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Systemic Gene Transfer to Skeletal Muscle Using Reengineered AAV Vectors

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Abstract

Gene therapy of musculoskeletal disorders warrants efficient gene transfer to a wide range of muscle groups. Reengineered adeno-associated viral (AAV) vectors that selectively transduce muscle tissue following systemic administration are attractive candidates for such applications. Here we provide examples of several lab-derived AAV vectors that display systemic tissue tropism in mice. Methods to evaluate the efficiency of gene transfer to skeletal muscle following intravenous or isolated limb infusion of AAV vectors in mice are discussed in detail.

Keywords

AAV; Reengineering; Capsid; Tropism; Muscle; Gene transfer; Isolated limb infusion; Gene therapy

1. Introduction

Musculoskeletal disorders include a broad spectrum of diseases that often share clinical symptoms involving muscle weakness, damage, and degeneration. For instance, the muscular dystrophies (MD) are a group of inherited muscle disorders characterized by progressive muscular degeneration. Diseases including Duchenne, Becker, limb girdle, myotonic, and others are included within this disease group (1, 2). A wide variety of neuromuscular diseases affecting the central nervous system such as cerebral palsy, spinal muscular atrophy, and congenital myasthenia gravis also share similarities with musculoskeletal complications seen in MD (3, 4). Another prominent example is lysosomal storage disease, a heterogeneous group of inherited metabolic diseases resulting from defects in the degradation or transport of several distinct by-products of cellular turnover (5, 6). The various subtypes are characterized by multisystemic involvement with neurodegenerative features and musculoskeletal complications often seen in most severe forms of the disease.

Gene therapy of musculoskeletal disorders is a promising strategy, wherein corrective gene replacement can abrogate disease symptoms. For instance, promising preclinical studies using truncated forms of the dystrophin gene in mouse models of muscular dystrophy have been reported (7–10). Correction of glycogen storage disorders such as Pompe disease by efficient expression of enzymes such as acid alpha-glucosidase in skeletal muscle has also been shown (11, 12). However, a significant hurdle towards achieving efficient gene transfer in skeletal muscle is the paucity of vectors that can selectively transduce a broad spectrum of muscle groups following systemic administration.

We recently generated a panel of synthetic adeno-associated viral (AAV) vectors by reengineering a previously identified heparan sulfate receptor footprint on AAV serotype 2 (13). This approach yielded several chimeric AAV capsids displaying systemic muscle tropism following intravenous administration in mice (Table 1). In particular, an AAV2/AAV8 chimeric (AAV2i8) with a unique capsid surface footprint readily traversed the blood vasculature and selectively transduced cardiac and whole body skeletal muscle tissues with high efficiency. Unlike other AAV serotypes, which are preferentially sequestered by the liver, AAV2i8 displays reduced hepatic tropism. The unique pharmacokinetic and pharmacodynamic features of AAV2i8 might be particularly relevant for systemic gene delivery in the treatment of musculoskeletal disorders. The current report describes methods for evaluating the systemic muscle tropism of AAV vectors following intravenous or isolated limb infusion in mice.

2. Materials

2.1. Generating Reengineered AAV2 Vectors

2.1.1. Mutagenesis

1. Plasmid containing AAV2 capsid gene coding for VP1, VP2, and VP3 proteins (see Note 1).
2. Primers for site-directed mutagenesis (Table 2) (IDT, Coralville, IA, USA).
3. Quikchange II site-directed mutagenesis kit[®] (Stratagene, La Jolla, CA, USA).
4. Molecular biology/PCR grade water.
5. Biometra Thermal Gradient Cyclor (or other equivalent).
6. Electroporation-competent *E. coli* DH10B (Stratagene) (see Note 2).
7. Ampicillin LB agar plates for selection.
8. Plasmid DNA Miniprep and Maxiprep kits (Qiagen, Valencia, CA, USA).

2.1.2. AAV Vector Production—Several detailed protocols for the production of recombinant AAV vectors packaging different transgene cassettes have been reported (14, 15). Specific reagents for generation of reengineered AAV2 vectors relevant to the current report include:

1. Plasmid containing reengineered AAV2 capsid genes.
2. Plasmid containing AAV inverted terminal repeats (ITRs) flanking the chicken beta-actin (CBA) promoter-driven firefly luciferase (Luc) cassette.

2.2. Evaluating Systemic Gene Transfer to Skeletal Muscle Using AAV Vectors

2.2.1. Intravenous (Tail Vein) Administration of AAV Vectors

1. Balb/c male or female mice (see Note 3).

¹Different plasmid reagents have been established for production of naturally occurring AAV isolates as well as lab-derived strains. For this specific application, the pXR2 plasmid backbone or pACG backbones described earlier are appropriate templates (16, 17). Alternative templates containing capsid gene encoding for VP1, VP2, VP3 proteins of AAV serotype 2 can also be utilized for site-directed mutagenesis. It is important to note that primer sequences might vary accordingly.

²Stratagene recommends utilization of Electroten[®] blue electroporation-competent *E. coli* for transformation following site-directed mutagenesis. We have successfully obtained mutant clones at a high frequency (50% or more) using the DH10B strain. Chemically competent *E. coli* may also be utilized, although the transformation efficiency might be lower.

³Previous studies have demonstrated gender differences in transduction efficiency of different AAV vectors in mice (18, 19). In particular, certain AAV serotypes tend to show higher transduction levels in the male liver following intravenous administration. Other tissue types do not appear to demonstrate marked differences in transduction efficiency.

2. 28–30G insulin syringes (BD, Franklin Lakes, NJ, USA).
3. AAV Vector suspended in 1× phosphate-buffered saline (PBS).
4. 70% isopropyl alcohol to cleanse tail vein injection site.
5. Heat lamp or warm water (~48°C) to increase circulation and dilate the tail vein.
6. Plastic mouse restrainer for tail vein injection (Baintree Scientific, MA, USA).

2.2.2. Isolated Limb Infusion of AAV Vectors

1. Balb/c male or female mice (see Note 4).
2. AAV suspended in 1× PBS.
3. Polyethylene PE-10 tubing (BD).
4. 30G needles (Thermofisher, Waltham, MA, USA).
5. High-pressure syringe pump (Harvard Apparatus).
6. Isofluorane.
7. Heat pad (Baintree Scientific).
8. Mineral oil or other eye lubricant.
9. Betadine and 70% isopropyl alcohol.
10. Sterile scissors and forceps (Baintree Scientific).
11. Sterile cotton swabs.
12. 5–0 silk suture (Baintree Scientific).
13. Small rubber-bands.
14. Clippers with #40 blade (Baintree Scientific).

2.2.3. Live Animal Bioluminescence Imaging (See Note 5)

1. Balb/c male or female mice from Subheadings 2.2.1 or 2.2.2.
2. Xenogen Lumina[®] live animal bioluminescence imaging system and software (Caliper Life Sciences, CA, USA).
3. Isofluorane.
4. Luciferin substrate (Nanolight, Pinetop, AZ, USA or Caliper Lifesciences, CA, USA).
5. 28G insulin syringes (BD).

2.2.4. Harvesting Different Muscle Groups

1. Balb/c male or female mice from above sections.
2. Dissecting scissors and forceps.
3. 25G needles (1/2 in.).

⁴Gender differences may be seen as noted earlier. In addition, for isolated limb infusion studies, any mouse strain such as C57/B16 or other relevant strain may be used. Balb/c mice (white coat) are typically utilized for applications involving live animal imaging.

⁵Although the Xenogen live animal imaging system allows monitoring kinetics and dynamics of gene expression, quantitative analysis of luciferase expression in harvested tissue/organs is a well-established alternative to this approach. Accordingly, we have included procedures for quantitation of bioluminescence in tissue lysates as an alternative for live animal imaging.

4. 20–60 mL luer-lock syringes.
5. 1× PBS.
6. 3.8% sodium citrate or 0.5 mM EDTA (anticoagulant).
7. 2 mL microcentrifuge tube.
8. 2.5% Avertin or anesthetic of choice (Sigma, St. Louis, MO, USA).

2.2.5. Tissue Lysate Luciferase Expression Assay

1. TissueLyser II (Qiagen) (see Note 6).
2. 96-Well flat bottom, white polystyrene assay plates.
3. 96-Well flat bottom, clear polystyrene assay plates.
4. Assay-grade Luciferin substrate (Promega, Madison, WI, USA).
5. 2× Passive Lysis Buffer (Promega).
6. Victor 2[®] Luminometer (Perkin Elmer, Waltham, MA, USA).
7. Bradford protein assay reagent (Bio-Rad, Hercules, CA, USA).
8. Eppendorf 5424 microcentrifuge.

3. Methods

3.1. Generating Reengineered AAV2 Vectors

3.1.1. Mutagenesis (See Note 7)

1. Setup reactions for site-directed mutagenesis as outlined in the Instruction Manual for the Quikchange II site-directed mutagenesis kit.
2. Setup thermal cycling parameters as follows: denaturation at 95°C for 30 s; annealing at 55°C for 1 min; extension at 68°C for 7 min for a total of 25–30 cycles.
3. Upon completion of thermal cycling, cool the reaction at 4°C followed by treatment with *DpnI* to digest plasmid template.
4. Transform using electroporation-competent DH10B followed by plating of transformed cells onto LB Amp agar plates and incubate overnight at 37°C.
5. Subject individual clones amplified overnight to plasmid DNA isolation using a miniprep kit and submit for DNA sequencing to validate modified capsid gene sequence.

3.1.2. AAV Vector Production—Several detailed protocols for the production of recombinant AAV vectors packaging different transgene cassettes have been reported. No modifications to published methods are required for the generation of reengineered AAV2 vectors (see Note 8).

⁶The Qiagen TissueLyserII[®] is ideally suited for processing tissue and rapid generation of tissue lysates. Alternatively, tissues can be minced/ground using dissecting scissors or homogenized using a small mortar and pestle, respectively.

⁷We have optimized PCR conditions for carrying out mutagenesis of different AAV capsid genes. However, reaction conditions for site-directed mutagenesis are also outlined by the manufacturer (Stratagene Quikchange[®] kit). Primer design and reaction conditions might require further optimization for different genes.

3.2. Evaluating Systemic Gene Transfer to Skeletal Muscle Using AAV Vectors

3.2.1. Intravenous (Tail Vein) Administration of AAV Vectors

1. Allow mice to sit under heat lamp for 1–3 min, or submerge tail in warm water for 30 s to 1 min to allow the tail vein to dilate.
2. Restrain the mouse with a plastic restrainer and wipe tail with 70% ethanol.
3. Insert needle almost parallel to the left or right lateral tail vein.
4. Ensure that the bevel of the needle is facing upward and inject the vector dose slowly.
5. Injection quality is considered good if the vein clears. However, if injection fluid accumulates subcutaneously, remove needle and reinject at a proximal site on the tail vein.

3.2.2. Isolated Limb Infusion of AAV Vectors

1. Remove hub from one of the 30G needles and insert into the end of the PE-10 tubing. This constitutes the injection end. Use another 30G needle and insert to the opposite end (leaving hub intact for syringe attachment). The length of tubing can vary based on space between the test animal and syringe pump setup.
2. Load the syringe with 200, 500 μ L, or 1 mL of vector dose ranging from 10^9 to 10^{11} particles per animal.
3. Anesthetize mice using 2.5% isoflurane delivered through a nose-cone apparatus. Alternatively, intraperitoneal injection of low-dose Avertin or preferred anesthetic can be used.
4. Once mice are completely anesthetized, apply rubber band around the base of the hind limb (Fig. 1) to occlude blood flow.
5. Tape foot down to surgical board to secure and shave hair around incision site using clippers.
6. Sterilize with three rotations of Betadine and 70% isopropyl alcohol using sterile swabs.
7. Make a 1 cm incision to expose the medial saphenous vein (Fig. 1).
8. Insert needle until bevel is inside of the vein.
9. Infuse at 1 mL/min.
10. After injection is complete, leave needle in for another minute until pressure has decreased and entire dose has been delivered (see Note 9).
11. Suture skin together and apply antibiotic ointment.
12. Allow mice to recover on heat pad until awake and moving around.

⁸It should be noted that reengineered AAV2 vectors, relevant to the current study, do not bind heparan sulfate (13). Therefore, heparin affinity column chromatography is not suitable for purification of aforementioned AAV vectors. Standard purification procedures involving cesium chloride ultracentrifugation have been described (14, 15).

⁹For high-pressure isolated limb infusions, it is important to leave the catheter tip in vein for another minute after injection is complete to minimize leakage/back-flow. For high volume injections, fluid leakage from the injection site is likely. Such can be minimized by optimizing injection conditions and intravenous residence time of the catheter for differently sized mice (20).

3.2.3. Live Animal Bioluminescence Imaging (See Note 5)

1. Anesthetize mice using 2.5% isoflurane using Xenogen anesthesia setup.
2. Once completely anesthetized, inject 120 μ L of a 25 mg/mL luciferin substrate per 20 g body weight through intraperitoneal injection and wait 5 min.
3. Place animals in individual nose-cones within imaging chamber and reduce isoflurane to 1.5%.
4. After 5 min, acquire image and obtain overlaid bioluminescent and grayscale mouse images at 5 s, 1, and 5 min exposure time.
5. Allow mice to recover on a heat pad until fully awake.

3.2.4. Harvesting Different Muscle Groups

1. Anesthetize mice with 2.5% Avertin (or anesthetic of choice).
2. Once mice are completely anesthetized, make a midline incision from the bottom (between the hind limbs) all the way up to the second rib.
3. Separate muscle and skin with needles or retractors, and cut diaphragm to expose the heart.
4. Once heart is exposed, insert 25G needle (attached to a 20–60 mL leur-lock syringe containing 20 mL 1 $\times$ PBS with 100 μ L of 3.8% sodium citrate or 0.5 mM EDTA) into the left ventricle. Begin perfusion followed by incision of right atrium of the heart to allow outflow of perfusate.
5. Upon completion of perfusion, euthanize the mouse via cervical dislocation and remove specific muscle groups (five sets of fore and hind limb skeletal muscle, diaphragm, abdominal, intercostal muscles, and other organs, if warranted) (Fig. 2). Be sure to rinse with 1 $\times$ PBS before storage in 2 mL micro-centrifuge tubes.
6. Tissues may be prepared for luciferase assay immediately, or frozen at -80°C for up to 6 months.

3.2.5. Luciferase Expression Assay with Tissue Lysates

1. For use with TissueLyser II – add sterile, stainless steel beads to 2 mL microcentrifuge tubes and run at 10 Hz for 2 intervals of 10 s each (see Note 6).
2. Take approximately 50 μ L (~15–25 mg of tissue homogenate) and place in a separate tube for viral DNA isolation, if required.
3. Add 150 μ L of 2 $\times$ passive lysis buffer to new sample and mix by pipetting several times.
4. Allow sample to sit on ice for 5 min, then mix by pipetting.
5. Centrifuge samples at $2,350 \times g$ for 3 min.
6. Take 50 μ L of supernatant and place in the 96-well, flat bottom, white polystyrene plates. Tissue lysates from untreated mice are used as background controls (see Note 10).

¹⁰For luciferase assays, presence of minor amounts of tissue debris in the supernatant will not interfere with the assay. It is critical to skip rows and columns (i.e., place samples in alternating wells) to avoid overlap in signal between wells. This step is critical to avoid false positives.

7. Add 100 μ L of assay-grade luciferin substrate to each sample well and obtain readout using the Victor 2[®] luminometer within 10 min of substrate addition.
8. To determine protein concentration of supernatant, add 1 μ g to a 96 well, flat bottom, clear polystyrene assay plate. Add 200 μ L of Bio-rad protein assay reagent (diluted 1:5 using sterile dH₂O) to each well, mix by pipetting, and incubate at room temperature for 5 min. Obtain protein concentrations using a standard curve generated using different concentrations of BSA (Sigma, St. Louis, MO) and normalize tissue lysate luciferase expression levels to relative light units (RLU)/ μ g of cellular protein (Fig. 2).

References

1. Guglieri M, Straub V, Bushby K, Lochmüller H. Limb-girdle muscular dystrophies. *Curr Opin Neurol.* 2008; 21:576–584. [PubMed: 18769252]
2. Deconinck N, Dan B. Pathophysiology of duchenne muscular dystrophy: current hypotheses. *Pediatr Neurol.* 2007; 36:1–7. [PubMed: 17162189]
3. Farrar MA, Johnston HM, Grattan-Smith P, Turner A, Kiernan MC. Spinal muscular atrophy: molecular mechanisms. *Curr Mol Med.* 2009; 9:851–862. [PubMed: 19860664]
4. Meriggioli MN, Sanders DB. Autoimmune myasthenia gravis: emerging clinical and biological heterogeneity. *Lancet Neurol.* 2009; 8:475–490. [PubMed: 19375665]
5. Aldenhoven M, Sakkars RJ, Boelens J, de Koning TJ, Wulffraat NM. Musculoskeletal manifestations of lysosomal storage disorders. *Ann Rheum Dis.* 2009; 68:1659–1665. [PubMed: 19822711]
6. Mah C, Cresawn KO, Fraites TJ Jr, Pacak CA, Lewis MA, Zolotukhin I, Byrne BJ. Sustained correction of glycogen storage disease type II using adeno-associated virus serotype 1 vectors. *Gene Ther.* 2005; 12:1405–1409. [PubMed: 15920463]
7. Duan D. Myodys, a full-length dystrophin plasmid vector for Duchenne and Becker muscular dystrophy gene therapy. *Curr Opin Mol Ther.* 2008; 10:86–94. [PubMed: 18228186]
8. Muir LA, Chamberlain JS. Emerging strategies for cell and gene therapy of the muscular dystrophies. *Expert Rev Mol Med.* 2009; 25:e18. [PubMed: 19555515]
9. Zhu T, Zhou L, Mori S, Wang Z, McTiernan CF, Qiao C, Chen C, Wang DW, Li J, Xiao X. Sustained whole-body functional rescue in congestive heart failure and muscular dystrophy hamsters by systemic gene transfer. *Circulation.* 2005; 112:2650–2659. [PubMed: 16230483]
10. Tang Y, Cummins J, Huard J, Wang B. AAV-directed muscular dystrophy gene therapy. *Expert Opin Biol Ther.* 2010; 10:395–408. [PubMed: 20132060]
11. Pastores GM. Musculoskeletal complications encountered in the lysosomal storage disorders. *Best Pract Res Clin Rheumatol.* 2008; 22:937–947. [PubMed: 19028373]
12. Mah C, Pacak CA, Cresawn KO, Deruisseau LR, Germain S, Lewis MA, Cloutier DA, Fuller DD, Byrne BJ. Physiological correction of Pompe disease by systemic delivery of adeno-associated virus serotypes 1 vectors. *Mol Ther.* 2007; 15:501–507. [PubMed: 17245350]
13. Asokan A, Conway JC, Phillips JL, Li C, Hegge J, Sinnott R, Yadav S, DiPrimio N, Nam HJ, Agbandje-McKenna M, McPhee S, Wolff J, Samulski RJ. Reengineering a receptor footprint of adeno-associated virus enables selective and systemic gene transfer to muscle. *Nat Biotechnol.* 2010; 28:79–82. [PubMed: 20037580]
14. Choi, VW.; Asokan, A.; Haberman, RA.; Samulski, RJ. *Curr Protoc Mol Biol.* Vol. Chapter 16. 2007. Production of recombinant adeno-associated viral vectors for in vitro and in vivo use; p. 25
15. Grieger JC, Choi VW, Samulski RJ. Production and characterization of adeno-associated viral vectors. *Nat Protoc.* 2006; 1:1412–1428. [PubMed: 17406430]
16. Zolotukhin S, Potter M, Zolotukhin I, Sakai Y, Loiler S, Fraites TJ Jr, Chiodo VA, Phillipsberg T, Muzyczka N, Hauswirth WW, Flotte TR, Byrne BJ, Snyder RO. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods.* 2002; 28:158–167. [PubMed: 12413414]

17. Rabinowitz JE, Rolling F, Li C, Conrath H, Xiao W, Xiao X, Samulski RJ. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol.* 2002; 76:791–801. [PubMed: 11752169]
18. Pañeda A, Vanrell L, Mauleon I, Crettaz JS, Berraondo P, Timmermans EJ, Beattie SG, Twisk J, van Deventer S, Prieto J, Fontanellas A, Rodriguez-Pena MS, Gonzalez-Aseguinolaza G. Effect of adeno-associated virus serotype and genomic structure on liver transduction and biodistribution in mice of both genders. *Hum Gene Ther.* 2009; 20:908–917. [PubMed: 19419275]
19. Davidoff AM, Ng CY, Zhou J, Spence Y, Nathwani AC. Sex significantly influences transduction of murine liver by recombinant adeno-associated viral vectors through an androgen-dependent pathway. *Blood.* 2003; 102:480–488. [PubMed: 12637328]
20. Hagstrom JE, Hegge J, Zhang G, Noble M, Budker V, Lewis DL, Herweijer H, Wolff JA. A facile nonviral method for delivering genes and siRNAs to skeletal muscle of mammalian limbs. *Mol Ther.* 2004; 10:386–398. [PubMed: 15294185]

Fig. 1.

Schematic of isolated limb infusion procedure for evaluation of systemic gene transfer to skeletal muscle using AAV vectors. (*Left*) Cartoon shows the venous structure of the hind limb. (*Right*) The great saphenous vein is utilized for vector administration. The catheter tip is inserted into the distal portion of the great saphenous vein. As the syringe pump injects the virus, the entire saphenous vein will clear, including all branching veins that feed from different hind limb muscle groups. The tourniquet will prevent any leakage from the saphenous vein into the vena cava, which will in turn preclude systemic organ transduction. This schematic was previously published in ref (20) and has been reproduced in accordance with the rights and permissions policy of Nature Publishing Group.

Fig. 2.

Relative luciferase expression levels in different skeletal muscle groups following isolated limb infusion of AAV2i8 (*black bars*) and AAV8 (*gray bars*) vectors in mice. Different volumes of injection, 200 µL (**a**, low), 500 µL (**b**, medium), and 1 mL (**c**, high), were administered into the saphenous vein as described in the main text. All studies were carried out in triplicate. *Error bars* indicate one standard deviation. Note that AAV2i8 vectors display increased gene transfer efficiency at low volume of injection, while AAV8 vectors transduce certain skeletal muscle groups with higher efficiency at high injection volume.

Table 1Summary of tissue tropisms and relative transduction efficiencies of reengineered AAV vectors^a

AAV mutant(s)	Amino acid sequence	Transduction efficiency (muscle)	Transduction efficiency (liver)	Tropism
AAV2	585-RGNRQA-590	+	+++	Liver
AAV8	585-QQNTAP-590	++++	++++	Systemic
2i1; 2i6	585-SSSTDP-590	–	–	N/A
2i3a/3b	585-SSNTAP-590	–	–	N/A
2i4	585-SNSNLP-590	–	–	N/A
2i5	585-SSTTAP-590	–	–	N/A
2i9	585-SAQAQA-590	–	–	N/A
2i8; 2irh43; 2irh49–53,57,58,64	585-QQNTAP-590	++++	+	Systemic muscle
2i10; 2irh40	585-QANTGP-590	++	+	Systemic muscle
2irh38	585-QTNTGP-590	++	+	Systemic muscle
2irh2	585-QTNGAP-590	++	+	Systemic muscle
2i11; 2i12; 2irh32–34	585-NATTAP-590	++	+	Systemic muscle

^a Transduction profiles were derived from live animal imaging, luciferase expression, and vector genome biodistribution data; (+) one relative log unit

Table 2

Nucleotide sequences of primers utilized in site-directed mutagenesis for reengineering AAV2 capsids

Name	Primer sequence
2i1; 2i6	5'-ctaccaacctccagagtagcagcacagatccagctaccgcagatg-3'
2i3b	5'-ctaccaacctccagagtagcaacacagcaccagctaccgcagatg-3'
2i4	5'-ctaccaacctccagagtagtaacagcaacctaccagctaccgcagatg-3'
2i5	5'-ctaccaacctccagagtagcaccacagcaccagctaccgcagatg-3'
2i7	5'-ctaccaacctccaggcagccaacacagcagcagctaccgcagatg-3'
2i8; 2irh43, 2irh49–53, 57,58, 64	5'-ctaccaacctccagcaacagaacacagcaccagctaccgcagatg-3'
2i9	5'-ctaccaacctccagagtgcccaggcacaagcagctaccgcagatg-3'
2i10, rh40	5'-tctaccaacctccagcaggcaaacacgggtcctgctaccgcagat-3'
2i11, 12, rh32–34	5'-tctaccaacctccagaacgccaccactgccccgctaccgcagat-3'
2irh2	5'-tctaccaacctccagcagacaaacgggtcctgctaccgcagat-3'
2irh38	5'-tctaccaacctccagcagacaaacgggtcctgctaccgcagat-3'