IndelCheck™ CRISPR/TALEN insertion or deletion detection system

Cat. No. IC001
Cat. No. IC002
Cat. No. IC003
Cat. No. IC004
Cat. No. IC005
Cat. No. IC006
Cat. No. IC007
Cat. No. IC008

User Manual

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USER MANUAL

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I. Introduction

CRISPR/TALEN introduced double-stranded breaks (DSBs) at desired target sites can be repaired by nonhomologous end joining (NHEJ), which is error prone and typically results in small insertions or deletions (indels) near the DSB. The target region is PCR-amplified, and the PCR products are denatured and re-annealed to allow mismatched DNA to form, such as wildtype/indel mutation mismatches or indel mutation 1/indel mutation 2 mismatches.
T7 endonuclease I can recognize and cleave such mismatched DNA. By incubating the re-annealed PCR fragments with T7 endonuclease I, if two shorter bands of the predicted size are generated, it usually means that CRISPR/TALEN has successfully introduced indel mutations at the targeted chromosomal site. The IndelCheck™ mismatch cleavage assay (using T7 endonuclase I) can be used for **1) functional verification of sgRNAs and TALENs** (Figure 1), as well as **2) screening cell clones for knockout (KO) and knock-in (KI) modifications** (Figure 2).

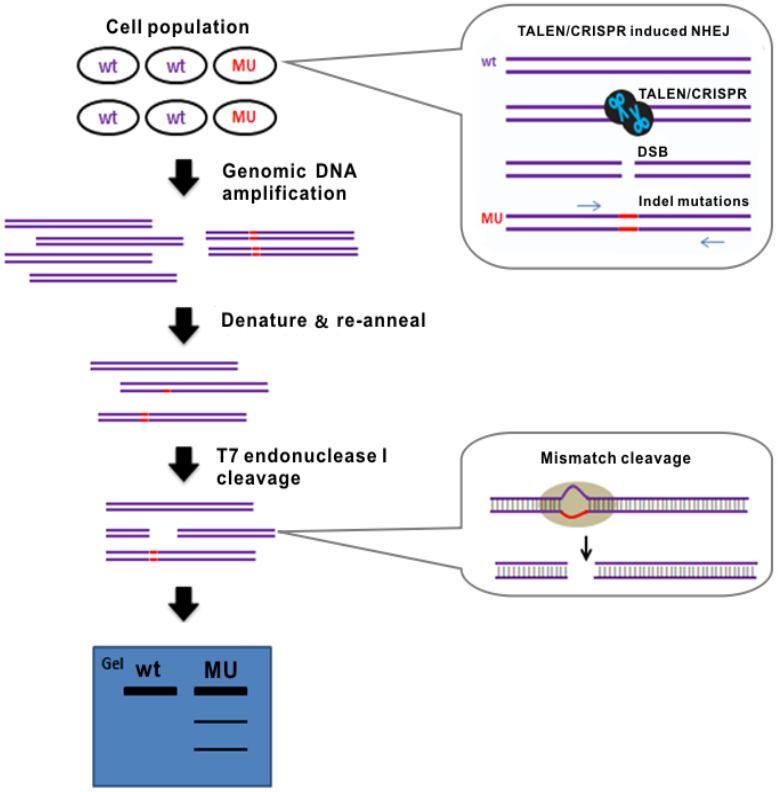


Figure 1. CRISPR or TALEN functional validation using the mismatch cleavage assay.

The IndelCheck™ CRISPR/TALEN insertion or deletion detection system (IC001, IC002) is a complete system designed to simplify your validation or screening workflow. It includes both the **Target site PCR kit (version 2.0)** (IC003, IC004) and the T7 Endonuclease I Assay kit (IC005-IC006). All components are optimized together for the best performance.

The **Target site PCR kit (version 2.0)** is optimized for robust amplification of the target site. With the proprietary lysis buffer included, genomic DNA isolation is no longer required. The kit contains the high efficiency and high fidelity SuperHeRo DNA polymerase, which produces both blunt-end and sticky-end PCR products compatible with a variety of sequencing vectors. GeneCopoeia also provides the target site PCR primer design and synthesis. Please visit our website for more details.

The T7 Endonuclease I Assay kit (version 2.0) contains T7 endonuclease I, which detects and cleaves heteroduplex DNA. T7 Endonuclease I cuts at the first, second or third phosphodiester bond at the 5' terminal of the junction. This kit also contains positive controls for both target PCR and indel mismatch cleavage.

The **Target Site PCR Cloning kit** (IC007, IC008) includes T4 DNA ligase, a blunt-end vector, and sequencing primers for cloning and sequencing target site PCR products, for sequence verification of the target site in the genome. (Figure 2). The blunt-end vector contains a lethal gene between the insertion sites to minimize background, vector-only ligation.

Advantages

- Complete system to simplify your CRISPR/TALEN validation and knockout clone screening
- Robust amplification for the target site PCR. No genomic DNA isolation is required
- Easy to use T7 endonuclease I assay with optimized conditions and positive control

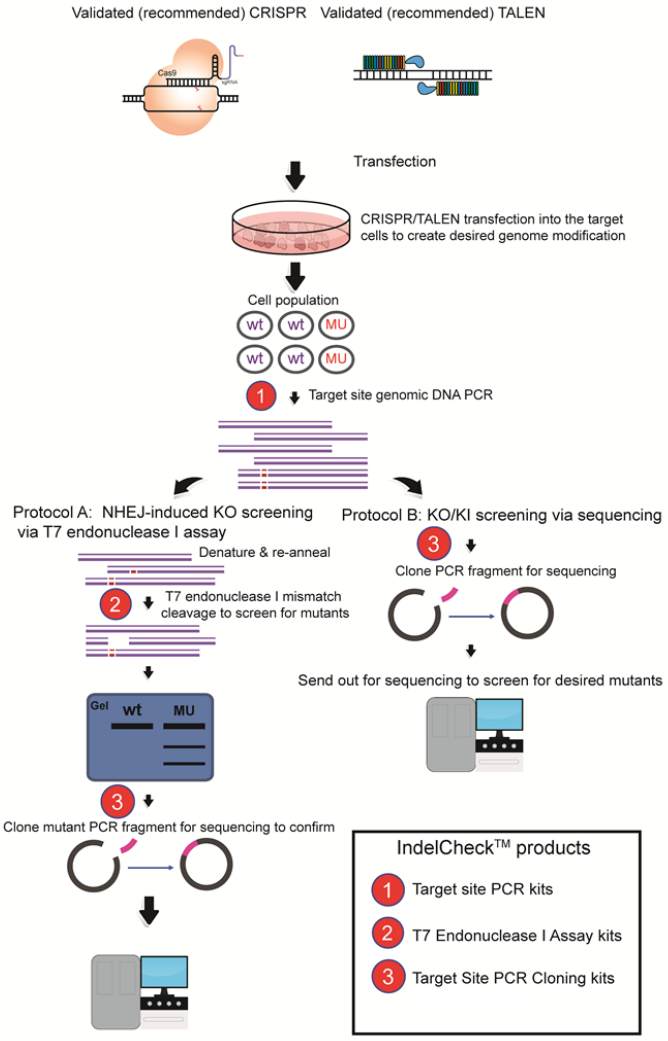


Figure 2. Using the IndelCheck™ system to screen for cell clones carrying desired CRISPR- or TALEN-mediated genomic modifications.

II. Contents and Storage

IndelCheck™ CRISPR/TALEN insertion or deletion detection system (IC001, IC002)

The T7 endonuclease I assay kit is available in two sizes:

50-Reaction Kit (Catalog No. IC005)

200-Reaction Kit (Catalog No. IC006)

Content	Quantity 50 reactions 200 reactions	Shipping temperature	Storage temperature
Digestion reagents			
T7 Endonuclease I (2 U/μl)	50 μl 50 μl×4	Ice pack	-20℃ Stable for at least 12 months
10× T7EN Buffer	100 μl 100 μl×4	Ice pack	-20℃ Stable for at least 12 months
Control reagents			
Control template & primer mix	100 μl 100 μl×4	Ice pack	-20℃ Stable for at least 12 months

The Target site PCR kit (version 2.0) **is available in two sizes:**

50-Reaction Kit (Catalog No. IC003)

200-Reaction Kit (Catalog No. IC004)

Content	Quantity 50 reactions 200 reactions	Shipping temperature	Storage temperature
Lysis Buffer	1300 μl 1300 μl×4	Ice pack	-20℃ Stable for at least 12 months
2 × SuperHero PCR Mix	650 μl 650 μl×4	Ice pack	-20℃ Stable for at least 12 months

The Target Site PCR Cloning kit is available in two sizes:

20-Reaction Kit (Catalog No. IC007)

100-Reaction Kit (Catalog No. IC008)

Content	Quantity 20 reactions 100 reactions	Shipping temperature	Storage temperature
5 × Ligase Buffer	40 µl 40 µl×5	Dry ice and Ice pack	-20℃ Stable for at least 12 months
T4 DNA Ligase (200 U/µl)	20 µl 20 µl×5	Dry ice and Ice pack	-20℃ Stable for at least 12 months
Blunt-end linear vector (20 ng/µl)	20 µl 20 µl×5	Dry ice and Ice pack	-20℃ Stable for at least 12 months
Control Insert (40 ng/µl)	20 µl 20 µl×5	Dry ice and Ice pack	-20℃ Stable for at least 12 months
Forward Sequencing Primer (20 µM)	250 µl 250 µl×5	Dry ice and Ice pack	-20℃ Stable for at least 12 months
Reverse Sequencing Primer (20 µM)	250 µl 250 µl×5	Dry ice and Ice pack	-20℃ Stable for at least 12 months

Important note:

Store all components at –20℃ upon receipt.

Materials required but not supplied

The following materials are required but not supplied:

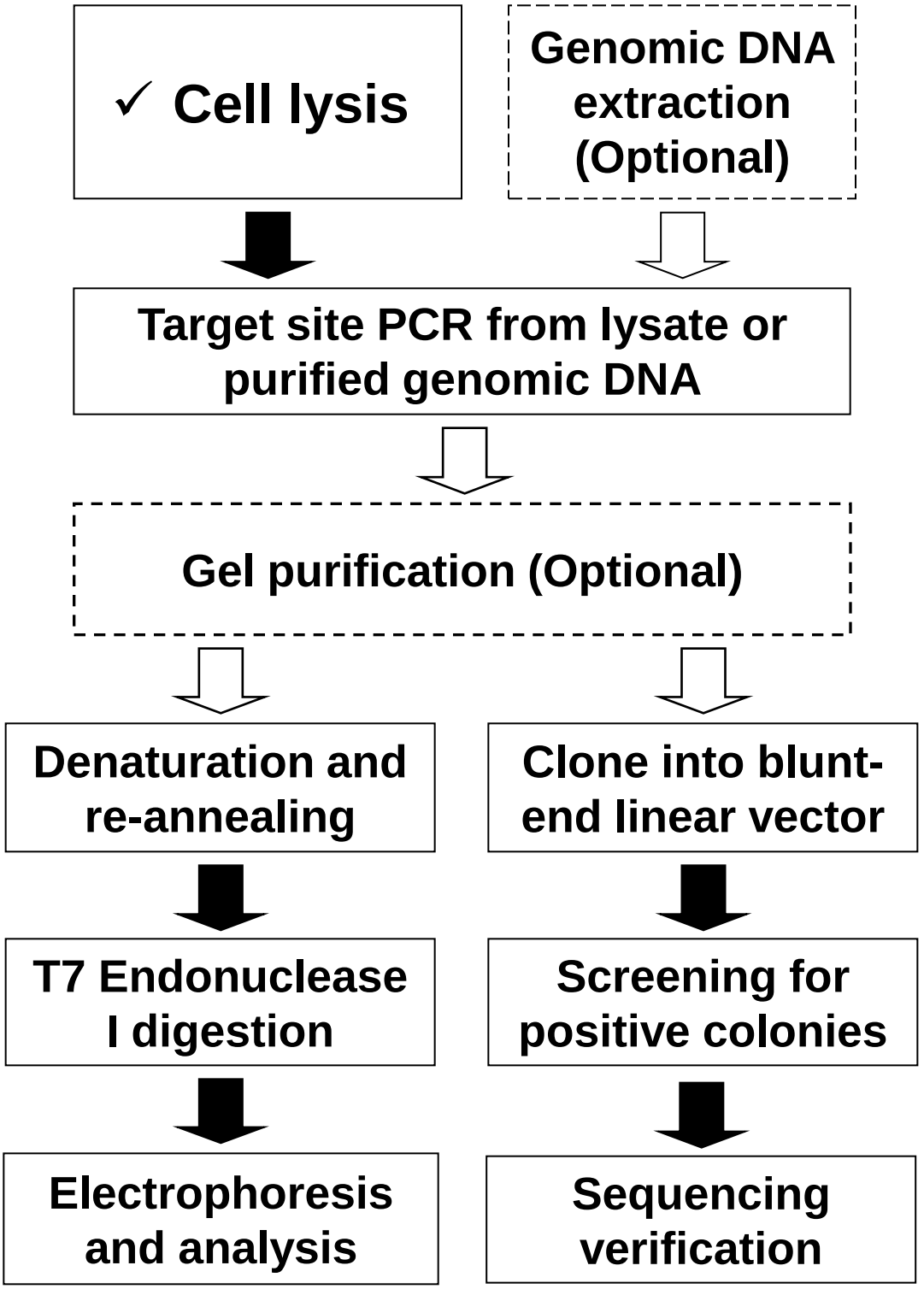
ddH_2O. Avoid using autoclaved H_2O. The recycled steam in some autoclaves can introduce contaminants that may interfere with PCR.

Target site PCR primers specific to target site(s) with Tm ≥ 60° C The primers should flank the TALEN or sgRNA target site and generate an amplicon of approximately 500-800 bp with the TALEN or sgRNA target site offset from the center by approximately100 bp. Make sure the primers are specific for the intended site. Avoid using primers that contain inosine. Avoid to have potential SNP sites or sequence differences between alleles in your target region.

NOTE: We highly recommend you to obtain sequence information of the target site of you cell line before primer design and any other experiment. It is possible for mammallian cells, which are usually dipoid, to have sequence differences between alleles. This may cause false positive results when using T7 endonuclease I to digest negative controls. Such sites should be avoided when designing target PCR primers. See Appendix 4. for further instructions on checking potential SNP sites using online tools.

*GeneCopoeia also provides design and synthesis services for sequence-specific target site PCR primers.

IV. Protocol Overview



IV. Experimental Procedures

This section provides instructions for validating CRISPR sgRNA or TALEN chromosomal cleavage activity using the IndelCheck™ CRISPR/TALEN insertion or deletion detection system.

In addition to the steps shown in the protocol overview, we also provide brief instructions for PCR primer design and genomic DNA extraction. If you stop before completing all the steps, please store your PCR or digestion products at -20℃ until the next step. Avoid repeated freeze-thaw cycles.

We recommend that first time users perform a positive control PCR reaction using the control reagents in the kit. This PCR product can serve as a control for denaturation and re-annealing, as well as mismatch digestion steps.

1. Primer Design

1) Target site PCR primers should have a Tm value of no less than 62℃

2) For optimum results, the amplicon size range should be approximately 500~800 bp.

3) Design primers so that the CRISPR sgRNA or TALEN target site is offset from the center of the PCR product by approximately 100 bp. This ensures being able to readily resolve the cleavage products on the gel.

2. Sample Preparation

- Option 1: Genomic DNA extraction
    a) Harvest cells (no fewer than ~10^6 cells per well).
    b) Extract genomic DNA using your method of choice or following the provided protocol of extraction kit manufacturer. Make sure the concentration of genomic DNA solution is above 25 ng/μL.
- Option 2: Cell lysate preparation
    a) Collect cells from cell culture dish, 6- well plate or 96-well plate. Centrifuge at 3000 rpm at 4℃ for 5 min and carefully remove the supernatant.
    b) Add 300 μL 1×PBS. Pipette gently to suspend cells. Centrifuge at 3000 rpm at 4℃ for 5 min and remove the supernatant.
    c) Add 300 μL 1×PBS and resuspend cells. Sample the suspension to calculate the cell number if necessary. Centrifuge at 3000 rpm at 4℃ for 5 min and remove the supernatant as completely as possible. Proceed to lyse or store the pellet at -80℃.
    d) Add 25 μL Lysis Buffer and lyse cells at 65° C for 15 min, then 95℃ for 10 min. Quickly put it on ice afterward.

**The volume of Lysis Buffer can be adjusted basing on the cell number. At least 50,000 and no more than 5×10^5 cells are recommended for use in 25 μL Lysis Buffer. For confluent cells of a well of 6-well plate, add 200 μL~600 μL Lysis Buffer. For confluent cells of a well of 96-well plate, add 50-100 μL Lysis Buffer. For amplifying fragment > 1 kb, we suggest prolonging the cell lysis at 65 ° C for 40 min, but no more than 1 hr.*

However, It is not not necessary to obtain complete cell lysis in most experiments. The remaining cells can be stored at -80℃ or for continued culture.

e) Frozen centrifuge at 12000 rpm for 1 min.

**Too much floc after centrifuge suggests too little lysis occurred. After transferring the suspension to another tube, the precipitate can be resuspended by adding another 25 μL Lysis Buffer.*

f) Proceeded to PCR reaction with Target PCR kit. The cell lysate can be stored at 4 ℃ for no more than one week or -20℃ for several months until use.

3. Target PCR and product processing

1) Target PCR

a) Thaw 2 × SuperHero PCR Mix on ice. For PCR from extracted genomic DNA, prepare a Master Mix with target PCR primers flanking the insert as follows:

Item	Amount	
genomic DNA	50-200	ng
Forward and reverse primers (5μM each)	1.25	μl
2 × SuperHero PCR Mix	12.5	μl
ddH_2O	to 25	μl
Final	**25**	**μl**

b) For PCR from lysate, prepare a Master Mix with target PCR primers as follows:

Item	Amount
lysate	1* µl
Forward and reverse primers (5 µM each)	1.25 µl
2 × SuperHero PCR Mix	12.5 µl
ddH_2O	to 25 µl
Final	**25 µl**

**To avoid insufficient PCR amplification, adjust the volume of cell lysate based on the cell number. to ensure that no less than 2000 copies of template are present in the reaction. For example, for HT1080 cells, which contains 2 copies of each chromosome, at least 1000 lysed cells are needed in a PCR reaction. To get a bright and clear band on agarose gel, about 10,000 lysed cells are needed.*

NOTE: See Appendix 3. for PCR system for the control mix.

c) Proceed with PCR using the following program

Temperature	Time	Cycles
94℃	5 min	1
94℃	30 s	35
58℃	30 s	
72℃	1 min	
72℃	5 min	1

NOTE: PCR should produce a sufficiently high yield of a SINGLE amplified band of the correct size. If you are using PCR reagents from other manufacturers, we strongly recommend use of a high fidelity DNA polymerase to reduce the amount of base misincorporation during PCR and subsequent false positives. If you are using IC003 or IC004, the PCR product can be directly used as the substrate for T7 Endonuclease I digestion.

2) Purification or gel extraction of correct-sized band from non-specific PCR background. See Appendix 2 for using gel purification to optimize cleavage of non-specific amplicons

4. Denaturation and re-annealing

PCR produces fragments containing both wild-type and mutant target sequences. The DNA double chains are upzipped 95℃, and then gradually anneal to room temperature, resulting in forming mismatched heterozygous DNA (such as wild-type / insertion deletion mutant mismatch, or mutant 1/ mutation 2 mismatch).

1) For purified genomic PCR product:

DNA (>25 ng/μl)	200~500	ng
10 × T7EN Buffer	2	μl
Nuclease-free water	Add up to 19	μl
Total	**19**	**μl**

For PCR products amplified with GeneCopoeia target PCR kit, combine the following

Unpurified PCR product	200~500	ng (5~19 μl)
Nuclease-free water	Add up to 19	μl
Total	**19**	**μl**

2) Mix and centrifuge for a few seconds.

3) Heat at 95℃ for 5 min.

4) Reanneal by allowing the denatured PCR products to cool down to RT.

5. Cleavage with T7 Endonuclease I

1) Add 1 μL of 2 U/μL T7 Endonuclease I.

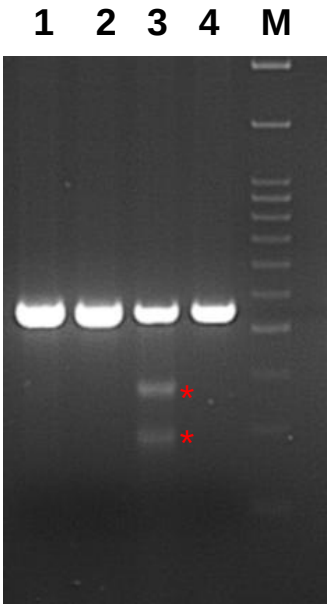
2) Incubate at 37℃ for 20-60 min.

6. Gel analysis

1) Add 1/10 volume of 10 × loading buffer [with 0.1% SDS] to each reaction and mix thoroughly.

2) Load half of the mixture into the well of 2% agarose/EtBr gel and run in TAE or TBE buffer.

3) Also load a 100 bp DNA ladder (#M01010A) as a size reference marker in one of the adjacent wells. Run the gel at 5 V/cm – 11 V/cm until the bromophenol blue reach 2/3 of the length of the gel.



Gene	PCR Product	T7 Endonuclease I	
NR0B1	429	147	282

Figure 2. T7 Endonuclease I assay.

Lane 1: PCR product from negative control cells, digested with T7 Endonuclease I.

Lane 2: PCR product from negative control cells, undigested.

Lane 3: PCR product from sample cells, digested with T7 Endonuclease I. Digestion yields 3 bands: 1 unmodified + 2 cleavage products of predicted sizes (red asterisks).

Lane 4: PCR product from sample cells, undigested.

Lane M: 100 bp plus DNA ladder (#M01010A).

NOTE: See Appendix 3. for T7 endonuclease I assay of control.

7. Sequenceing verification (following step 3)

1) Purification of PCR products (optional)

Confirm the quality of PCR products by agarose gel electrophoresis, which should show a single band with no non-specific products. Remove 5-10 µL from each PCR reaction for agarose gel electrophoresis to verify the quality and quantity of your PCR products. GeneCopoeia recommends gel-purifying the products before ligation if the PCR products do not appear as single bands on an agarose gel.

2) Cloning into the blunt-end linear vector

Set up the following 10 µL ligation reaction:

Reagent	Volume	Final Concentration
5× Ligase Buffer	1 µl	1×
Blunt-end linear vector	1 µl	20 ng/µl
PCR product	1 µl	≥ 30 ng/µl*
ddH2O	2-5 µl	——
T4 DNA Ligase	up to 10 µl	200 U/µl
Total	**10 µl**	

** Add the PCR product according to the concentration in the table. If the agarose gel electrophoresis band is not bright enough, GeneCopoeia recommends quantifying the PCR product first, then increasing the amount of the target fragment to ensure the minimum amount of the PCR product is not less than 30 ng.*

Place the ligation reaction in a 25 ℃ water bath for 1 hr.

3) **Transformation of ligation products**

-Thaw competent *E. coli* cells on ice. Place the required number of microcentrifuge tubes on ice and dispense 100 µL of competent cells into each tube.

-Gently mix 2 µL of each ligation reaction with the competent cells (the expected transformation efficiency is 1×10^9). Incubate on ice for 30 min.

-Heat shock cells by placing the tubes into a 42 ℃ water bath for 45 s. Immediately place the tubes on ice for 3 min.

-Add 400 µL of SOC (or LB) medium and incubate at 37 ℃ for 1 hr with shaking at 200 rpm.

- Plate 200 µL of each transformation onto LB plates containing ampicillin (recommended concentration is 100 µg/mL). Incubate plates overnight (12 to 16 hr) at 37 ° C.

4) **Screening for positive colonies**

Randomly pick approximately 10 colonies from each plate for PCR verification. Use the Forward sequencing primer (20 µM)/Reverse sequencing primer (20 µM) mix in the kit to detect the positive colonies. Perform PCR reactions according to the protocol provided by the manufacturer of your PCR kit.

An example PCR reaction is shown below:

Reagent	Volume	Final Concentration
2× PCR buffer	12.5 µl	1×
Forward sequencing primer (20 µM)	1 µl	0.8 µM
Reverse sequencing primer (20 µM)	1 µl	0.8 µM
dNTP(25 mM)	0.2 µl	0.2 mM
Enzyme(5 U/µl)	0.2 µl	1 U/µl
ddH_2O	10.1 µl	——
colony	——	——
Total	**25 µl**	

Temperature	Time	Cycles
94℃	5 min	1
94℃	30 s	30
58℃	30 s	
72℃	1 min*	
72℃	5 min	1

** The extension time should be adjusted according to the length of the fragment. The extension rate of Taq DNA polymerase is approximately 1000 bp/min.*

5) **Sequencing verification**

- Inoculate a single positive colony in 5 mL of LB liquid media containing ampicillin (recommended concentration is 100 μg/mL). Incubate overnight at 37° C with shaking at 220 rpm.

- Extract plasmids according to the protocol provided by the manufacturer of your extraction kit.

- Use the Forward sequencing primer (20 μM)/Reverse sequencing primer (20 μM) mix in this kit to perform sequencing verification.

6) **Ligation reaction of control insert (positive control)**

If this is the first time you are using the Target Site PCR Cloning kit, GeneCopoeia recommends performing the control insert (40 ng/μl) reaction to help you evaluate your results.

The ligation reaction of control insert is performed as follows.

Reagent	Volume	Final Concentration
5× Ligase Buffer	2 μl	1×
Blunt-end linear vector	1 μl	20 ng/μl
Control Insert	1 μl	40 ng/μl
T4 DNA Ligase	1 μl	200 U/μl
ddH_2O	5 μl	——
Total	**10 μl**	

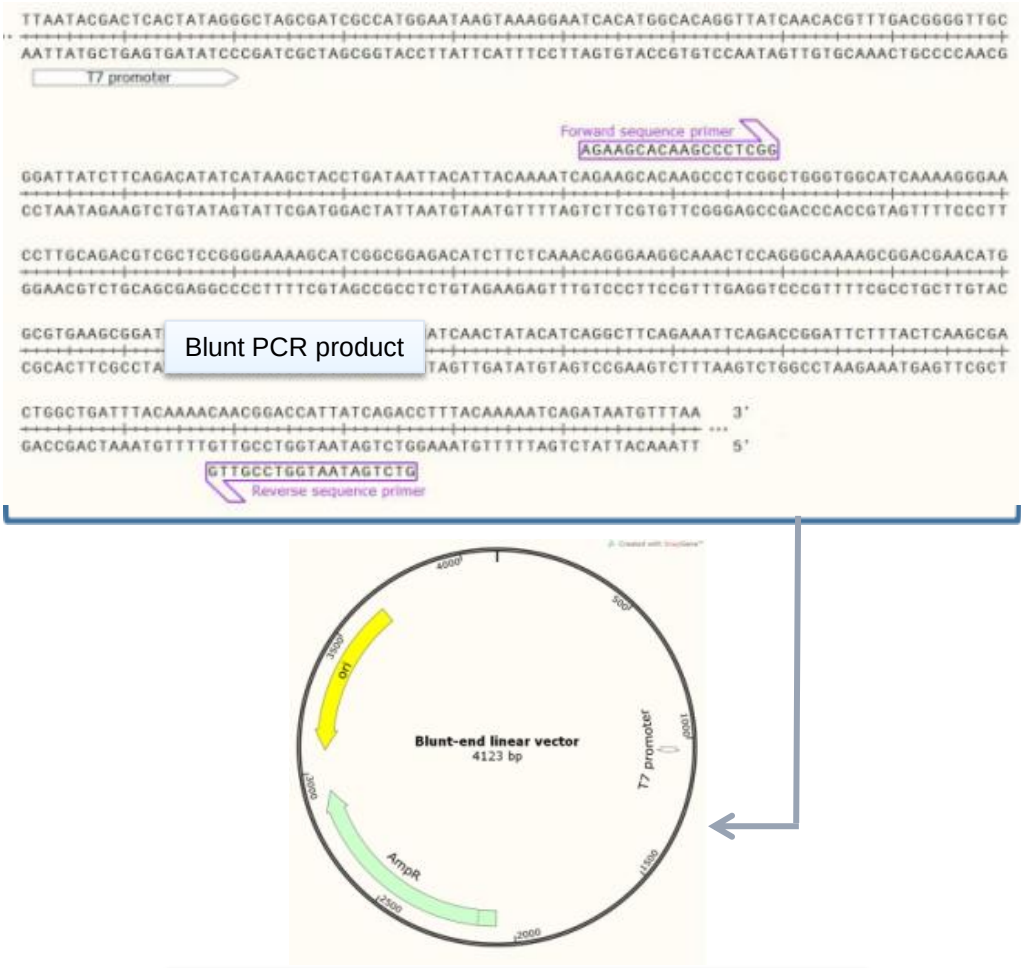
-Place ligation reaction in a 25℃ water bath for 1 hr.

-Thaw competent *E. coli* cells on ice. Place the required number of microcentrifuge tubes on ice and dispense 100 µl of competent cells into each tube.

-Gently mix 2 µL of each ligation reaction with the competent cells (the expected transformation efficiency is 1×10^9).

-Refer to steps V.3 and V.4 for transformation and screening procedures.

Partial vector sequence and primer sequence information for blunt-end linear vector are shown below:



Forward Sequencing Primer: 5'-agaagcacaagccctcgg-3'

Reverse Sequencing Primer: 5 '-gtctgataatggtccgttg-3'

VI. Notes and Troubleshooting

Cleavage troubleshooting

Problem	Possible Causes	Recommended Solution
Non-specific cleavage bands are observed	Non-specific PCR amplification	• Do gel purification to ensure that your amplicons are single bands (See Figure 4 in Appendix). • Optimize PCR primers on untreated genomic DNA or cell lysates. • Optimaze PCR condition.
No cleavage products observed	Low T7 Endonuclease I activity	• If no cleavage is observed for all samples including the positive control, add $MnCl_2$ at a final concentration of 10mM to enhance T7 Endonuclease I activity.
Nonspecific nuclease activity is observed	Incorrect reaction temperature	• Ensure that samples are incubated at 37℃.
	Reaction time too long	• Advoid treating with T7 Endonuclease I more than 1.5 hour.
	Poor annealing operation	• Perform denaturalization and annealing in heated water. Let the reaction cool down naturelly with the water. • Perform the denaturalization and annealing step in a PCR machine. as follows: (1)95℃ 5min (2)94℃(-2℃/cycle), 10-20 sec (3)93℃(-2℃/cycle), 10-20sec and go to step (2),34 cycles
	PCR is introducing mutations	• Be sure to use a high-fidelity polymerase for PCR amplification
DNA bands are too weak to be observed	Low positive rate of modification	• If possible, optimize the conditions of your genome editing experiment (e.g., design new CRISPR sgRNA or TALENs).
	Insufficient amount of DNA loaded on gel	• Be sure to load enough DNA to enable ready visualization on the gel. Also, load equal amounts of total PCR product DNA in each lane.

Target site PCR troubleshooting

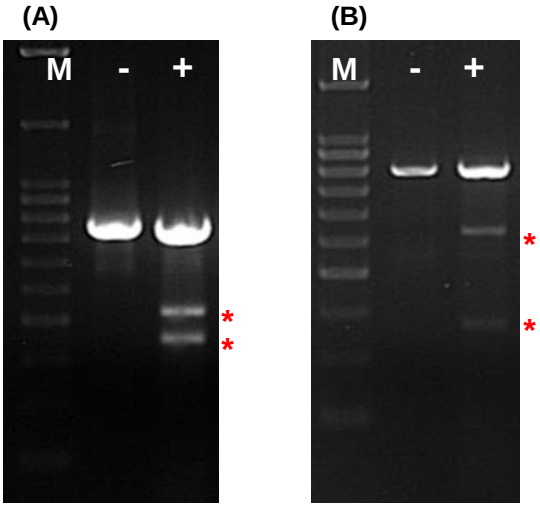
Problem	Possible Causes	Recommended Solution
No expected bands	Suboptimal PCR conditions	• Analyze the sequence of your target. If the GC content is lower than 40%, do not add Enhancer in the PCR reaction.
	Concentration of PCR template is too low	• Extract and purify genomic DNA to better control the template concentration.
	Incomplete lysis	• Remove PBS as completely as possible before adding Lysis Buffer, or the remaining PBS will dilute the lysis buffer. • Caluculate the cell number by hemocytometer or cellometer before lysing the cells. Adjust the volume of lysis buffer according to the cell number.
	Poor PCR primer design	• Check if the primers match with the template correctly.
Non-specific bands	Suboptimal PCR conditions	• Increase the annealing tempture to 0~5℃ above the Tm value.
	Poor PCR primer design	• Check the design of PCR primers for possible non-specific binding. If necessary, redesign primers to improve specifity.
	Too much polymerase	• Decrease the volume of polymerase to 0.2 μL (1 U)
Smear	Concentration of PCR template is too high	• Dilute template 2 or more fold and repeat the PCR.
	Too much polymerase	• Decrease the volume of polymerase to 0.2 μL (1U)

Target site PCR cloning troubleshooting

Problem	Possible Causes	Recommended Solution
Few or no colonies, due to ligation failure or low efficiency	Inactivate ligase	• Use the Control Insert provided in the kit to set up a positive control group, in order to access the activity of T4 DNA Ligase.
	Lack of ATP or Mg^{2+} in the ligation system	• Use the 5 × Ligase Buffer provided in the kit. This buffer contains a sufficient amount of ATP and Mg^{2+} . If you hope to tweak the reaction system, set up control groups according to the protocol in the Apprendix for potential troubleshooting.
	Overly high concentration of total DNA in the ligation system	• Overly high concentration of total DNA in the ligation system results in large fragments of linear DNA instead of closed circular molecules. Adjust the DNA concentration in the ligation system. • Set up a positive control group. The positive control reagents provided in the kit are optimized, and can be used as a reference for optimization of the sample group.
	Non blunt-end PCR product	• The vector provided in Target Site PCR Cloning kit is blunt-ended. It cannot be used for cloning sticky-end PCR products. • We strongly recommend you use the Target Site PCR kit to amplify the target sequence. The SuperHero plymerase produces a mixture of blunt-end and sticky-end PCR products, which can be easily cloned into both blunt-end and sticky-end vectors.
Too much bacterial growth	Contamination	• Set up a negative control with no ligation product added for contamination troubleshooting.
	No antibiotic in plates	• Add the antibiotic. 50 μg/ml ampicillin for the blunt-end vector provided by the kit.
	False positives	• The vector provided in the kit contains a lethal gene between the cloning sites to minimize false positives. If you are using other vectors, please set up a positive control for potential troubleshooting.
	Overly high ligation efficiency	• Consider reducing the amount of ligation product used in transformation, or reducing the T4 DNA ligase and/or shorten the ligation time for optimization. Set up control groups for potential troubleshooting.

VII. Appendix

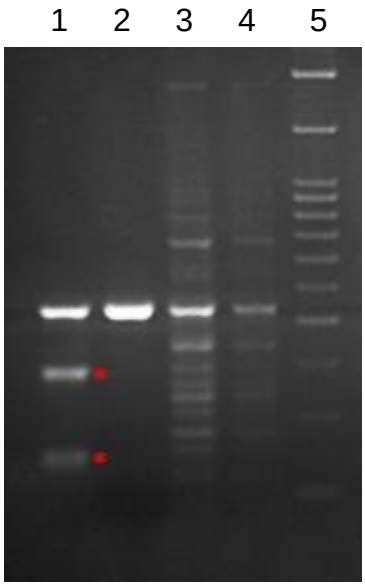
1. Example of using IndelCheck™ CRISPR/TALEN indel detection system to validate CRISPR sgRNA or TALEN cleavage activity



Panel	Target Gene	PCR Product	T7 Endonuclease I	
A	NR4A1	775	428	347
B	ESRRA	791	267	524

Figure 3. T7 Endonuclease I digestion products of amplicons derived from human genomic DNA. Control cells (-) should only have a larger band corresponding to the uncut genomic amplicon. Sample cells (+) transfected with indicated Cas9-sgRNA have a larger band and smaller bands corresponding to the DNA fragments resulting from the cleavage of the genomic amplicon by T7 Endonuclease I.

2. Example of using gel purification to optimize cleavage of non-specific amplicons



Target Gene	PCR Product	T7 Endonuclease I	
NR0B1	429 bp	127 bp	302 bp

Figure 4. Cleavage comparisom between gel-purified and unpurified non-specific target site PCR products.

Lane1: Gel extracted PCR product, digested by T7 Endonuclease I

Lane2: Gel extracted PCR product, undigested

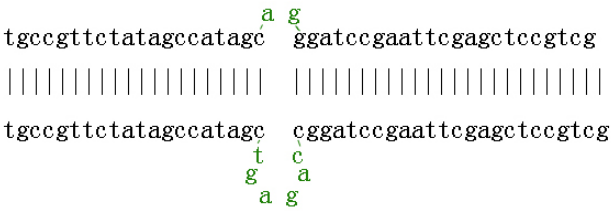
Lane3: Unpurified PCR product with non-specific bands, digested by T7 Endonuclease I

Lane4: Unpurified PCR product with non-specific bands, undigested

Lane5: 100bp plus DNA ladder (#M01010A)

3. Procedures for control target PCR and T7 endonuclease I assay using the control template & primer mix

The kit provides a positive control of the target site PCR and T7 endonuclease I assay. The positive control is composed of a control template and primer mix containing forward and reverse primers. Double-stranded DNA fragments containing two types of sequences are produced by PCR amplification. After denaturation and annealing, there is a certain probability to producing heterologous double-stranded DNA containing the following structure, which will be detected and cleaved by the T7 endonuclease I.



1) Control PCR

a) Prepare control PCR using the following system.

Item	Amount
Control template & primer mix	4 μl
2 × SuperHero PCR Mix	12.5 μl
ddH_2O	8.5 μl
Final	25 μl

b) Proceed with PCR using the following program.

Temperature	Time	cycles
94℃	5 min	1
94℃	30 s	35
58℃	30 s	
72℃	1 min	
72℃	5 min	1

2) Denaturation and re-annealing

a) For PCR products amplified with GeneCopoeia target PCR kit, combine the following

Unpurified PCR product	200~500	ng(5~19 μl)
Nuclease-free water	Add up to 19	μl
Total	**19**	**μl**

b) Mix and centrifuge for a few seconds.

c) Heat at 95℃ 5 min.

d) Reanneal by allowing the denatured PCR products cool down to RT.

3) Digest with T7 Endonuclease I

a) Add 1μL of 2 U/μL T7 Endonuclease I.

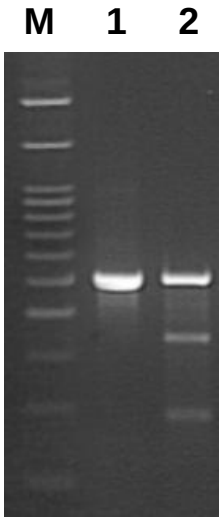
b) Incubate at 37℃ for 20-60 min.

4) Gel analysis

a) Add 1/10 volume of 10 × loading buffer [with 0.1% SDS] to each reaction and mix thoroughly.

b) Load half of the mixture into the well of 2% agarose/EtBr gel and run in TAE or TBE buffer.

c) Also load a 100 bp DNA ladder (#M01010A) as a size reference marker in one of the adjacent wells. Run the gel at 5 V/cm until the bromophenol blue has migrated 2/3 of the length of the gel.



Gene	PCR Product	T7EI	
control	520	180	330

Figure 3. T7 Endonuclease I assay result for control

Lane M: 100bp plus DNA ladder (#M01010A).
Lane 1: unpurified PCR product from control,undigested
Lane 2: unpurified PCR product from control, digested

4. Procedures for control target site PCR cloning using the Control insert:

1) The ligation reaction of control insert is performed as follows.

Reagent	Volume	Final Concentration
5× Ligase Buffer	2 μl	1×
Blunt-end linear vector	1 μl	20 ng/μl
Control Insert	1 μl	40 ng/μl
T4 DNA Ligase	1 μl	200 U/μl
ddH_2O	5 μl	——
Total	**10 μl**	

2) Place ligation reaction in a 25℃ water bath for 1 hr.

3) Thaw competent E. coli cells on ice. Place the required number of microcentrifuge tubes on ice and dispense 100 μl of competent cells into each tube.

4) Gently mix 2 μL of each ligation reaction with the competent cells (the expected transformation efficiency is 1×10^9). Incubate on ice for 30 min.

5) Heat shock cells by placing the tubes into a 42℃ water bath for 45 s. Immediately place the tubes on ice for 3 min.

6) Add 400 μL of SOC (or LB) medium and incubate at 37℃ for 1 hr with shaking at 200 rpm.

7) Plate 200 μL of each transformation onto LB plates containing ampicillin (recommended concentration is 100 μg/mL). Incubate plates overnight (12 to 16 hr) at 37℃.

8) Randomly pick approximately 10 colonies from each plate for PCR verification. Use the Forward sequencing primer (20 μM)/Reverse sequencing primer (20 μM) mix in the kit to detect the positive colonies. Perform PCR reactions according to the protocol provided by the manufacturer of your PCR kit.

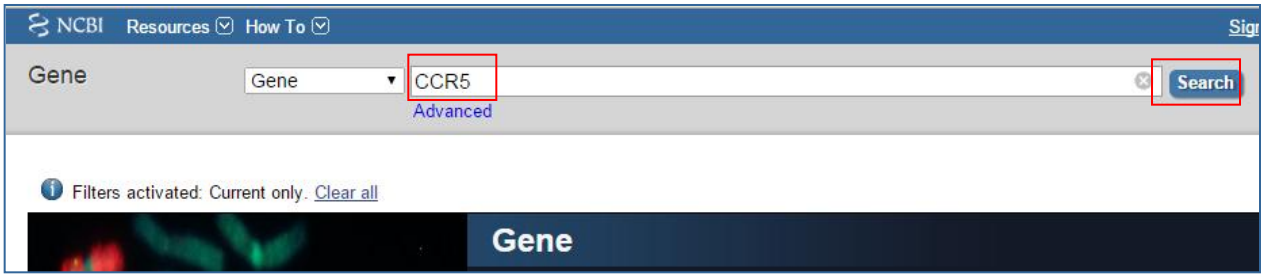
4. Search for potential SNP sites using online tools

Mammalian cells contain at least 2 copies of every chromosome, one copy from the mother, and the other from the father. So although most stretches of the chromosome will be identical to one another, it is possible to have some variations between them-in the forms of SNPs or other base pair differences in your target region sequence. Also, some tumor genes, such as P53, tend to have lots of mutations. So even in the negative control, it is possible to get T7 cutting from PCR product denaturion and reannealing. This is why when designing target primers, your design strategy should include avoiding SNPs in the first place.

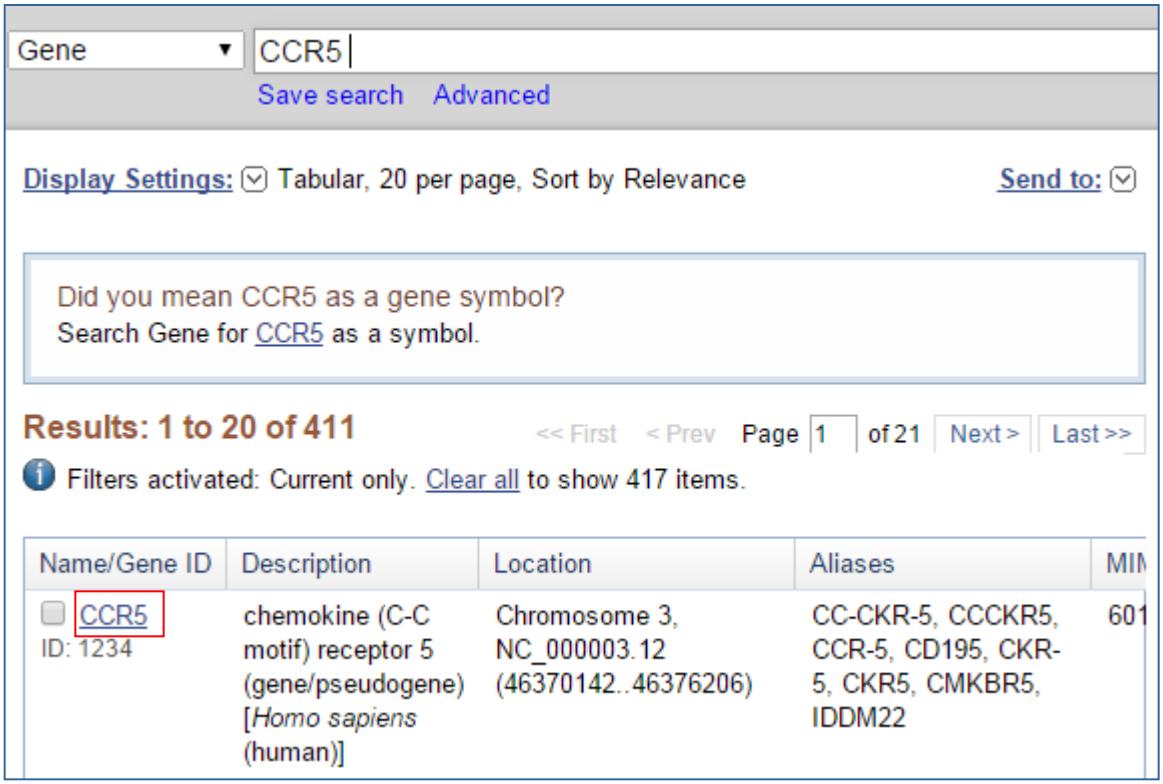
We highly recommend you obtain sequence information of the target site of your cell line before primer design and any other experiment. NCBI provides a tutorial for searching for SNP information of a gene. We offer a brief description of the process here.

●BY GENE NAME

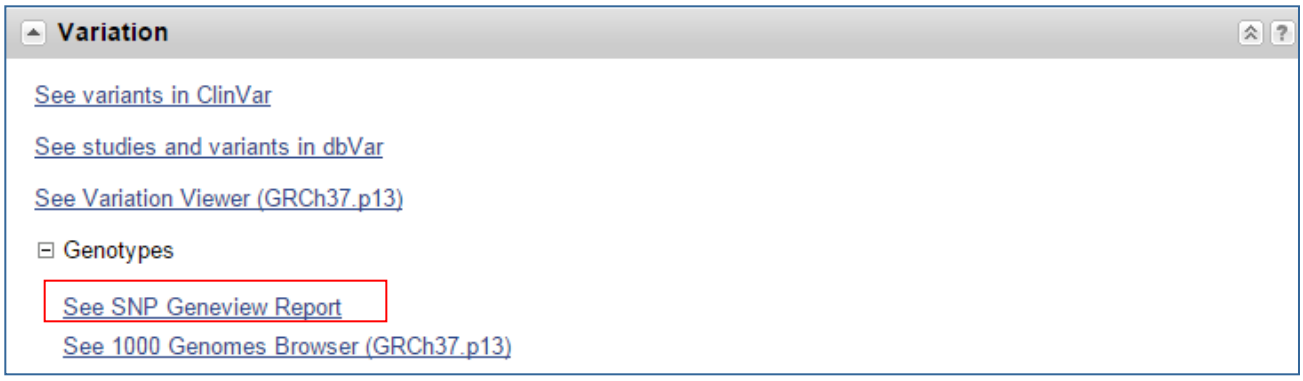
1. Search the Gene database with the gene name. If you know the gene symbol and species, enter them as follows: tpo[sym] AND human[orgn]

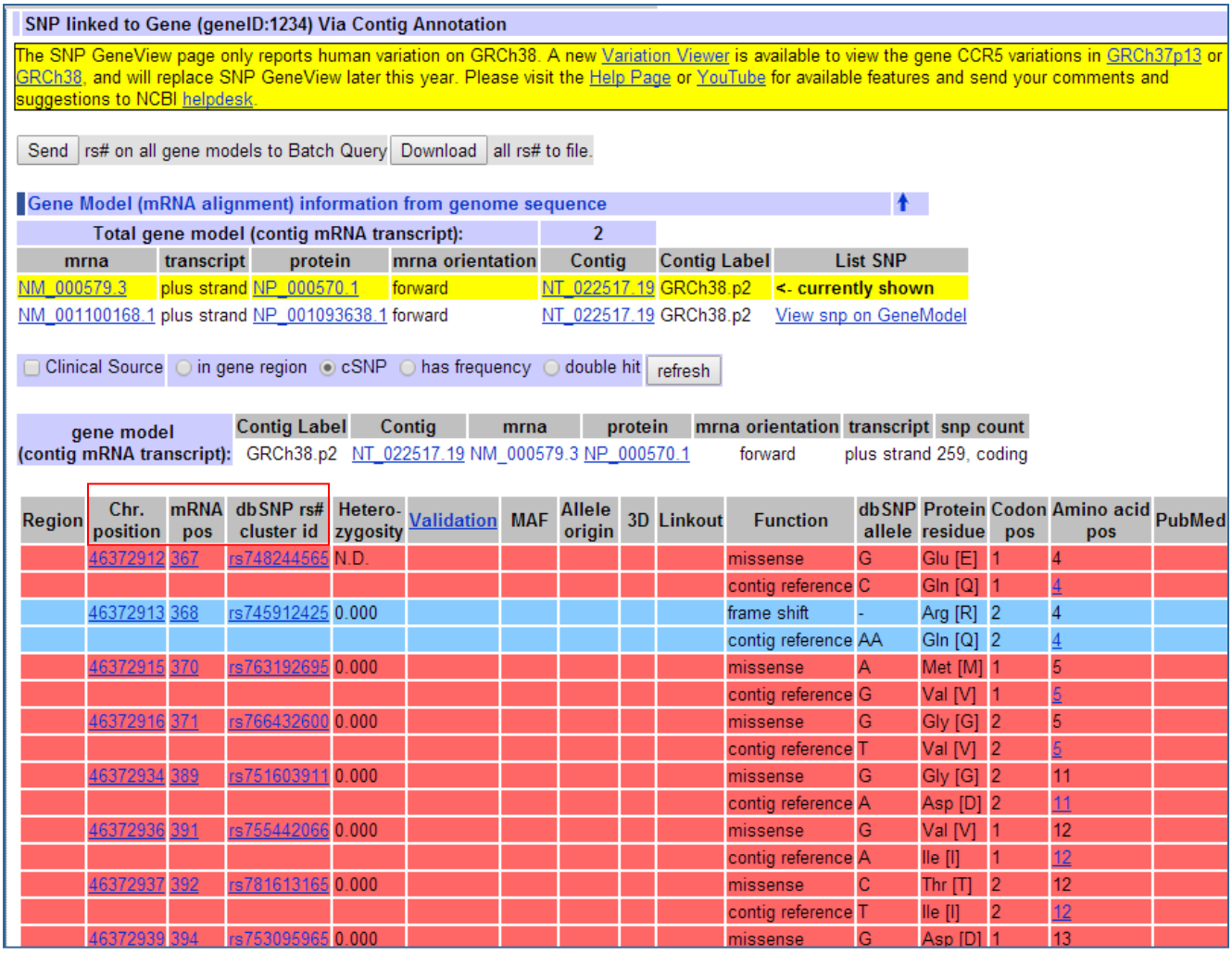


2. Click on the desired gene.



3. In the list of links on the right, click "SNP: GeneView". If the link is not present, no SNPs are currently linked to this gene.
4. For human genes, another option is to go to the variation section (Click on Variation in the table of contents in the upper right), and follow links to Variation Viewer for either the GRCh37/hg19 or GRCh39/h38 assemblies, to the 1000 Genomes Browser, ClinVar and more.





SNP linked to Gene (geneID:1234) Via Contig Annotation

The SNP GeneView page only reports human variation on GRCh38. A new Variation Viewer is available to view the gene CCR5 variations in GRCh37p13 or GRCh38, and will replace SNP GeneView later this year. Please visit the Help Page or YouTube for available features and send your comments and suggestions to NCBI helpdesk.

Send rs# on all gene models to Batch Query Download all rs# to file.

Gene Model (mRNA alignment) information from genome sequence

Total gene model (contig mRNA transcript):				2		
mrna	transcript	protein	mrna orientation	Contig	Contig Label	List SNP
NM_000579.3	plus strand	NP_000570.1	forward	NT_022517.19	GRCh38.p2	<- currently shown
NM_001100168.1	plus strand	NP_001093638.1	forward	NT_022517.19	GRCh38.p2	View snp on GeneModel

Clinical Source in gene region cSNP has frequency double hit refresh

gene model (contig mRNA transcript):	Contig Label	Contig	mrna	protein	mrna orientation	transcript	snp count
	GRCh38.p2	NT_022517.19	NM_000579.3	NP_000570.1	forward	plus strand	259, coding

Region	Chr. position	mRNA pos	dbSNP rs# cluster id	Hetero-zygosity	Validation	MAF	Allele origin	3D	Linkout	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos	PubMed
	46372912	367	rs748244565	N.D.						missense	G	Glu [E]	1	4	
										contig reference	C	Gln [Q]	1	4	
	46372913	368	rs745912425	0.000						frame shift	-	Arg [R]	2	4	
										contig reference	AA	Gln [Q]	2	4	
	46372915	370	rs763192695	0.000						missense	A	Met [M]	1	5	
										contig reference	G	Val [V]	1	5	
	46372916	371	rs766432600	0.000						missense	G	Gly [G]	2	5	
										contig reference	T	Val [V]	2	5	
	46372934	389	rs751603911	0.000						missense	G	Gly [G]	2	11	
										contig reference	A	Asp [D]	2	11	
	46372936	391	rs755442066	0.000						missense	G	Val [V]	1	12	
										contig reference	A	Ile [I]	1	12	
	46372937	392	rs781613165	0.000						missense	C	Thr [T]	2	12	
										contig reference	T	Ile [I]	2	12	
	46372939	394	rs753095965	0.000						missense	G	Asp [D]	1	13	

Figure 4. Example of SNP Geneview Report on NCBI. Check column "mRNA pos" for your SNP site of interest. Check column "Chr, position" or "db SNP rs# cluster id" for the sequence of the site of interest.

VIII. Limited Use License and Warranty

Limited use license

Following terms and conditions apply to use of the IndelCheck™ CRISPR/TALEN insertion or deletion detection system. If the terms and conditions are not acceptable, the Product in its entirety must be returned to GeneCopoeia within 5 calendar days. A limited End-User license is granted to the purchaser of the Product. The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use. The Product must not be resold, repackaged or modified for resale, or used to manufacture commercial products or deliver information obtained in service without prior written consent from GeneCopoeia. Use of any part of the Product constitutes acceptance of the above terms.

Limited warranty

GeneCopoeia warrants that the Product meets the specifications described in the accompanying Product Datasheet. If it is proven to the satisfaction of GeneCopoeia that the Product fails to meet these specifications, GeneCopoeia will replace the Product. In the event a replacement cannot be provided, GeneCopoeia will provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to GeneCopoeia within 30 days of receipt of the Product. GeneCopoeia’s liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. GeneCopoeia’s liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. GeneCopoeia does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

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