USER GUIDE

GeneArt® Genomic Cleavage Detection Kit

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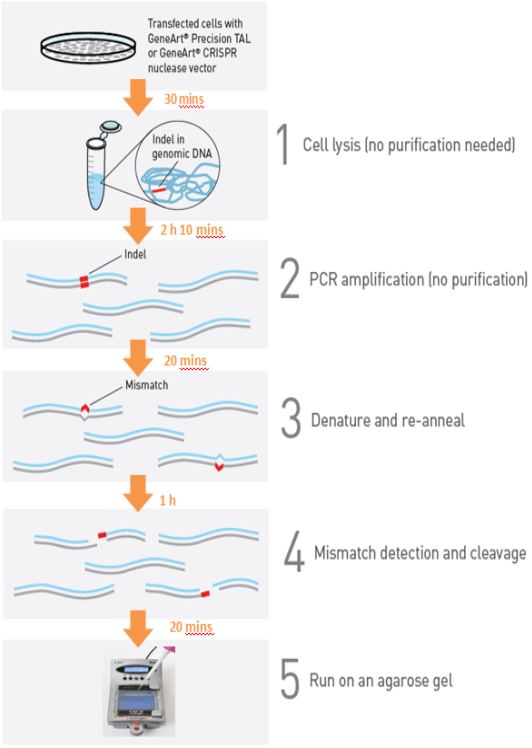
Product information

Product description

Introduction

The GeneArt® Genomic Cleavage Detection Kit provides a simple, reliable, and rapid method for the detection of locus specific cleavage of genomic DNA. The assay uses genomic DNA extracted from cells transfected with constructs expressing engineered nucleases such as Transcription activator-like effector nuclease (TALEN), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9, or Zinc-finger nuclease (ZFN). Following cleavage, genomic insertions or deletions (indels) are created by the cellular repair mechanisms. Loci where the gene-specific double-strand breaks occur are amplified by PCR. The PCR product is denatured and re-annealed so that mismatches are generated as strands with an indel re-annealed to strands with no indel or a different indel. The mismatches are subsequently detected and cleaved by Detection Enzyme and then the resultant bands are analyzed by gel electrophoresis and band densitometry.



Kit contents and storage

Contents

The following reagents included with GeneArt® Genomic Cleavage Kit are enough for 20 reactions. Store the kit at –20°C

Component	Quantity
Cell Lysis Buffer	1 mL
Protein Degrader	96 µL
AmpliTaq Gold® 360 Master Mix	1 mL
Water	1 mL
Detection Enzyme	20 µL
10X Detection Reaction Buffer	40 µL
Control Template & Primers	10 µL

Required materials not included with the kit

- 2% E-Gel® EX Gel, E-Gel® iBase™ Power System (recommended) or user-prepared 2% agaraose gel and electrophoresis system
- UV transilluminator
- Gel image analysis software
- PureLink® PCR Purification Kit (optional)
- 360 GC Enhancer (optional)
- Loading buffer without dye for user-prepared gels

Methods

General guidelines

Guidelines

For the first time user, we recommend using the Control Template & Primers in the kit to perform a control PCR reaction. This PCR product can also serve as a control for denaturing and re-annealing, and enzyme digestion steps.

Primer design

Primers

Genomic DNA at the locus being investigated must be PCR amplified prior to detection. Follow the recommended guidelines to ensure optimal amplification and subsequent detection.

- For best results, use primers with T_m >55°C.
- Design primers that are 18–22 bp in length and have 45–60% GC content.
- For efficient amplification, design primers to yield amplicon lengths between 400 and 500 bp.
- Design primers so that the potential cleavage site is not in the center of the amplicon and the detection reaction will yield two distinct product bands.

Harvest cells

Harvest

1. Spin down cells transfected with TALEN, CRISPR, or ZFN constructs at 200*g* for 5 minutes at 4°C.
2. Carefully remove supernatant and proceed to lysis or store pellet at –80°C.

   Note: The quality of the results is strongly influenced by transfection conditions, i.e. good cell viability and transfection efficiency. A fluorescent transfection marker is recommended for estimating the transfection efficiency of desired cell types.

Cell lysis and DNA extraction

Lysis and extraction

1. Mix 50 µL Cell Lysis Buffer with 2 µL Protein Degrader in a microcentrifuge tube.
2. Add 50 µL of Cell Lysis Buffer/Protein Degrader mix to each cell pellet and resuspend the pellet.
3. Transfer all of the resuspended cell pellet to a PCR tube.
4. Run the following program in a thermal cycler:

Temp	Time
68°C	15 min
95°C	10 min
4°C	Hold*

*Following completion, immediately proceed to PCR amplification or store at –20°C.

Note: It is important that at least 50,000 and no more than 2 x10^6 cells are used in 50 µL Cell Lysis Buffer. Lysate from less than 50,000 cells will be insufficient for PCR amplification and greater than 2 x10^6 cells will inhibit the PCR reaction. The volume of lysis buffer can be adjusted based on cell number.

PCR amplification

PCR reaction

Briefly vortex cell lysate.

Add the following components to a PCR tube:

Component	Sample	Control
Cell lysate	2 µL	—
10 µM F/R primer mix	1 µL	—
Control Template & Primers	—	1 µL
AmpliTaq Gold® 360 Master Mix	25 µL	25 µL
Water	22 µL	24 µL
Total	**50 µL**	**50 µL**

Note: For best results, add 1–10 µL of 360 GC Enhancer per 50 µL PCR reaction when amplifying GC rich loci.

PCR parameters

Run the PCR reaction with the following conditions:

Stage	Temp	Time	Cycles
Enzyme activation	95°C	10 min	1 X
Denature	95°C	30 sec	40 X
Anneal	55°C (T_m)	30 sec	
Extend	72°C	30 sec	
Final extension	72°C	7 min	1 X
Hold	4°C	Hold*	1 X

*Following completion of PCR reaction, proceed to next step or store at –20°C

Note: The annealing temperature can be adjusted based on the primers used. Adjust the extension time based on the size of amplicon (60 second for each kb). For best results, amplify approximately 500 bp of the target locus.

Verify PCR product

1. Load 3 µL of PCR product with 10 µL water on a 2% agarose gel (For best results, run 2% E-Gel® EX Gel on E-Gel® iBase™ Power System for 30 minutes at low voltage).

   Note: A DNA ladder can be run in parallel to estimate the DNA concentration by visual inspection. Fill unused wells with 10 µL water.

2. If a single band of the correct size is present, with an intensity similar to 50 ng of 400 bp band in the mass ladder, proceed to the denaturing and re-annealing step, or store the PCR product at –20°C for later use.

   Note: A single band of expected size is crucial for obtaining accurate cleavage detection. Optimize the PCR conditions including primers, annealing temperature, and amount of lysate volume until you obtain good quality PCR products. Refer to **PCR Troubleshooting** (page 11) if a single clear band is not present.

Cleavage assay

Set up denaturing and re-annealing reaction

This step serves to randomly anneal the PCR fragments with and without indels to form heterogeneous DNA duplexes.

1. Combine 1–3 µL of PCR product with 1 µL 10X Detection Reaction Buffer in a PCR tube.
2. Bring to a volume of 9 µL with Water.
3. Briefly centrifuge in a microfuge to ensure that no bubbles are present.

   Note: The amount of unpurified DNA and the total amount of DNA in the cleavage reaction affects the cleavage efficiency and specificity. Decrease the amount of input DNA to increase the cleavage efficiency. For the positive control reaction, use 1 µL of control PCR product in a 10 µL reaction.

   Note: The amplified PCR product can be used in cleavage assay without further purification. However, to increase accuracy, purify the PCR product (PureLink® PCR Purification Kit, recommended) and use the same quantity of purified DNA for each reaction. Use 50 ng to 100 ng of DNA for each reaction.

   Note: It is good practice to include a negative enzyme control for each sample, i.e. make duplicate re-annealing reactions for each sample to be analyzed in order to distinguish the background bands from the expected cleavage product.

Run re-annealing reaction

Place the PCR tube from above in a thermal cycler with a heated-lid and run the following program:

Stage	Temp	Time	Temp/time
1	95°C	5 min	—
2	95°C–85°C	—	–2°C/sec
3	85°C–25°C	—	–0.1°C/sec
4	4°C	—	Hold*

*Following completion of re-annealing reaction, immediately proceed to enzyme digestion or store reaction at –20°C.

Enzyme digestion

In this step the heteroduplex DNA containing the insertion, deletion, or mismatched DNA (indel) is cleaved by the Detection Enzyme, allowing for quantification of the percent of gene modification using gel analysis software.

1. Add 1 µL Detection Enzyme to all test samples, mix well.
2. Add 1 µL Water to all negative control samples, mix well.
3. Incubate at 37°C for 1 hour.
4. Vortex briefly and spin down.
5. Place at 4°C.

   Note: Do not allow the reaction to incubate at 4°C for more than 2 hours. Otherwise, add 1 µL of 100 µM EDTA to each reaction and store samples at –20°C.

Gel analysis

1. Immediately load the entire 10 µL sample on a 2% E-Gel® EX Gel with 10 µL Water. Run a sizing standard such as a 1 kb DNA ladder in parallel.

   Note: Avoid any bubbles in the well as this generates an uneven band and might skew the cleavage measurements.

   Note: For user-prepared gels, use loading buffer without dye to avoid interference with band intensity measurements.
2. Run 2% E-Gel® EX Gel on an E-Gel® iBase™ Power System for 30 minutes at low voltage.
3. View gel using a UV transilluminator. Refer to Figure 1 (Appendix A) for example gel image.

   Note: In some instances there is a band visible above the parent amplicon after denaturing, and re-annealing. For example in Figure 1 (Appendix A), when no Detection Enzyme is added to Target 1 the heteroduplex runs slower than the parent band.
4. Use a gel imaging system to take an image and determine the relative proportion of DNA contained in each band using desired gel analysis software. Refer to (Appendix A) Figure 2 and Figure 3 for examples of gel analysis using AlphaImager® Software and microfluidic electrophoresis bioanalyzer, respectively.
5. Use the following equation to calculate the cleavage efficiency:

   Cleavage Efficiency= $1-[(1-\text{fraction cleaved})^{1/2}]$

   Fraction Cleaved= sum of cleaved band intensities/(sum of the cleaved and parental band intensities)

   Note: Digestion of hybrid control PCR from control template and primers produces two cleavage products of 225 and 291 bp in size. The cleavage efficiency for the control is calculated as: [sum of cleaved band intensities/(sum of cleaved and parental band intensities)]× 100%.

   Note: The sensitivity of the DNA fragment detection in gels varies depending on the dyes for DNA staining (ethidium bromide or SYBR® Green), the wavelength of the UV transilluminator, and different commercial digital image systems. Optimize the imaging conditions to achieve the best ratio of signal to background signal.

Troubleshooting

PCR troubleshooting

DNA band appearance	Possible cause	Recommendation
Smear	Lysate is too concentrated	Dilute lysate 2-fold to 4-fold and repeat the PCR reaction
Too faint	Lysate is too dilute	Double the amount of lysate in the PCR reaction. **Note:** Do not use more than 4 µL of lysate in the PCR reaction. Lysate can inhibit the PCR reaction.
Disparity in band intensity between amplicons	Lysate concentrations vary between samples	Purify the PCR products with PureLink® PCR Purification Kit. **Note:** For best results in comparing samples, purify PCR and use the same quantity of DNA in each cleavage assay. 50 ng to 100 ng of DNA is enough for each reaction.
No PCR product	Poor PCR primer design	Re-design primers that are 18–22 bp, have 45–60% GC content, and a 52–58°C T_m range.
	High GC-rich region	Add 1–10 µL of 360 GC Enhancer in a 50 µL reaction and repeat the PCR amplification.

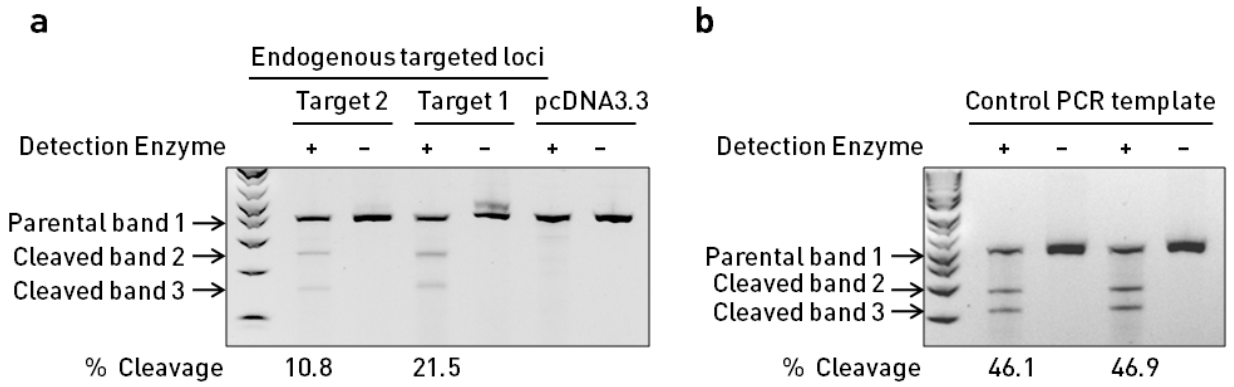
Cleavage troubleshooting

Problem	Possible cause	Recommendation
No cleavage band visible	Nucleases unable to access target sequence or unable to cleave at target site Transfection efficiency too low	Design new targeting strategy at nearby sequences.
	Genomic modification too low	Optimize transfection protocol.
	Omitted denaturing and re-annealing step	Use kit Control Template & Primers to verify the kit components and protocol.
Difficulty in analyzing gel data	Background interferes with the measurements of cleaved bands	Re-design the PCR primers to produce a distinct cleaved binding pattern
Non-specific cleavage bands	Intricate mutations at the target site Digestion incubation too long Too much Detection Enzyme added Non-specific cleavage by Detection Enzyme for certain target loci.	Re-design PCR primers to amplify target sequence. Use lysate from mock transfected cells or cells transfected with irrelevant plasmids as negative control to distinguish background from specific cleavage.

Appendix A

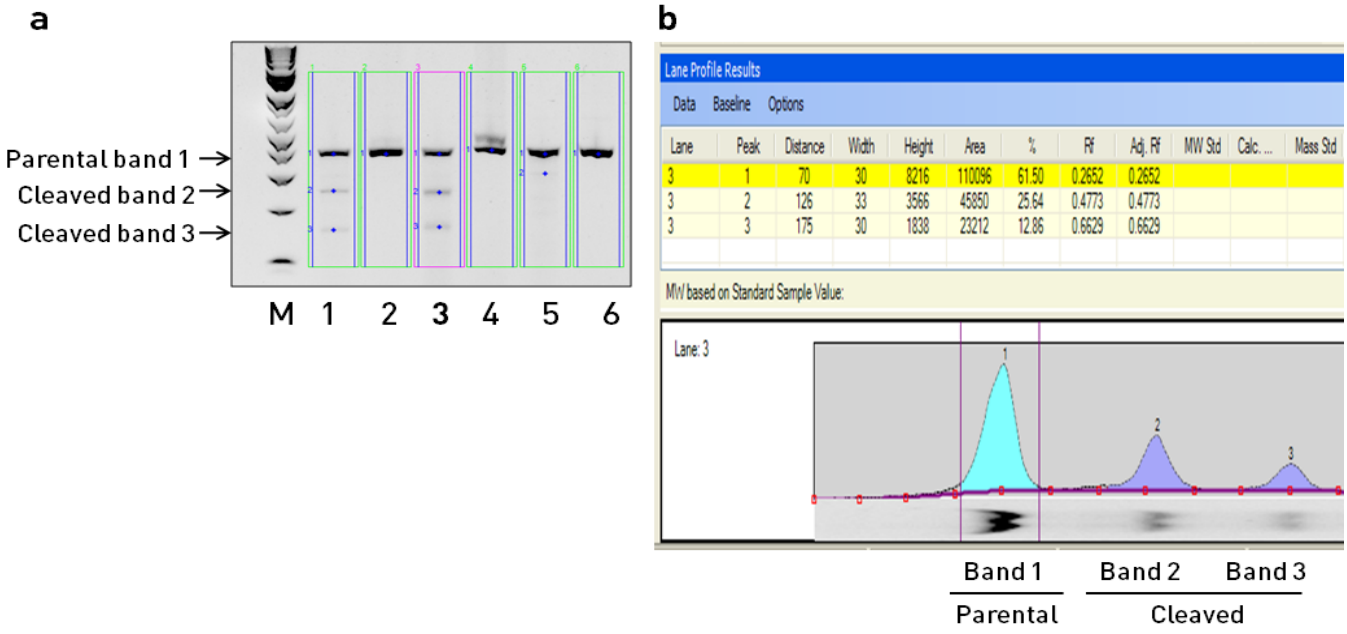
Example of Genomic Cleavage Detection Assay

Figure 1 Example of Genomic Cleavage Detection Assay gel



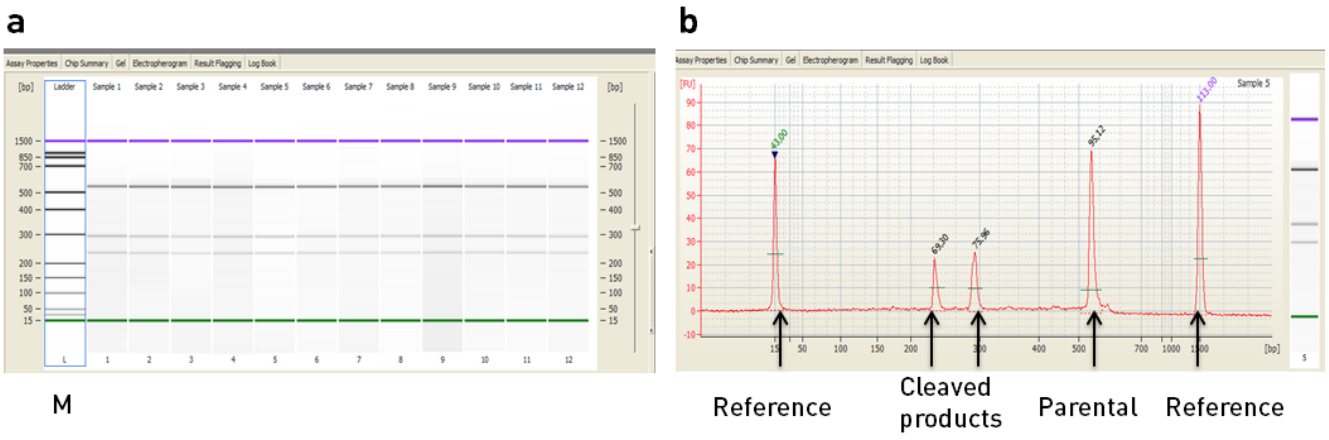
(a) Gel image of Genomic Cleavage Detection Assay using transfected cells. 293 FT cells were transfected with GeneArt® CRISPR All-In-One vectors targeting different regions of the human AAVS1 locus (Target 1 and Target 2) using Lipofectamine® 2000. A negative control sample for gene modification was also prepared by transfecting with pcDNA3.3. The above samples were PCR amplified using the same set of primers flanking the region of interest. After re-annealing, samples were treated with and without Detection Enzyme and run on a 2% E-Gel® EX Gel. **(b) Gel image of Genomic Cleavage Detection Assay using Control Template & Primers.** After re-annealing samples were treated with and without Detection Enzyme and run on a 2% E-Gel® EX Gel.

Figure 2 Gel image analyzed by image software



(a) Highlighted lane profiles from Genomic Cleavage Detection Assay gel analyzed by AlphaImager® Software. Lane profiles with relevant bands were selected and all visible bands automatically detected. M=molecular weight. **(b) Example lane profile results and chromatogram for lane 3.** The lane profile results were used to determine the fraction cleaved. The cleavage product consists of two bands, which combined total the fraction cleaved. In Lane 3, 61.5% of the total DNA in the lane is contained in parental band 1, while 25.64% and 12.86% of the total DNA is present in cleaved bands 2 and 3, respectively. The sum of the cleaved bands, 0.385 (38.5%) is the total fraction cleaved. Gene Modification Efficiency (test sample) = 1- ((1-fraction cleaved) $^{1/2}$) = 1- ((1- 0.385)$^{1/2}$) = 0.215. Approximately 21.5% of DNA in Lane 3 is genetically modified.

Figure 3 Gel image analyzed by microfluidic electrophoresis



(a) Gel image analyzed with Agilent 2100 Bioanalyzer microfluidic electrophoresis. The digestion reactions of re-annealed control PCR products from Control Template & Primers were run on a bioanalyzer. M=molecular weight. **(b) An example of electropherograms analyzed using 2100 Expert software.** Reference peaks shown at 15 bp and 1500 bp flank the peaks for cleaved and parental DNA. The concentration of each DNA fragment was automatically calculated based on the fluorescence intensity and the peak area of each individual DNA fragment.

Appendix B

Accessory products

Additional products

Additional reagents that may be used are available at **www.lifetechnologies.com**.

Item	Cat. no.
2% E-Gel® EX Gel	G401002
E-Gel® iBase™ Power System	G6400
AmpliTaq® 360 Buffer, 25 mM $MgCl_2$, and 360 GC Enhancer	4398848
PureLink® PCR Purification Kit	K3100-02

Documentation and support

Obtaining SDSs

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/support**.

Note: For the SDSs of chemicals not distributed by Life Technologies Corporation, contact the chemical manufacturer.

Obtaining Certificates of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

Obtaining support

For the latest services and support information for all locations, go to **www.lifetechnologies.com/support**

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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