Services for Genome Editing Knockout & Knockin Rabbit and Guinea Pig Model

Renova Life Inc. (RLI) provides the genome editing knockout and knockin rabbit and guinea pig service worldwide.

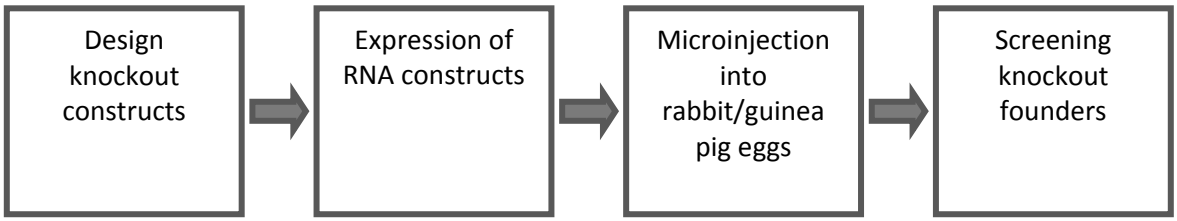
Rabbits are phylogenetically closer to humans, comparing to mice. Because of the anatomical, physiological, genetic and biochemical similarities between the rabbit and the human, this species is preferentially used in pulmonary, cardiovascular and metabolic studies, including airway obstructive disease, embolic stroke, arteriosclerosis, cholera, cystic fibrosis, neoplasia, diabetes, acute respiratory distress syndrome, malignant lymphoma, acquired immunodeficiency syndrome and hypercalcemia of malignancy. As a classical experimental animal species, rabbit has several other advantages over some other animal species (e.g., monkey, pigs). It has a short gestation period (30-31 days), large litter size (4-12/litter) and can be housed conveniently in an indoor facility. In addition to the use as an animal model, rabbit is also used for drug screening, antibody production, and the production of therapeutic proteins (bioreactors). Some proteins can be properly synthesized in rabbit milk, but not in other animal systems (e.g. cattle or goats) due to the post translation processing.

The guinea pig (Cavia porcellus) is a species of rodent belonging to the family Caviidae and the genus Cavia. They are used in research, primarily as models for human medical conditions such as juvenile diabetes, tuberculosis, scurvy, and pregnancy complications. Guinea pigs were popular laboratory animals until the later 20th century; about 2.5 million guinea pigs were used annually in the U.S. for research in the 1960s. In the past, they were widely used to standardize vaccines and antiviral agents; they were also often employed in studies on the production of antibodies in response to extreme allergic reactions, or anaphylaxis. Less common uses included research in pharmacology and irradiation. In 2004, the U.S.'s National Human Genome Research Institute announced plans to sequence the genome of the domestic guinea pig. The guinea pig was most extensively implemented in research and diagnosis of infectious diseases. Common uses included identification of brucellosis, Chagas disease, cholera, diphtheria, foot-and-mouth disease, glanders, Q fever, Rocky Mountain spotted fever, and various strains of typhus. They are still frequently used to diagnose tuberculosis, since they are easily infected by human tuberculosis bacteria. Because guinea pigs are one of the few animals

which, like humans and other primates, cannot synthesize vitamin C, but must obtain it from their diet, they are ideal for researching scurvy. The guinea pig model proved a crucial part of vitamin C research. Complement, an important component for serology, was first isolated from the blood of the guinea pig. Guinea pigs have an unusual insulin mutation, and are a suitable species for the generation of anti-insulin antibodies. Present at a level 10 times that found in other mammals, the insulin in guinea pigs may be important in growth regulation, a role usually played by growth hormone. Additionally, guinea pigs have been identified as model organisms for the study of juvenile diabetes and, because of the frequency of pregnancy toxemia, of pre-eclampsia in human females.

We can quickly knockout or knockin a gene in the rabbits and guinea pigs, with a consideration of nuclease-mediated genome editing technologies as an alternative, since the homologous recombination-based knockout approach is not available due to lack of authentic embryonic stem cells in these two species. Several types of artificially constructed nucleases (*e.g.*, TALEN, Cas9 and ZFN) can be engineered to recognize and cleave arbitrary sequences. When such nucleases (or their DNA or mRNA precursors) designed to target a specific site in the rabbit/guinea pig genome are microinjected into fertilized eggs, cleavage at the target site followed by imperfect repair can result in small deletions (and insertions, more rarely) of one or more base pairs. If the cut site is in the coding region of a gene, this will result in frameshift mutations downstream of the site, generating a knockout. The specific point mutations in the repair template can be introduced at the nuclease cleavage site, when a repair template with homology to the target site is present during the repair process, thus generating a knockin. Genome editing using TALEN or CRISPR/Cas9 can generate a knockout or knockin in as little as ~3 months in rabbits, and 5-6 months in guinea pigs.

Flow Chart of Gene Targeting Services in Rabbit and Guinea Pig



Service Description

Animals

New Zealand white rabbit and Hartley short hair guinea pig will be used for generating nuclease-mediated knockout or knockin animals by TALEN and CRISPR-Cas9 technology in both species.

Strategy and vector design

We will design a nuclease-mediated strategy for the clients by providing which the gene is like to knockout or with which point mutation is desired to knockin in targeted gene.

This includes identification of the type of nuclease (TALEN or CRISPR/Cas9) most suitable for your goals. If CRISPR-Cas9 technology is applied, we will design the optimal targeted sites in the gene for a higher efficiency, but minimize off-target activity by trunked guide RNA via CRISPR/Cas9 technology. If the client has experience on construct designing, we will be more than happy to discuss a better solution and discount for knockout and knockin in rabbit and guinea pig model. For each targeted gene, we will design vectors against two or three target sites in the gene to ensure success. Genotyping assays based on PCR and sequencing will also be designed for the screening of knockout or knockin founder rabbits and guinea pigs.

Microinjection of TALEN and CRISPR-Cas9 mRNA into rabbit/guinea pig eggs
DNA vectors that express the desired nucleases will be constructed. To assure the success of knockout and knockin in these two species, we will perform prior experiments before the real experiment. We will test the efficiency of vectors in rabbit and guinea pig cell line and select the best one for embryo work. When optimal vector is selected in cell lines, RNA microinjection will be performed in fertilized eggs and injected embryos will be cultured *in vitro* into blastocyst stage for further genome screening. This experiment can be assured that the constructs will work for generating KO and KI rabbits and guinea pigs after embryo transfer.

RNA: Nuclease mRNA will be transcribed *in vitro*. Relative capping and polyadenylation modification to mRNA will be completed to ensure its proper translation into protein in mammalian cells and embryos.

Pronuclear and or cytoplasmic injection to obtain founders: According experimental design, nuclease mRNA and/or donor DNA will be injected into fertilized eggs, followed by transferring injected eggs into the synchronized recipient to obtain F0 offspring. We guarantee a minimum of 3 knockout or knockin founders. According to our experience, more positive founders will be generated by CRISPR-Cas9 gene editing technology. We may request the bonus if client needs more founders after completion of the project. Usually, we will inject 150-200 eggs per optimal target site (selected from cells and cultured embryos) to achieve our target, in any case, we will target two or more sites with microinjection of 400-500 eggs per gene to reach our promise and guarantee.

If client prepares nuclease expression vectors (or mRNA products), it is recommended that 2-3 sites per gene is targeted in order to increase the chance of success. The verification of efficiency in cell lines are required.

Determining Knockout/Knockin founders
PCR and further sequencing will be performed to screen out knockout or knockin founder animals. For more convincing of screening results, we prefer client to identify the positive founders with the tissues collected from newborns, although we can provide the service for knockout and knockin screening upon the approval from the client. Animals carrying frame shift deletions/insertions on at least one allele are considered knockout founders. In some cases, an animal may be found to have both alleles of the target site mutated, but the chances for survival will be decreased in those double knockout animals.

Breeding service of founders

Breeding service will be a separate project to knockout/knockin project in rabbit and guinea pig model. Since the breeding project is longer than 6-8 months and the risk of uncontrollable factors is high during growth period of founder animals and breeding, we will discuss with the clients in details for a better breeding strategy. Usually, we will raise the founder animals to sex maturity (rabbit female, 5-6 months, rabbit males, 9-10 months; guinea pig male and female, about 3 months), and then we breed the founders to wild type animals, and screen the offspring to obtain positive F1 offspring.

Service guarantee

When the nuclease expression vectors are designed and constructed by RLI, we will fully refund service charge if founder(s) fails to be generated. When the vectors are provided by the client, we cannot guarantee the generation of knockout or knockin animals, but when client provides the convincing data in cultured cells, and further confirmed by our embryo microinjection study, we will discuss with client for the guarantee in a scientific and professional manner. We will discuss the risk of knockout important gene on the X chromosome or any critical genes affecting or detrimental to fetal development.

Service Charge and Turnaround Time

Project overview with a full service

Milestones	Service		Price	Turnaround time
1	Strategy and vector design		Free	Two weeks
2	Nuclease expression vector construction for **knockout**		$3,000	5-6 weeks
	Nuclease expression vector construction for **knockin**		$3,500	5-6 weeks
3	mRNA preparation and vitrification		$2,000	2-3 weeks
4	Generating **knockout** founders by TALEN or CRISPR/Cas9 micro-injection into fertilized eggs and ET	Rabbit	$75,000	2-3 months
		Guinea pig	$70,000	4-6 months
	Generating knockin founders by TALEN or CRISPR/Cas9 micro-injection into fertilized eggs and ET	Rabbit	$95,000	2-3 months
		Guinea pig	$85,000	4-6 months
5	Identifying **knockout** founders		$3,500	3-5 weeks
	Identifying **knockin** founders		$4,500	4-6 weeks
6	Breeding founders to obtain F1		Consulting RLI	6-8 months

Price Discount
For the second project from old client, or a new project introduced by old client, the service charge will be provided with a discount of 15% off. We encourage the clients to promote our services and reputation.

Shipping
Animals and related products are shipped from our China CRO animal facility in Jiangsu Province, China (about 100 miles north of Shanghai).The client shall be responsible for transportation of transgenic animals from RLI China subsidiary facility to designated location by clients, including the charges derived from quarantining, health paper, transportation and related costs. RLI will provide all assistance and efforts to complete the delivery. RLI will use professional animal transportation company "World Courier" to ship live animals worldwide.

IPs and Confidentiality
Client has all rights to own animals and related products generated from the service. Any service information is highly confidential without the permission from client.

If client provides own vectors, the intellectual property (IP) is absolutely belong to the client. RLI will not look into any of IPs without consulting and approval from the client.

Inquiries and Quote Requests
Please email info@renovalife.com or fax to 301-576-5078 to inquire about our transgenic or gene editing services in rabbits and guinea pigs.

SUCCESS STORIES

Specific Genome Editing by TALEN and CRISPR-Cas9 technology Transcription activator-like effector nucleases (TALENs) are artificial restriction enzymes generated by fusing a TAL effector DNA binding domain to a DNA cleavage domain.TAL Nuclease has revolution the array of genome editing based on its ability to create a site-specific double-stranded DNA break (DSB). The break is then subsequently repaired by cellular machinery, through either homology-dependent repairs or non- homologous end joining (NHEJ), that creates a deletion mutation or a knock-in site for introducing site-specific DNA sequences of interest.Clustered, regularly interspaced, short palindromic repeat (CRISPR) RNA-guided nucleases (RGN) can robustly induce genome editing. Repair of RGN-induced double-stranded breaks by non-homologous end-joining or homology repair introduces insertion or deletion mutations or specific sequence alterations. The Streptococcus pyogenes Cas9 nuclease (Cas9) cleaves the intervening spacer sequence directed by a single guide RNA (gRNA) with a 20 nucleotides (nt) target complementarity region at its 5' end.

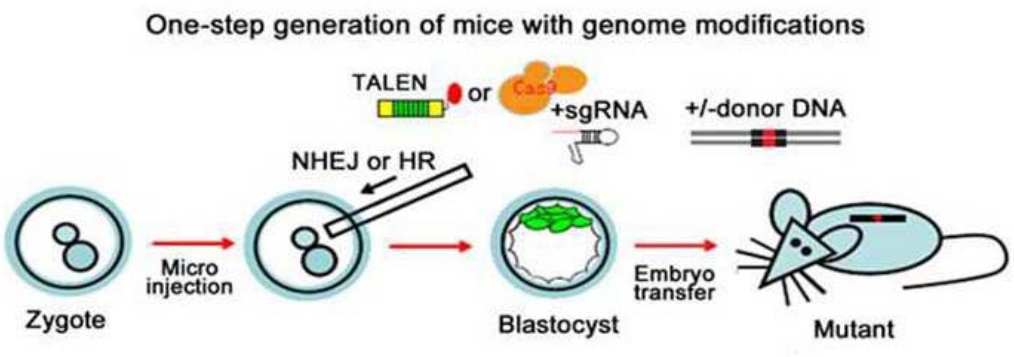
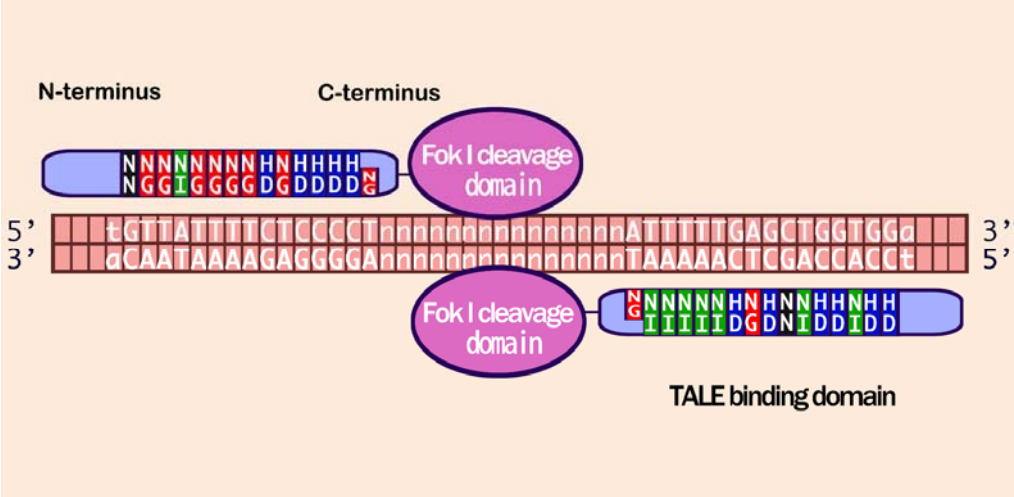


Table 1 describes the different strategies and technologies for gene targeting.

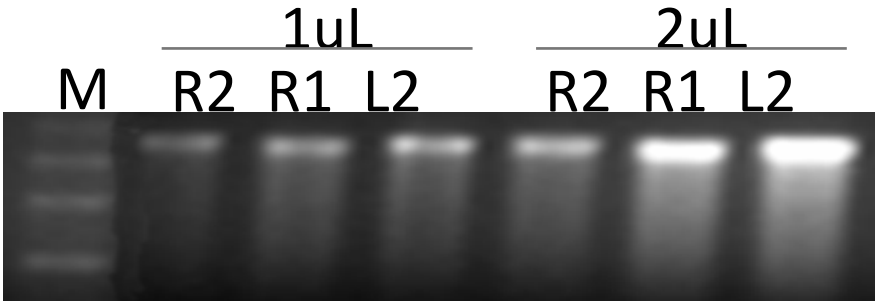
Table 1. Comparison of different gene targeting technologies

Property/Tools	ESCs-HR	ZFN	TALEN	CRISPR/Cas9
Type of recognition	HR	Protein-DNA	Protein-DNA	RNA-DNA
Methylation sensitive	sensitive	sensitive	sensitive	Not sensitive
Mutuation efficiency	Very low	high	high	Very high
Off-target effects	none	Less	Less	High
Multiplexing	Rarely used	Rarely used	Rarely used	Capable
Cost	High	Very high	High	Low

Story 1. Generating factor *FVIII* knockout animals with TALEN



TALEN expressed vector at 1 μL and 2 μL was examined at 1% agarose get, the intensity of RNA and its degradation were monitored prior to microinjection shown in the figure below.



We generated factor *FVIII* knockout mice by TALEN technology with the efficiency of 2.56-3.57% (Table 2).

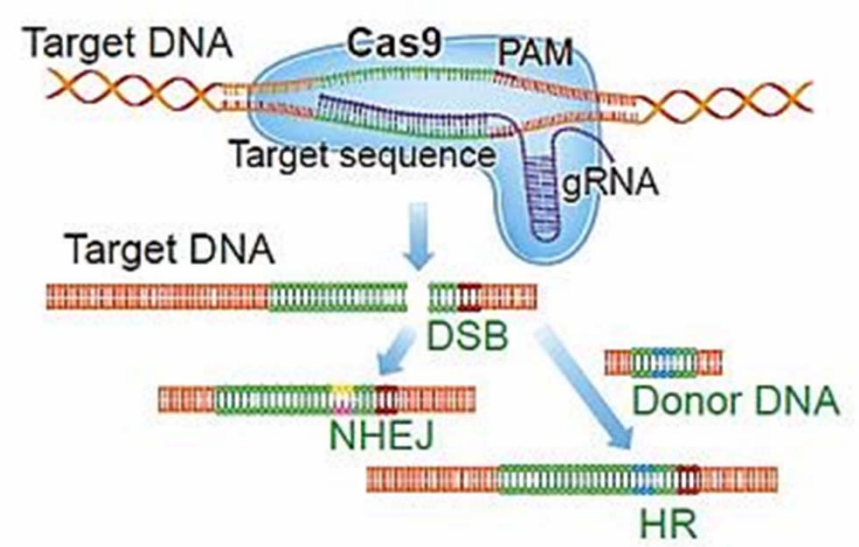
Table 2. Generating factor FVIII knockout mice with TALEN genomic editing

Talens pairs	Embryos injected	Newborn	Indels detection	Mutant mouse / embryos transferred (%)	Founder Name
L1+R2	96	0	0	0	
L1+R3	171	28	1（3.57%）	0.58	H32 ♂
L2+R1	120	20	0	0	
L2+R2	229	39	1（2.56%）	0.44	Q83 ♂
L2+R3	171	26	0	0	

Story 2. Generating factor *FVII* knockout animals with CRISPR-Cas9

Several strategies to improve specificity of the Cas9 system have been reported, such as the paired Cas9 nickase approach in which two gRNAs target adjacent sites on opposite DNA strands and each recruit a Cas9 nickase that nicks DNA instead of cutting both strands. This method can reduce off-target modifications at sites induced by single gRNA-guided Cas9.

Fu et al. reported that truncated gRNAs (tru-gRNAs) improved Cas9 nuclease specificity in U2OS.GFP and FT-HEK293 cells by shortening the gRNAs to 17/18 nt. They found that 5’-end nucleotides are not required for standard gRNA (std-gRNAs, 20 nt) activity and compensate for mismatches at unwanted positions along the gRNA target DNA interface, as shorter gRNAs are more sensitive to mismatches and therefore exhibit higher specificity. Here, we investigated the activity and specificity of tru-RGNs in inducing coagulation factor VII (FVII) gene mutations in murine cells and its efficiency in generating gene knockout (KO) mice by zygote RNA microinjection.



We generated factor *FVII* knockout mice by CRISPR-Cas9 technology with an efficiency as high as 78.9% (15/19) (Table 3).

Table 3 Generating *FVII* KO mice with gRNA and Cas9 mRNA co-injection

gRNAs	No. injected embryos	No. embryos transferred	No. recipients	No. newborns	Mutant alleles per mouse		Mutant mouse / total mouse tested (%)	Mutant mouse / embryos transferred (%)
					1(allve)	2(allve)		
F7-1	78	71	2	18	1 (1)	0(0)	1/18(5.6)	1.4
tF7-1	98	98	3	38	1(1)	18(0)	19/34(55.9)	19.4
F7-2	110	106	4	24	2(2)	6(0)	8/22 (36.4)	7.5
tF7-2	85	67	2	20	15(15)	0(0)	15/19(78.9)	22.4
F7-3	80	77	2	6	2(2)	0(0)	2/6 (33.3)	2.6
tF7-3	130	115	3	20	8(8)	0(0)	8/20(40)	6.9

Detection FIIV mutation frequencies by T7EI assay and subsequent sequencing (Table 4).

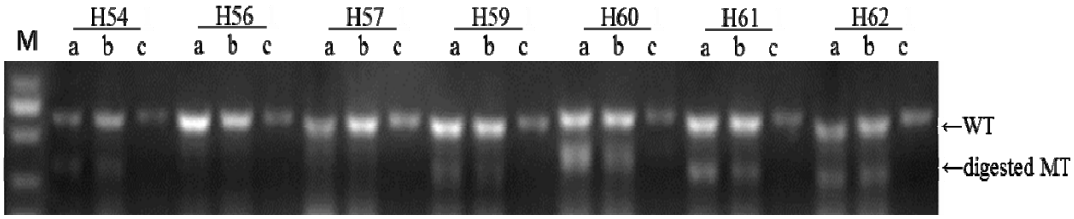


Table 4. Gene mutations in factor FVII knockout mice induced by CRISPR-Cas9

Founder	Target sequences (5'-3')	Indels
RGNs F7-1		
WT	A A A G G C G T G C C A A C T C A C T C C T G G A G G A G C T T T G G C C C G G C T C T C T G G A G A G A G A	
O68	A A A G G C ---------- T T T G G C C C G G C T C T C T G G A G A G A G A	-24 bp
RGNs tF7-1		
WT	C T G T T T C T C A G T T T T C A T A A C C C A G G A G G A A G C A C A T G G T G T C C T A C A C A G G C A A A G G C G T G C C A A C T C A C T C C	
I18	T T G T T T T T C A G T T T T C A T A A C C C A G G A G G A A G C A C A T G G T G T C C T A C A C A G G C A A A G G C G T G C C A A C T C A C T C C	C→T, C→T
RGNs F7-2		
WT	C C T A C A C A G G C A A A G G C G T G C C A A C T C A C T C C T G G A G G A G C T T T G G C C C G G C T C T	
I31	C T T A C A C A G G C A A A G G C G T G C C A A C T C A C T C C T G G A G G A G C T T T G G C C C G G C T C T	C→T
I34	C T T A C A C A G G C A A A G G C G G G C C A A C T C A C T C C T G G A G G A G C T T T G G C C C G G C T C T	C→T, T→G
RGNs tF7-2		
WT	A G G C G T G C C A A C T C A C T C C T G G A G G A G C T T T G G C C C G G C T C T C T G G A G A G A G A G T G C A A T	
H66	A G G C ---------- C C G G C T C T C T G G A G A G A G A G T G C A A T	-30 bp
H67	A G G C G T G C C A A C T C A C T C ---------- T C T G G A G A G A G A G T G C A A T	-22 bp
H68	A G G C G T G C C A A C T C A - - - - - G A G G A G C T T T G G C C C G G C T C T C T G G A G A G A G T G C A A T	-6 bp
H69	A G G C G T G C C A A C T ---------- T T G G C C C G G C T C T C T G G A G A C A G T G C A A T	-16 bp
H71	A G G C T T G - - - - - - - G C C C C T ---------- C C - - - - T C T C T G G A C A G A G T G C A A T	-25 bp
H72	A G G C G T G C C A A C T C ---------- T C T G G A G A G A G T G C A A T	-27 bp
H73	A G G C G T G C C A A C T C A C T C C T G A A G - A G C T T T G G C C C G G C T C T C T G G A G A G A G T G C A A T	-1 bp
H74	A G G C G T G C C A A C T C A C T C C T T G G A G G A G T T T T G G C C C G G C T C T C T G G A G A G A G T G C A A T	-6 bp
H75	A G G C G T G C C A A C T C A C T C C ---------- T T T G G A C C G G T T C T C T G G A T A T A G T G C A A T	-16 bp
H76	A G G C G T G C C A A C T C A C T C A C T T T ∧ G G A G C T T T G G C C C G G C T C T C T G G A G A G A G T G C A A T	+8 bp
	AATATGGA	
H78	A G G C G T G C C A A C T C A C T ---------- T G G G A G A G ---- G G - T C T C ---------- A G T G C A A T	-27 bp
H79	A G G C G T G C A A A C T C ---------- T T T A G C T C T G C T C ------- A C - - A G T G C A A T	-29 bp
H82	A G G C G T G C C A A C T C A C C T - C ---------- A A C T T T G G A C C G G C T C T C T G G A T A T A G T G C A A T	-13 bp
H83	A G G C G T ----------	-167 bp
H84	A G G C G T G C C A A C T C A C T ---------- C C G G T C T C A G G A T A G A G A G T G C A A T	-27 bp
RGNs F7-3		
WT	C C T G G A G G A G C T T T G G C C C G G C T C T C T G G A G A G A G T G C A A T G A G G A A C A C C T G G	
I66	C C T G G A G G A ---------- A G T G C A A T G A G G A A C A C C T G G	-24 bp
I68	C C T G G A G ---------- A G A G A G T G C A A T G A G G A A C A C C T G G	-24 bp
RGNs tF7-3		
WT	C C T G G A G G A G C T T T G G C C C G G C T C T C T G G A G A G A G A G T G C A A T G A G G A A C A G T G C	
H54	C C T G G A G G A G C T T T G G C C C G G C T C T C T G G A G A G A G A G T G C A A T G A C G A A G A G A G C	G→C, C→G, T→A
H59	C C T G G A G G A G C T T T G G C C C G G C T T C T C G G A A A A A A A A G G G A A A G G A G A A A C A T T G C	+1 bp
H60	C C T G G A G G A G C T T T G G C C T G G C T C T C T G G A G A G A G A G T G C A A T G A G G A A C A G T G C	C→T
H62	C C T G G A G ---------- A G A G T G C A A T G A G G A A C T G T G C	-24 bp

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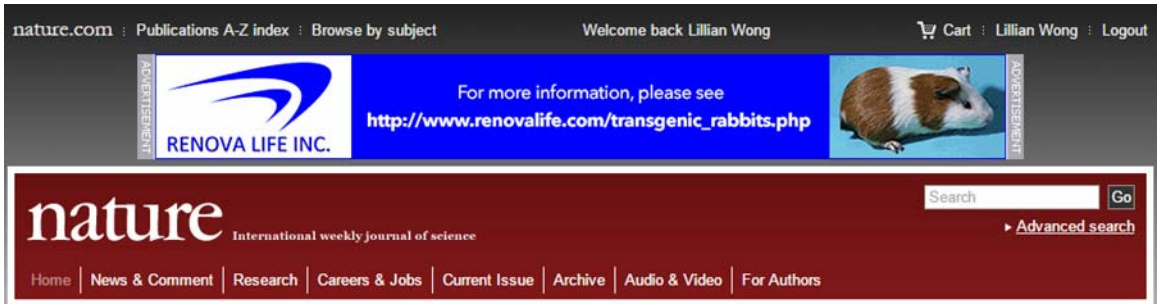
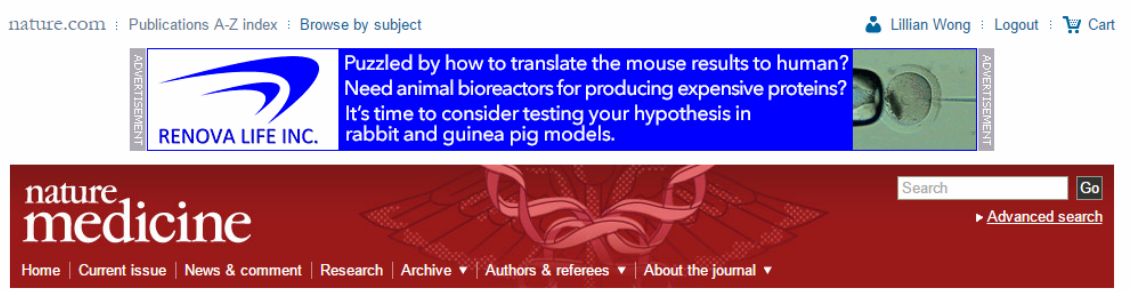
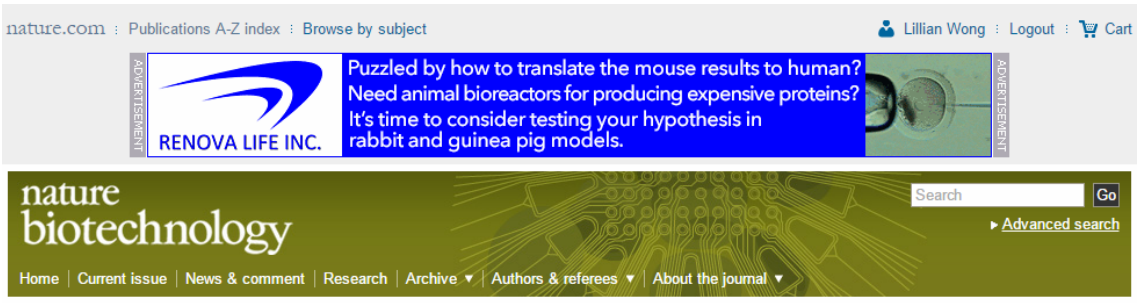
To our knowledge, RLI. is the only company providing transgenic rabbit and guinea pig platform worldwide. We are promoting our service in the world prestigious journals of **Nature**, **Nature Medicine** and **Nature Biotechnology**. Please see the banners below.







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Web: http://www.renovalife.com/transgenic_rabbits.php