treatment of the required PCR products or vectors. Furthermore, special and expensive vectors and bacterial strains are required in some cases, such as the In-Fusion Cloning Kit manufactured by Takara Bio Inc. and the Gateway Cloning Kit (Invitrogen). Therefore, novel, streamlined, and cost-effective approaches for gene cloning are the need of the day. Research is currently focused on the development of *in vivo* cloning techniques (Spiliotis, 2012; Ulrich et al., 2012). These techniques take advantage of the endogenous *Escherichia coli* enzymatic activities; the PCR products and vectors are modified to incorporate the identical sequences at their respective 5'- and 3'-ends, and are subsequently digested with single or double restriction endonucleases (Spiliotis, 2012). The PCR products and linearized vectors are then simply co-transformed into *E. coli*, thereby bypassing the need for T4-ligase treatment, and greatly simplifying the steps of gene cloning. However, the major impedance to this approach is the difficulty in digesting all plasmids using restriction enzymes, especially when employing double digestions in one solution. The transformation efficiency of residual closed circular DNA is approximately 100-1000 fold (Hanahan, 1983) higher than that of linear DNA (PCR products and linearized vectors). Therefore, a very small amount of uncleaved, closed circular vector plasmid can result in the production of a large amount of unwanted transformants in selective medium. Furthermore, as vectors have different multiple cloning sites (MCS), designing the necessary number of primers with different vector-homologous sequences when cloning multiple genes to different vectors is a tedious and expensive process.

Therefore, a low-cost and high-throughput cloning method is needed to accommodate the high volume of cloning required in systematic molecular biology analyses, such as the analysis of protein-protein interactions by GST pull-down assays, defining protein subcellular localization using fluorescent tracers, and investigating the various cell signaling pathways by manipulating

the gene expression. To address this need, we have optimized the restriction-ligation-free (RLF) method for vector construction, and examined the efficacy of this approach on high-throughput cloning in multiple vectors. The RLF method utilizes the precise DNA repair and recombination systems in *E. coli* (Kobayashi and Takahashi, 1988) to bypass the *in vitro* restriction and ligation enzyme reactions, which are commonly included in routine cloning procedures. RLF cloning allows the seamless cloning of PCR products into multiple PCR-generated vectors. This new approach requires only two PCR steps to amplify the interested genes and vectors, and is free of the limitations presented by vectors, strains, digestion, ligation, and recombinases, and any additional steps (apart from the PCR amplification step).

MATERIAL AND METHODS

Experiment scheme

As shown in Figure 1, RLF cloning requires two separate PCR steps to amplify the vector(s) and insert. Short DNA fragments, or homologous arms, were added to both ends of the PCR products. The forward primers for the insert and vector bear the same homologous arm, and reverse primers for the insert and vector share another homologous arm. These homologous arms can be flexibly designed. Following PCR, the insert and vector (flanked by homologous arms) are mixed and co-transformed into *E. coli*. A typical RLF cloning procedure can be completed in 6 h (Figure 2). This approach is particularly useful when cloning multiple target genes, as there is no need to custom design different restriction digestions for different vectors.
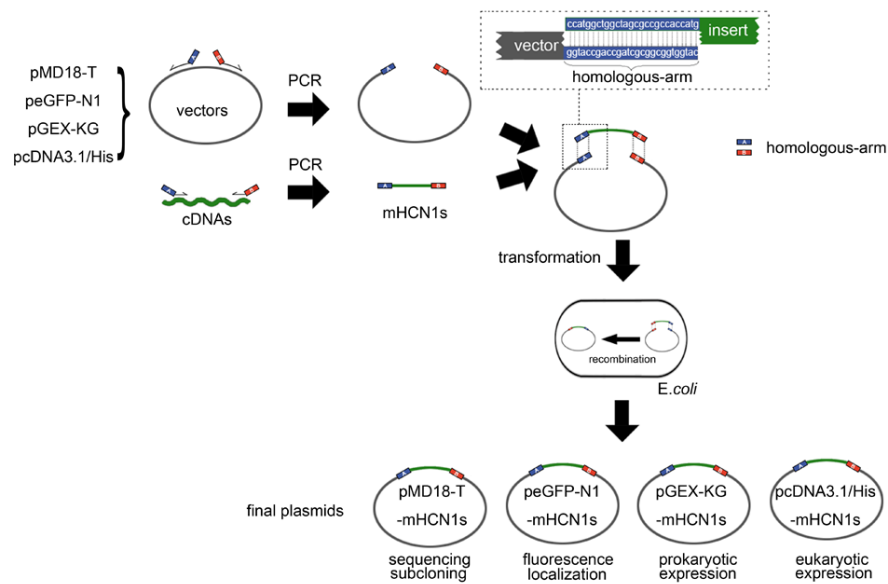

Figure 1. General scheme for the RFL cloning method. PCR products were amplified from vectors and cDNA. Note that the two fragments have the same flanking primer sequences, which were referred to as "homologous arm" sequences. The insert and vector sequences in the homology region hybridize with each other, subsequently forming a partial tetrameric-chain-complex. The vectors used to generate PCR products contain different selectable markers (ampicillin and kanamycin resistance).

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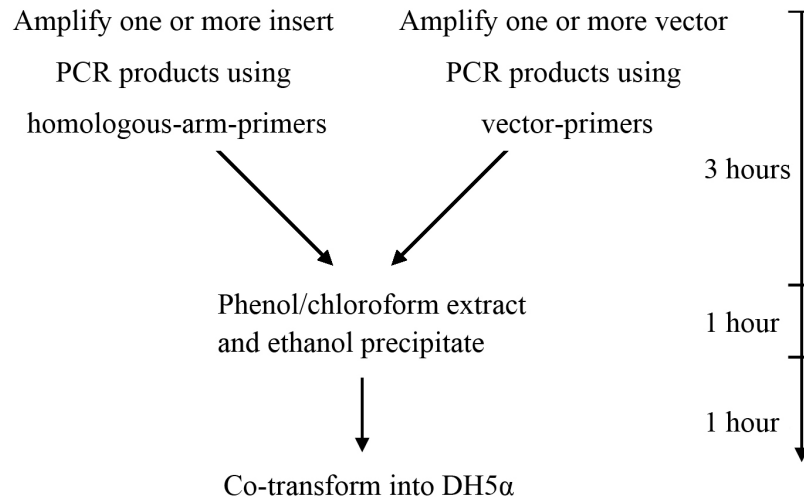

Figure 2. Timeline of a typical complete RFL cloning experiment. Several insert genes and vectors can be amplified simultaneously. As this method does not require digestion and ligation by any enzymes, different target genes can be universally cloned into vectors in less than 6 h utilizing the precise *Escherichia coli* DNA repair and recombination response.

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Vectors and primers

Four vectors, pMD18-T (Takara Bio Inc., Otsu, Japan), peGFP-N1 (Invitrogen, Carlsbad, CA, USA), pGEX-KG (GE Healthcare, Buckinghamshire, UK), and pcDNA3.1/His (Invitrogen), were used in this study. The cDNA of mouse hyperpolarization-activated cyclic nucleotide-gated action channel1 (mHCN1; NM_010408, 2733 bp) was used as the insert template. The primers were synthesized by Genewiz (Genewiz Biotechnology Suzhou, China).

Several primers were designed to generate a series of truncated mHCN1 PCR products (114-2787 bp) to determine whether the insertion size would influence the rate of recombination. All primers consisted of a 27-nucleotide homologous arm at the 5'-end, and a segment complimentary to the template DNA at the 3'-end. All primers utilized in this study are listed in Table 1.

PCR and transformation

PCR was performed in a 50 µL solution containing 0.1 ng vector (template), 200 nM primers, and 2 U Fast PFU DNA polymerase (SinoBio Biotech, Shanghai, China). The PCR was

run under the following conditions: an initial denaturation step at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for an appropriate time calculated using the amplification speed of 2 kb/min; and a final extension at 72°C for 5 min. The PCR products were purified by agarose gel electrophoresis and recovered using the DNA Gel Extraction Kit (Axygen, Union City, CA, USA) according to the manufacturer protocols. Purified PCR products were dissolved in TE buffer [10 mM Tris-HCl, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 7.8]. The purity and concentration of total DNA was determined using a ND-2000C spectrophotometer (Thermo Scientific Fisher, Waltham, MA, USA). Two microliters of the vector (700 ng/μL, OD260/280 = 1.8) and 8 μL (1100 ng/μL, OD260/280 = 1.7) of the insert were mixed gently and subsequently transformed into chemically competent cells (*E. coli* XL Gold; Stratagene, La Jolla, CA, USA) prepared by the method described by Inoue et al. (1990) (competency > 10^9 CFU/μg). The colonies were screened for the presence of the inserted PCR products by colony PCR. A portion of a bacterial colony was picked using a sterile toothpick. The tip of the toothpick was dipped into 20 μL PCR mixture (SinoBio, Biotech, Shanghai, China) containing 100 nM check-primers (Table 1). The colony PCR was performed under the following conditions: an initial denaturation step at 94°C for 10 min; 35 cycles of denaturation at 94°C for 30 s, annealing at an appropriate temperature for 30 s, and extension at 72°C for an appropriate period of time; and a final extension at 72°C for 5 min. The presence or absence of inserts in the PCR products was assessed by electrophoresis.

Table 1. Forward (F) and reverse (R) primers used in the restriction-ligation-free (RLF) method.

Primer	Sequence*	Length
pMD18-T vector (F)	taggatccagcgtagagctgcggatccATCGTCGACCTGCAGGCAT	46
pMD18-T vector (R)	catggtggcggcgctagccagccatggATCTCTAGAGGATCCCCGG	46
pGEX-KG vector (F)	taggatccagcgtagagctgcggatccATTCTAGACTCCATGGGTCGA	48
pGEX-KG vector (R)	catggtggcggcgctagccagccatggCCGGGGATCCACGCGGAACCAG	49
peGFP vector (F)	taggatccagcgtagagctgcggatccGGCCGCGACTCTAGATCAT	46
peGFP vector (R)	catggtggcggcgctagccagccatggCTTGTACAGCTCGTCCATG	46
pcDNA3.1/His vector (F)	taggatccagcgtagagctgcggatccCACAGTGGCGGCCGCTCGA	46
pcDNA3.1/His vector (R)	catggtggcggcgctagccagccatggACCCATTTGCTGTCCACCAGTCAT	51
mHCN1 (F)	ccatggctggctagcgccgccaccatggAAGGCGGCGGCAAAC	43
mHCN1-60 (R)	ggatccgcagctctacgctggatccTAGACGCTGTTGCCATCGTCGC	47
mHCN1-120 (R)	ggatccgcagctctacgctggatccTACGGGGTCCCCAGGCGCTTG	46
mHCN1-240 (R)	ggatccgcagctctacgctggatccTAATACTGCCGCCGGGGCCCCT	47
mHCN1-480 (R)	ggatccgcagctctacgctggatccTACGTCTGCTCTGTGAAGAACGTGAT	51
mHCN1-960 (R)	ggatccgcagctctacgctggatccTAAACCCAGCAATCTGGTGGGAAGTC	51
mHCN1-2733 (R)	ggatccgcagctctacgctggatccTATAAATTCGAAGCAAAACGGGGT	49
pMD18-T check (F)	GAGCGGATAACAATTTCACACAGG	24
pMD18-T check (R)	CGCCAGGGTTTTCCCAGTCACGAC	24
peGFP-N1 check (F)	GGATCACTCTCGGCATGGACGA	22
peGFP-N1 check (R)	CCTCTACAAATGTGGTATGGCTGATTATG	29
pcDNA3.1/His check (F)	TAATACGACTCACTATAGGGAGAC	24
pcDNA3.1/His check (R)	TAGAAGGCACAGTCGAGGCT	20
pGEX-KG check (F)	CTGGTTCCGCGTGGATCC	18
pGEX-KG check (R)	TGAATTAAGCTTGAGCTC	18

*The 27 bp-long homologous arm sequences are denoted in lowercase letters, and the sequence that anneals with the templates are denoted in uppercase letters for all the primer sequences.

Functional validation

The expression of the inserts was examined by prokaryotic transformation and eukaryotic transfection of the 6 recombinant vectors (mHCN1-60, mHCN1-120, mHCN1-240, mHCN1-480,

mHCN1-960, and mHCN1-2733), in order to determine if the inserts exhibited normal functionalities in the 4 vectors (pMD18T, pGEX-KG, peGFP-N1, pcDNA3.1/His) created by RLF cloning. The pGEX-KG vector, which contains a fusion glutathione S-transferase (GST-tag) in the 5′-end of the inserts, was transformed into *E. coli* BL21 (DE3) cells for prokaryotic expression. *E. coli* BL21 (DE3) cells were cultured in Lysogeny broth media. Plasmid expression was induced by 0.8 mM isopropyl-β-d-thiogalactopyranoside (IPTG) at 37°C for 4 h.

The pEGFP-N1 and pcDNA3.1/His plasmids bearing the inserts were transfected into MCF-7 cells to detect the fluorescence localization and eukaryotic expression of the inserts. The cells were transfected as follows: MCF-7 cells were cultured in complete medium for 24 h, and subsequently in antibiotic-free and serum-free RPMI-1640 medium for 8 h. The resuspended cells were then transiently transfected with pEGFP-N1 plasmids (1 µg/mL per well) using the Turbofect transfection reagent (Hoffmann-LaRoche, Basel, Switzerland). The transfection reagent was removed after 8-9 h, and the cells were cultured in complete medium with antibiotics and serum for 24 h. The protein expression was analyzed by western blotting, as follows: MCF-7 cells with pcDNA3.1/His plasmids (bearing the inserts) were directly lysed with lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 25 mM glycerophosphate, 150 mM NaCl, 2 mM EDTA, 2 mM ethylene glycol tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and protease and phosphatase inhibitors) for 10 min on ice. The lysates were collected and centrifuged at 15,000 *g* for 15 min at 4°C. The supernatant was recovered, and the protein concentration measured using the protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA). The proteins were subsequently separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% SDS-PAGE), transferred to a polyvinylidene difluoride membrane, blocked in 5% milk, and incubated with anti-His antibody (Santa Cruz Biotechnology, USA) (1:3000) at 4°C overnight. The blots were incubated with secondary antibody at room temperature for 2 h, and subsequently washed with TBST. Antibody binding was visualized using the electrochemiluminescence kit (Thermo Scientific Pierce, Rockford, IL, USA).

RESULTS AND DISCUSSION

The influence of the insert size on transformant yield and recombination rates were defined by generating six PCR products containing differentially truncated mHCN1 (114 bp mHCN1-60, 174 bp mHCN1-120, 294 bp mHCN1, 534 bp mHCN1-480, 1014 bp mHCN1-960, and 2787 bp mHCN1-2733) using one forward primer and a panel of six reverse primers (Figure 3A). Four vectors were amplified by PCR using the vector-primers (Table 1). The amplified pMD18T, pEGPF, and pGEX are displayed in Figure 3B (pcDNA3.1/His is not shown). In order to determine the recombination rates following transformation into XL Gold *E. coli*, 16 colonies were randomly picked and tested by colony PCR using check-primers (Figure 3C). Transformant yields steadily increased with the decrease in insert size, while the recombination rates remained unaffected by the insert size (Table 2).

E. coli BL21 (DE3) cells harboring pGEX-KG (mHCN1-60, mHCN1-120, mHCN1-240, mHCN1-480, mHCN1-960, or mHCN1-2733) were induced by IPTG, and the expression of the GST-mHCN fusion proteins was subsequently analyzed by SDS-PAGE (Figure 4). The predicted molecular weights of the GST-mHCN recombinant fusion proteins were 28, 31, 35, 44, 61, and 126 kDa. The expression yields of mHCN1-120 (31 kDa), mHCN1-240 (35 kDa), mHCN1-480 (44 kDa), and mHCN1-960 (61 kDa) were high because of the properties of the fusion partner (GST-tag). However, mHCN1-2733 (126 kDa) was not expressed in the *E. coli* cells; this could be attributed to the 37 consecutive glutamines in the C-terminus of mHCN, which does not translate well in *E. coli* cells.
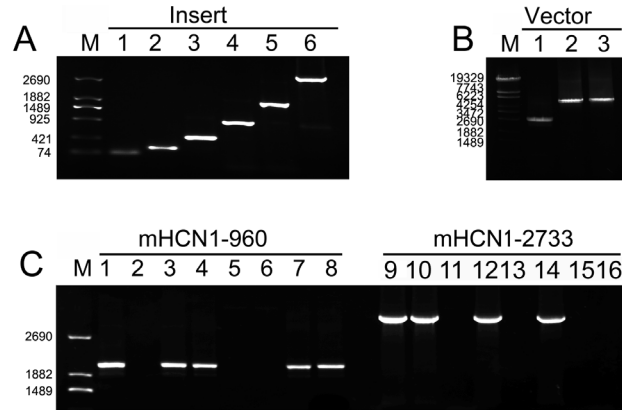
Figure 3. Agarose gel electrophoresis results for the inserts and constructs. **A.** Six different truncated PCR products of mHCN1. The fragments have a 27-bp homologous arm at both ends. The bands in *lanes 1-6* represent the PCR products (114, 174, 294, 534, 1014, and 2787 bp). *Lane M* indicates the marker. **B.** PCR products from three different vectors (pMD18-T, peGFP-N1, and pGEX-KG) using vector-primers, which include a 27-bp homologous arm overhang sequence with insert PCR products. *Lane 1* = pMD18-T. *Lane 2* = peGFP-N1. *Lane 3* = pGEX-KG. *Lane M* = marker. **C.** Results of the colony PCR screening for positive peGFP-N1 recombinants bearing mHCN1-960 and mHCN1-2733. *Lanes 1-8* = colony PCR screening for mHCN1-960-postive colonies. *Lanes 9-16* = colony PCR screening for mHCN1-2733-positive colonies. *Lane M* = marker.

Table 2. Effect of the vector length and type on the restriction-ligation-free method.

Vector	Length (kb)	Usage of vector	Resistance	mHCN1-60	mHCN1-120	mHCN1-240	mHCN1-480	mHCN1-960	mHCN1-2733
pMD18-T	2.7	Cloning	Amp	840 (81%)*ᐃ	872 (88%)	720 (69%)	648 (75%)	424 (88%)	256 (81%)
peGFP-N1	4.7	Tracing	Kan	632 (94%)	976 (88%)	392 (75%)	328 (88%)	272 (69%)	288 (75%)
pGEX-KG	5.0	Expression	Amp	616 (81%)	552 (88%)	496 (88%)	432 (75%)	224 (69%)	112 (56%)
pcDNA3.1/His	5.4	Expression	Amp	568 (88%)	456 (75%)	472 (81%)	232 (81%)	168 (88%)	56 (88%)

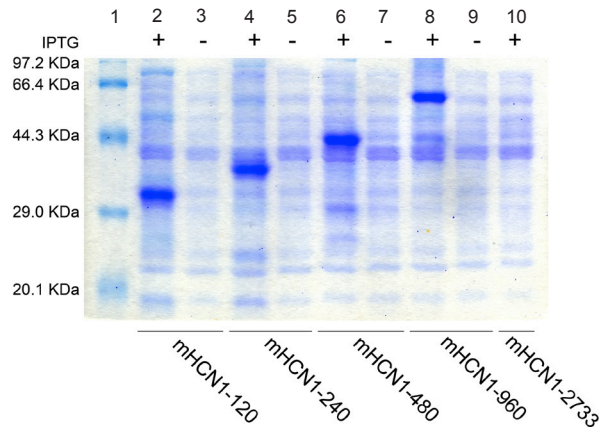*Number of total colonies; ᐃfraction of recombinants.

Figure 4. SDS-PAGE was used to identify the protein expression of pGEX-KG-mHCN1-120, -240, -480, and -960, generated by RFL cloning, in prokaryotic cells. *Lane 1* = marker; *lanes 3*, *5*, *7*, and *9* = before IPTG induction; *lanes 2*, *4*, *6*, *8*, and *10* = after IPTG induction.

The fluorescence localization (peGFP-N1) and eukaryotic expression (pcDNA3.1/His) of the recombinant vectors generated by RLF cloning were also analyzed. pEGFP-N1 vectors containing mHCN1-60 or mHCN1-2733 were transfected into MCF-7 cells (Figure 5); these two plasmids, generated by RLF cloning, were successfully expressed in the mammalian cells. Moreover, the His-tagged proteins mHCN1-480 (18 kDa), mHCN1-960 (36 kDa), and mHCN1-2733 (100 kDa) were expressed in cells transfected with recombinant pcDNA3.1/His vectors (detected by blotting; Figure 6). The His-tagged proteins mHCN1-60 (3 kDa), mHCN1-120 (5 kDa), and mHCN1-240 (9 kDa) were not detected because of their low molecular weight. These expression analyses revealed that recombinant vectors generated by RLF cloning are normally expressed in both prokaryotic and eukaryotic cells.
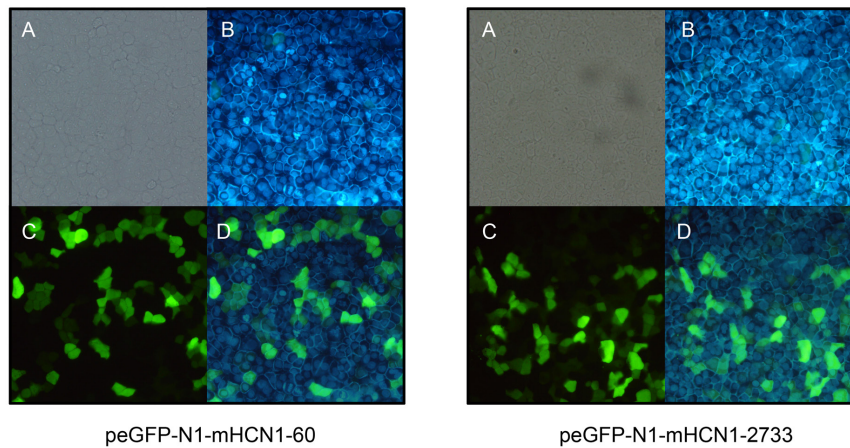

peGFP-N1-mHCN1-60   peGFP-N1-mHCN1-2733

Figure 5. Observation of peGFP-N1-mHCN1-60 and mHCN1-2733 expression in MCF-7 cells. The peGFP-N1-mHCN1s plasmids generated by RFL cloning are expressed in mammalian cells.peGFP-N1-mHCN1-60 and mHCN1-2733 presented a comparable transfection efficiency. **A.** Phase contrast images. **B.** Images showing GFP expression. **C.** DAPI images. **D.** Merged GFP and DAPI images.
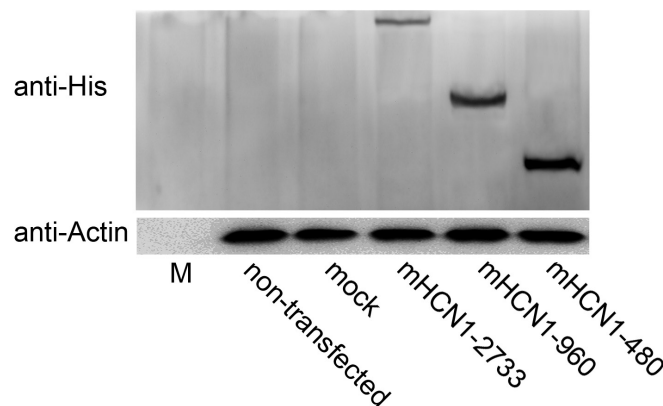

Figure 6. Expression of pcDNA3.1/His-mHCN1-480, -960 and -2733 in MCF-7 cells. The non-transfected and mock groups could not be detected by the anti-His antibody. The mHCN1-480, -960, and -2733-transfected groups presented specific bands. mHCN1-60, -120, and -240 cannot be detected because of their low molecular weights.

The RLF cloning approach has several advantages: 1) this approach dispels the need for enzymatic reactions, such as restriction enzyme digestions, alkaline phosphatase treatments, and T4 DNA ligase ligations, thereby reducing the cost and time required for cloning; therefore, this approach is especially suitable for high-throughput cloning experiments. 2) This method is simple, convenient, and rapid because of the minimal requirement of manual handling. 3) This approach enables the direct cloning of the insert into the vectors using an *in vivo E. coli* homologous recombination system. This is particularly useful in ensuring the correct reading frame of the insert. 4) The vector-primers can be designed for any site on the vector, which allows the insertion of genes of interest into multiple sites in the vector, as well as the modification of the elements in the vectors. 5) The method can be used to introduce mutations, such as deletions and/or insertions, into the target genes using vector-primers with homologous arms located inside the gene. 6) Only 2 homologous arms are used in the primers for multiple vectors and inserts, which minimizes the effort of designing homologous arms.

The selection of appropriate restriction enzymes with cutting sites only in the MCS of the vectors (and not in the inserted genes) is a challenging task, especially when cloning large genes. This is not a concern when using the RLF method, as this approach does not use restriction enzymes. One concern of RLF cloning is the possibility of introducing PCR-derived mutations, which can be minimized by the use of a high-fidelity DNA polymerase for PCRs.

The flexibility afforded by RLF in choosing inserts and vectors, as well as the seamless insertion, easy handling, and the high efficiency of cloning, make this method an ideal protocol for high-throughput recombinant vector construction.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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