INSTRUCTION MANUAL

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Authenticase™

NEB #M0689S/L

25/125 reactions

Version 1.0_8/23

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Description

Authenticase is a mixture of structure-specific nucleases capable of recognizing and cleaving outside mismatch and indel (insertion and/or deletion) regions, ranging from 1–10 basepairs (bp) on double-stranded DNA. The formulation has limited non-specific activity on homoduplex regions of DNA. Authenticase can be used as an error-correction reagent in oligo-based PCR gene assembly by enzymatically removing "mistakes" prior to the final renaturation and amplification step. Alternatively, Authenticase can replace T7 Endonuclease I in the mismatch detection assay used to assess the efficiency of genome editing.

Each Package Contains:

Materials for 25 reactions (NEB #M0689S) or 125 reactions (NEB #M0689L)

- 25/125 µl of Authenticase Mix
- 700 µl of Authenticase DNA Annealing Buffer (NEB #B2831)
- 500 µl of Authenticase Reaction Buffer (NEB #B2832)
- Mismatch Detection Assay Protocol
- DIY Gene Synthesis Protocol

Additional Materials Not Included:

- Q5® Hot Start High-Fidelity 2X Master Mix (NEB #M0494)

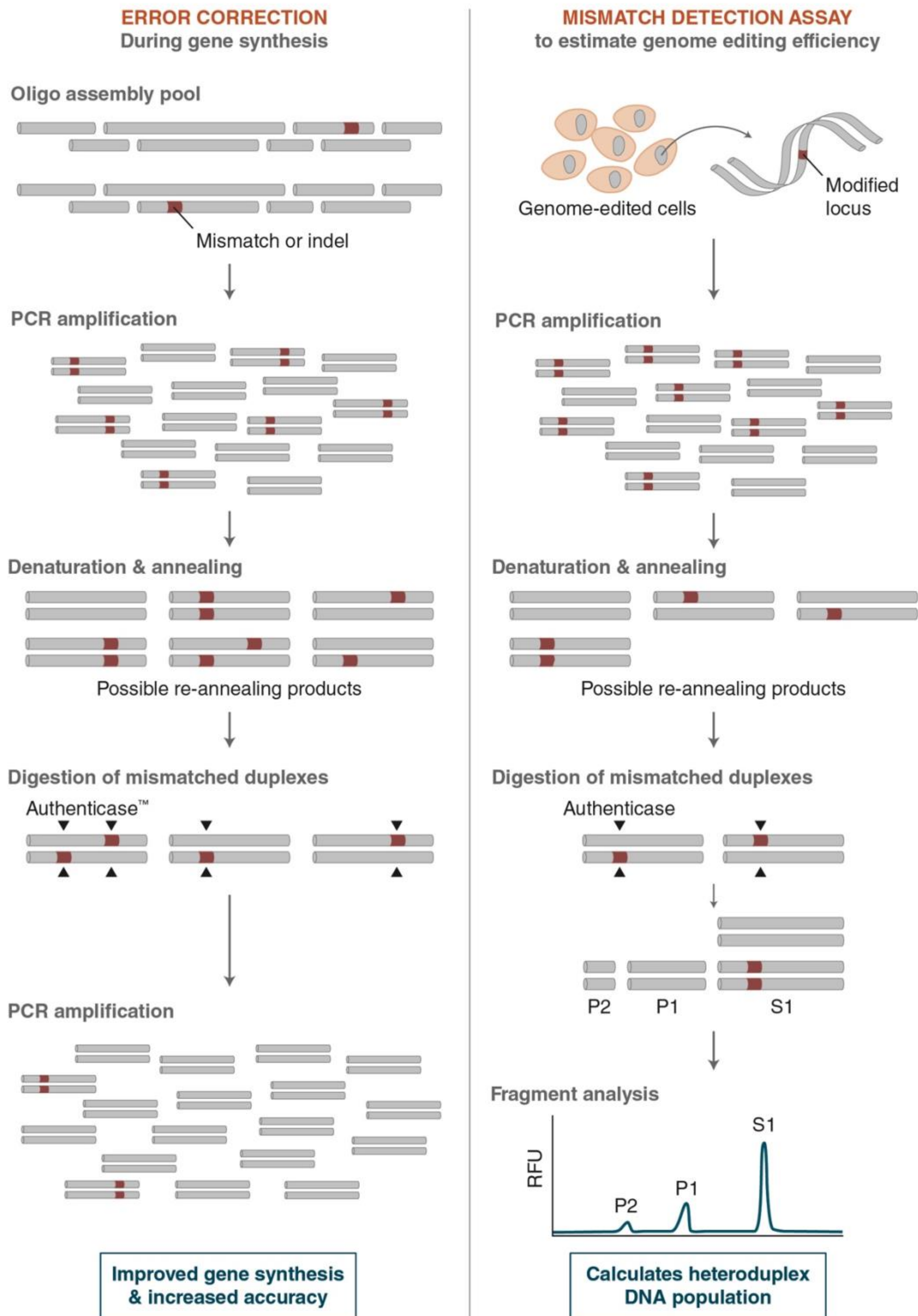
Supplied In:

10mM Tris-HCl, 500 mM NaCl, 1 mM Dithiothreitol, 0.1 mM EDTA and 50% Glycerol (pH 7.4 @ 25°C)

Applications of Authenticase

- **Error correction during gene synthesis:** reduction of errors in DNA fragments during DNA assembly-based gene synthesis
- **Mismatch detection assay:** to estimate genome editing efficiency

Figure 1: Applications of Authenticase



Application 1: Error Correction During Gene Synthesis

In many DIY gene synthesis workflows, users obtain fragments for assembly by purchasing synthesized dsDNA (e.g., gBlocks) or by preparing amplicons of overlapping oligos. Often times, these parts have residual errors arising directly from the oligonucleotides used to generate the fragments. Authenticase will reduce/remove the mismatch/indel (insertion/deletion) regions from amplicons that originate from errors incorporated during the chemical synthesis of oligonucleotides. The following protocol increases the population of correct fragments in the enzyme-corrected DNA pools and subsequently allows DNA polymerase amplification to enrich amplicons with more accuracy and efficiency. The combination of correction and enrichment steps enhances the quality of assembled gene synthesis, thereby producing higher numbers of transformed bacterial colonies with the desired correct DNA sequence.

Process efficiency gains are realized by requiring less colony-picking and sequencing of samples.

Figure 2. Error Correction During Gene Synthesis Workflow

Note: Authenticase is ONLY USED in the second section of this workflow. We provide the additional info and protocols to enable a more thorough workflow for error correction during DIY gene synthesis. These protocols were optimized and confirmed by NEB®.

1. Prepare heteroduplex DNA.

The products of the PCR reaction must be denatured and annealed to allow heteroduplex formation between PCR products with and without mutations.

1.1. Clean up dsDNA amplicons using a spin column (e.g., Monarch® PCR & DNA Cleanup Kit (5 µg) – NEB #T1030) and elute in a small volume (e.g., 12 µl).

1.2. Determine the dsDNA concentration.

1.3. Prepare annealing reaction with DNA concentration around ~40 ng/µl.

REAGENT	REACTION
PCR amplicon (800 ng)	X µl
5X Annealing Buffer	4 µl
Nuclease-free water	to 20 µl

1.4. Use a thermocycler to denature and anneal the sample, forming heteroduplex DNA.

CYCLE STEP	TEMP	RAMP RATE	TIME
Initial Denaturation	95°C		5 minutes
Annealing	95–85°C	–2°C/second	
	85–25°C	–0.1°C/second	
Hold	4°C		

2. Treat with Authenticase to digest DNA at mismatches and indels.

2.1. Setup Authenticase cleavage reaction on ice.

REAGENT	REACTION
Heteroduplex DNA from step 1.4. (200 ng)	5 µl
10X Authenticase Reaction Buffer	2 µl
Authenticase	1 µl
Nuclease-free water	12 µl
Total volume	**20 µl**

2.2. Incubate at 42°C for 60 minutes.

2.3. Add 1.7 µl 150 mM EDTA.

2.4. Heat samples at 95°C for 5 minutes. Tubes can be stored at –20°C until ready to use.

3. Amplify error-depleted population to increase percentage of error-corrected clones.

3.1. Setup reaction for correcting errors of samples from step 2.4.

REAGENT	REACTION
Heteroduplex DNA from step 2.4.	2 µl
Q5 Hot Start High-Fidelity 2x Master Mix	5 µl
Nuclease-free water	3 µl
Total volume	**10 µl**

3.2. Process reaction in a thermocycler to amplify the error-corrected pool.

CYCLE STEP	TEMP	RAMP RATE	TIME	CYCLES
Initial Denaturation	95°C		5 minutes	
Annealing	95–72°C	0.1°C/second		
Hold	72°C		10 minutes	
Denaturation	98°C		10 seconds	23
Annealing	64°C*		10 seconds	
Extension (for 500–1,000 bp)	72°C		30–50 seconds	
Final Extension	72°C		3 minutes	

* Please visit tmcalculator.neb.com to determine correct annealing temperature.

4. Enrich full-size gene of interest.

4.1. At this step, two nearly identical reactions (A&B) are created to amplify and enrich the full-length fragment of interest. The first reaction (Tube A) uses 2 µl of the error corrected pool from step 3.2. as template. The second reaction (Tube B) uses 2 µl from the first reaction (Tube A) as template to ensure an appropriate amount of amplification can be achieved.

4.2. Prepare 2 PCR reactions with 0.5 µM of forward/reverse primers, according to the table below. In the first, add 2 µl of template from step 3.2 to Tube A and mix properly. For the second, transfer 2 µl of Tube A reaction to Tube B. This will create 2 PCR reactions: one with 2 µl (Tube A) and a second with a lower amount (0.16 µl, Tube B) of template from step 3.2. Varying the template to primer ratio can help ensure at least one reaction is productive.

REAGENT	REACTION A	REACTION B	FINAL RXN. CONC.
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	12.5 µl	1X
10 µM Forward Primer	1.25 µl	1.25 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	1.25 µl	0.5 µM
Nuclease-free water	10 µl	10 µl	
Template DNA	2 µl	2 µl of Tube A mix	

4.3. Amplify full-length fragment of interest in a thermocycler.

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	2 minutes	
Denaturation	98°C	10 seconds	24
Annealing	64°C*	10 seconds	
Extension (for 500–1,000 bp)	72°C	30–50 seconds	
Final Extension	72°C	5 minutes	
Hold	4–10°C		

* Please visit tmcalculator.neb.com to determine correct annealing temperature.

5. Assemble DNA and transform.

5.1. Check the purity of PCR products from Tubes A and B (we recommend running 10% of the reaction on an agarose gel). Other methods such as Agilent® Bioanalyzer® or TapeStation® can suffice. Choose the PCR product with higher purity to proceed.

5.2. PCR amplicons are typically cleaned up by spin column prior to quantitation and cloning/DNA Assembly. Reactions can be directly used with reduced efficiency, if desired, although we recommend a cleanup step to ensure accurate quantitation can be performed and to remove potential inhibitors of enzymes used in the next steps of your workflow.

5.3. DNA is ligated or assembled into the destination vector of choice as part of the design strategy. NEBuilder HiFi DNA Assembly or Golden Gate Assembly methods (using NEBridge® reagents) can be used to assemble DNA.

5.4. Assembled DNA then can be transformed into competent *E. coli* (e.g., NEB® 5-alpha or NEB 10-beta) and propagated on rich agar plates with appropriate antibiotic selection.

5.5. Colonies are picked and analyzed for presence of correct clones:

Directly

5.5.1. Directly by colony PCR w/appropriate primers followed by agarose gel electrophoresis to confirm the amplicon, followed by Sanger sequencing (more details in supplemental protocol #2).

OR

Indirectly

5.5.2. By analysis of miniprep plasmid DNA after overnight culture, by restriction enzyme digest or Sanger DNA sequencing.

Application 2: Mismatch Detection Assay

Heterogeneous cell populations created by genome editing techniques (CRISPR, TALEN, ZFN, etc.) can be quickly screened using a mismatch detection assay to identify regions containing mismatches and/or indels. Authenticase cleaves heteroduplex regions of re-annealed PCR amplicons from target regions of edited genomes, including single base mismatches and indels not recognized by the commonly used T7 Endonuclease I protocol. By resolving the cleaved fragments on an agarose gel or Bioanalyzer, the proportion of uncut to cut fragments can be compared to provide an estimate of the efficiency of the genome editing event. By recognizing a more comprehensive set of structures, compared to T7 Endonuclease I, use of Authenticase can improve the accuracy of the mismatch detection assay.

NEB recommends designing PCR products around 700 bp with anticipated sizes of cleaved product around 450 and 250 bp respectively if Bioanalyzer will be used at the end to analyze genome editing efficiency.

For each amplicon, NEB recommends setting up three PCR reactions using the following templates:

a. gDNA from targeted cells (e.g., Cas9, TALEN or ZFN transfected cells)

b. gDNA from negative control cells (e.g., non-specific DNA transfected cells, WT targeted gene)

c. Water (i.e., no template control)

Note: Authenticase is ONLY USED in the second section of this workflow (Figure 2). NEB provides additional information and protocols to enable a more thorough workflow for mismatch detection during analysis of genome editing efficiency. These protocols were optimized and confirmed by NEB.

Two analytical methods are recommended to estimate the heterogeneity within a dsDNA pool generated by PCR amplification of an edited target region:

Method A is a conventional and popular setup (only requires amplifying DNA from edited cells) and provides a quick quantitative estimation (e.g., 53.1% mutation from a pool with 60% heterogeneous mutations (Case 1) and 27.9% for a pool with 60% homogeneous mutations (Case 2)).

Method B is a modified protocol (requires amplifying DNA from edited cells and wild type cells, respectively) that will provide a more accurate estimation of a pool with 60% heterogeneous mutation population (e.g., Case 1: 57.9% of Method B vs. 53.1% of Method A) including extreme cases where one type of specific mutation species comprises more than 50% of all the amplicons (as shown in Case 2: 47.7% of Method B vs. 27.9% of Method A).

Figure 3. Two methods for creating heteroduplex DNA for mismatch detection assay

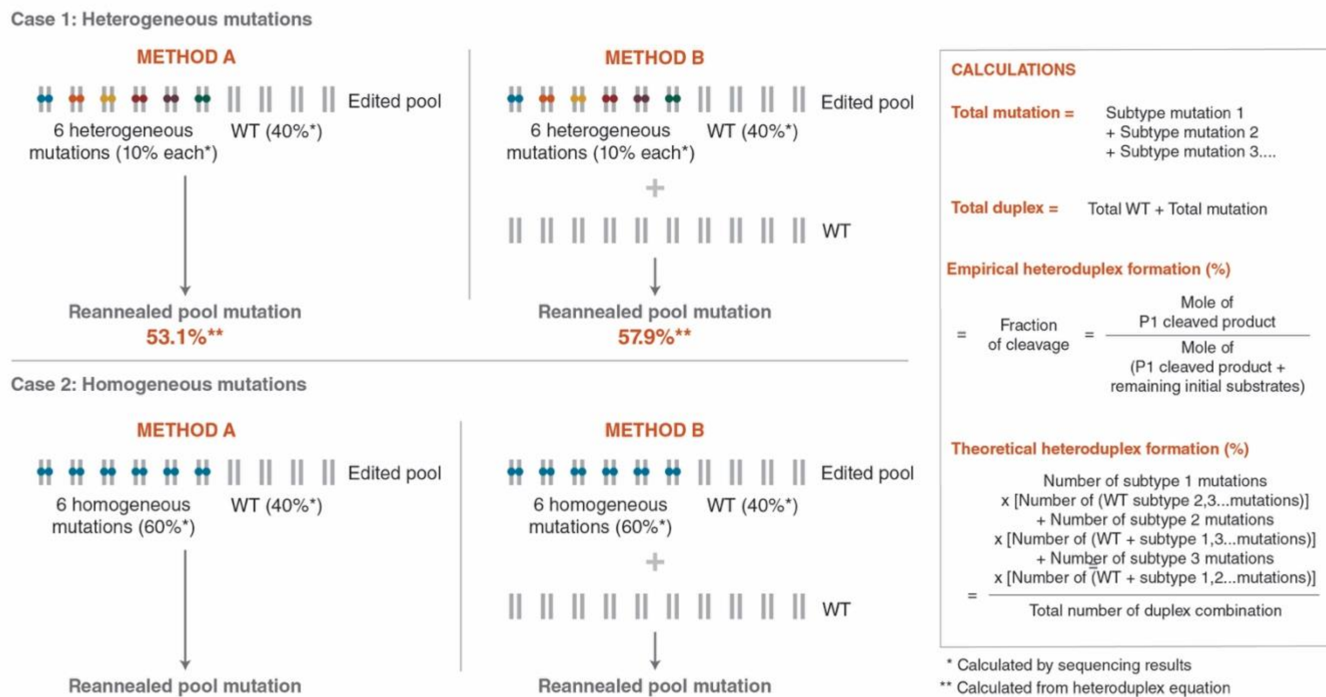


Figure 4. Mismatch Detection Assay Workflow

In practice, many users may choose to examine editing efficiency in target regions amplified from the edited cell population only (Method A). In cases where there is a dominant mutation (previously identified or suspected), having control gDNA from unedited cells can serve as a useful control and allow for an increased accurate calculation if Method B is followed. The following section provides guidance for amplification of both populations to enable this comparison.

1. Amplify target regions by PCR.

1.1. Thaw Q5 Hot Start High-Fidelity 2X Master Mix (purchased separately). Pulse-spin each component in microfuge prior to use.

1.2. Set up two 25 µl PCR reactions and use up to 500 ng of genomic DNA as templates. Reaction A is the experimental reaction with edited genomic DNA as template. Reaction B is the control reaction using gDNA from non-edited cells. Assemble the following reactions at room temperature:

REAGENT	REACTION A	REACTION B	FINAL RXN. CONC.
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	12.5 µl	1X
10 µM Forward Primer	1.25 µl	1.25 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	1.25 µl	0.5 µM
Template DNA (edited genome)	Variable		0.5–500 ng genomic DNA**
Template DNA (WT genome)		Variable	0.5–500 ng genomic DNA**
Nuclease-free water	to 25 µl	to 25 µl	

* NEB recommends designing primers to produce amplicons around 700 bp with anticipated sizes of cleaved product around 450 and 250 bp, respectively, if a Bioanalyzer will be used at the end to analyze the genome editing efficiency.

** To use cell lysate directly in PCR, lyse cells in QuickExtract™ using 50 µl cells in each well of a 96-well plate (~40,000 cells) according to the manufacturers' recommendation. Dilute the lysate 1:5 in TE and use 2.5 µl of the diluted lysate.

1.3. Gently mix the reaction. Collect all the liquid to the bottom of the tube with a brief spin. Transfer the tubes to a thermocycler and use the following conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	2 minutes	
Denaturation	98°C	10 seconds	35
Annealing	50–72°C*	5 seconds	
Extension (for 500–700 bp)	72°C	30 seconds	
Final Extension	72°C	2 minutes	
Hold	4–10°C		

* Please visit tmcalculator.neb.com to determine correct annealing temperature.

2. Heteroduplex DNA formation and digestion with Authenticase.

The products of the PCR reaction must be denatured and re-annealed to allow formation of heteroduplexes between PCR products with and without mutations. A rapid qualitative analysis can be done by reannealing unpurified PCR amplicons followed by Authenticase digestion. An existing protocol (https://www.neb.com/protocols/2016/03/17/t7-endonuclease-i-mutation-detection-with-the-engen-mutation-detection-kit-e3321) can be slightly modified to include replacing T7 Endo I with Authenticase using a 42°C reaction temperature and incubation time of 15 minutes.

Optional: If the efficiency of genome editing will be calculated, NEB recommends purification of the reactions prior to fragment analysis. One can either use enzymatic treatment with Thermolabile Exonuclease I (NEB #M0568) and Quick CIP (NEB #M0525) to remove remaining primers and dNTPs or a spin column (e.g., Monarch PCR & DNA Cleanup Kit (5 µg) –NEB #T1030) to purify the annealed dsDNA.

Method A: PCR amplicons from genome of edited cells

This method only requires PCR amplicons from the genome of edited cells to proceed.

2A.1 DNA cleanup

It is critical to accurately quantitate DNA concentrations used as input for the mismatch detection assay (Step 2A.3.). To prepare DNA for accurate quantitation, PCR reactions can be cleaned up by an enzymatic method (Step 2A.1.1.) or a column method (Step 2A.1.2.) prior to preparation of heteroduplex DNA formation.

2A.1.1. Enzymatic cleanup method

2A.1.1.1. Measure the dsDNA concentration by Qubit® DNA quantification method.

2A.1.1.2. Prepare samples for enzymatic cleanup and heteroduplex DNA formation.

REAGENT	REACTION
PCR reaction (unpurified)	18 µl
Thermolabile Exonuclease I (NEB #M0568)	1 µl
Quick CIP (NEB #M0525)	1 µl
Total Volume	**20 µl**

2A.1.1.3 Briefly spin down all tubes and incubate reactions at 37°C for 4 minutes followed by 80°C for 1 minute. Proceed to Step 2A.2. directly to generate heteroduplex DNA.

2A.1.2. Column cleanup method

2A.1.2.1. Clean up PCR products using Monarch PCR & DNA Cleanup Kit (5 µg – NEB #T1030) with elution volume of 12 µl. Measure the dsDNA concentration.

2A.1.2.2. Prepare annealing reaction with cleanup DNA.

REAGENT	REACTION
Cleanup PCR amplicons (400 ng)	1–16 µl
5X Annealing Buffer	4 µl
Nuclease-free water	to 20 µl

2A.1.2.3 Go directly to Step 2A.2. to generate heteroduplex DNA.

2A.2. Heteroduplex DNA formation

2A.2.1. Generate heteroduplex DNA products in a thermocycler using the following program:

CYCLE STEP	TEMP	RAMP RATE	TIME
Initial Denaturation	95°C		3 minutes
Annealing	95–85°C	2°C/second*	
	85–25°C	–0.1°C/second*	
Hold	4°C		

* Alternatively, if a thermocycler is not available with these ramp speeds, the sample can be heated to 95°C for 10 minutes and then allowed to cool slowly to room temperature.

2A.3. Digestion of heteroduplex DNA with Authenticase

2A.3.1. Digest heteroduplex DNA with Authenticase as follows:

REAGENT	ENZYMATIC-CLEANUP AMPLICONS		COLUMN-PURIFIED AMPLICONS	
	Reaction	Negative Control	Reaction	Negative Control
Annealed PCR amplicons (~200 ng)	1–12 µl*	1–12 µl*	10 µl	10 µl
10X Reaction Buffer	2 µl	2 µl	2 µl	2 µl
Authenticase	1 µl	0 µl	1 µl	0 µl
Nuclease-free water	16–5 µl	17–6 µl	7 µl	8 µl
Total Volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**

* The digestion reaction conditions have been optimized for up to 6 µl of the unpurified enzyme-treated Q5 Master Mix PCR reaction or 12 µl of unpurified One*Taq* PCR reaction containing up to 200 ng of amplified DNA. Increased amounts of PCR reaction and/or DNA may lead to inaccurate estimates of editing efficiencies.

2A.3.2. Mix well and briefly spin. Incubate each reaction at 42°C for 15 minutes. Stop the reaction with 1.7 µl of 150 mM EDTA. Proceed with fragment analysis or store at –20°C until ready.

Method B: PCR amplicons from genome of edited cells and WT cells

This method requires both PCR amplicons from the genome of edited cells and WT cells to proceed.

2B.1. DNA cleanup

Amount of DNA concentration is essential in the mismatch detection assay. PCR reactions can be cleaned up by an enzymatic method (Step 2B.1.1.) or a column method (Step 2B.1.2.) prior to preparation of heteroduplex DNA formation.

2B.1.1. Enzymatic cleanup method

2B.1.1.1. Measure the PCR amplicons concentration by Qubit® DNA quantification method.

2B.1.1.2. Prepare samples for enzymatic cleanup and heteroduplex DNA formation.

REAGENT	REACTION A	REACTION B
PCR amplicons from edited genome	18 µl	
PCR amplicons from WT genome		18 µl
Thermolabile Exonuclease I (NEB #M0568)	1 µl	1 µl
Quick CIP (NEB #M0525)	1 µl	1 µl
Nuclease-free water	to 20 µl	to 20 µl

2B.1.1.3. Briefly spin down all tubes and incubate reactions at 37°C for 4 minutes followed by 80°C for 1 minute.

2B.1.1.4. Prepare samples to make heteroduplex DNA by mixing 200 ng of reaction A (from edited gDNA template) and 200 ng of reaction B (from WT gDNA template). Assemble the reaction as follows and go to step 2B.2.:

REAGENT	REACTION
Enzymatic cleanup PCR samples from edited cells (200 ng)	X µl
Enzymatic cleanup PCR samples from WT cells (200 ng)	Y µl
Total Volume	**X + Y µl**

2B.1.1.5 Go to step 2B.2. to generate heteroduplex DNA.

2B.1.2. Column cleanup method

2B.1.2.1. Clean up PCR products using Monarch PCR & DNA Cleanup Kit (5 µg – NEB #T1030) with elution volume of 12 µl. Measure the dsDNA concentration.

2B.1.2.2. Prepare annealing reaction with cleaned up DNA.

REAGENT	REACTION
Column cleanup PCR samples from edited cells (200 ng)	X µl
Column cleanup PCR samples from edited cells (200 ng)	Y µl
5X Annealing Buffer	4 µl
Nuclease-free water	to 20 µl

2B.1.2.3. Go to step 2B.2. to generate heteroduplex DNA.

2B.2. Heteroduplex DNA formation

2B.2.1. Generate heteroduplex DNA products in a thermocycler using the following program:

CYCLE STEP	TEMP	RAMP RATE	TIME
Initial Denaturation	95°C		2 minutes
Annealing	95–85°C	2°C/second*	
	85–25°C	–0.1°C/second*	
Hold	4°C		

* Alternatively, if a thermocycler is not available with these ramp speeds, the sample can be heated to 95°C for 10 minutes and then allowed to cool slowly to room temperature.

2B.3. Digestion of heteroduplex DNA with Authenticase

2B.3.1. Digest heteroduplex DNA with Authenticase as follows:

REAGENT	ENZYMATIC-CLEANUP AMPLICONS		COLUMN-PURIFIED AMPLICONS	
	Reaction	Negative Control	Reaction	Negative Control
Annealed PCR amplicons (~200 ng)	(X + Y)/2 µl*		10 µl	
Annealed PCR product (~200 ng)		(X + Y)/2 µl*		10 µl
10X Reaction Buffer	2 µl	2 µl	2 µl	2 µl
Authenticase	1 µl	0 µl	1 µl	0 µl
Nuclease-free water	17 – (X + Y)/2 µl	18 – (X + Y)/2 µl	7 µl	8 µl
Total Volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**

* The digestion reaction conditions have been optimized for up to 6 µl of the unpurified enzyme-treated Q5 Master Mix PCR reaction or 12 µl of unpurified One*Taq* PCR reaction containing up to 200 ng of amplified DNA. Increased amounts of PCR reaction and/or DNA may lead to inaccurate estimates of editing efficiencies.

2B.3.2. Mix well and briefly spin. Incubate each reaction at 42°C for 15 minutes. Stop the reaction with 1.7 µl of 150 mM EDTA. Proceed with fragment analysis or store at –20°C until ready.

3. Analyze DNA fragments.

3.1 Gel or Fragment Analysis

3.1.1. Add 4 µl of Gel Loading Dye, Purple (6X, NEB #B7024) to the reaction and run on a 2% agarose gel stained with ethidium bromide.

3.1.2. Run the included DNA ladder or an appropriate DNA size marker alongside the sample for reference. Alternatively, samples can be analyzed using a fragment analyzer (e.g., Agilent Bioanalyzer or Advanced Analytical Technologies, Inc. (AATI) Fragment Analyzer). For the Agilent Bioanalyzer, remove 2 µl of enzyme-treated sample and mix 8 µl of water.

3.1.3. Analyze 1 µl of the 5X diluted sample on a high sensitivity Agilent DNA chip. This allows for detection of mutation populations down to 1 out of 80 copies based on 700 bp PCR amplicon design with cleaved product sizes of 450 bp and 250 bp. For the AATI Fragment Analyzer, 2 µl of the reaction can be used with the Standard Sensitivity NGS Fragment Analysis Kit (AATI Cat# DNF-473).

3.2 Efficiency Calculation

3.2.1. Calculate the estimated % modification using the following formula:

Method A

% Modification = 100 x [1 – ($\sqrt{1 - \text{fraction cleaved}}$)]

Method B

% Modification = {100 x [1 – $\sqrt{1 - \text{fraction cleaved}}$]} x 2

When calculating % modification for reactions with the control template where the starting material (S) is known, the equation (100 x fraction cleaved) can be used, where fraction cleaved (also known as % heteroduplex in Figure 4) = molarity of digested products (P1 and P2*)/(molarity of digested products + molarity of undigested band).**

*** Note: P2 does not factor into calculation.**

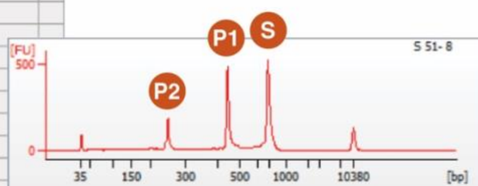
** Reference: Sentmanat, M.F., et al. (2018) *Sci Rep.* 8(1), 888.

Example of Calculation of Data from Bioanalyzer Results

An example of % efficiency sample data:

% Modification = 100 x [1 – ($\sqrt{1 - \text{P1}/(\text{P1} + \text{S})}$] = 100 x [1 – ($\sqrt{1 - 1728/(1728 + 1436)}$] = **32.6%**

		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125.00	5,411.3	Lower Marker
2		142	20.31	217.2	
3		151	7.99	80.2	
4		168	7.27	65.7	
5		175	16.05	138.8	
6		204	41.03	305.1	
7		216	22.38	156.7	
8		244	42.36	262.7	
9		251	228.18	1,378.0	P2
10		290	8.50	44.4	
11		306	15.55	77.1	
12		339	9.26	41.4	
13		350	9.10	39.4	
14		389	25.84	100.7	
15		414	19.85	72.6	
16		447	509.56	1,727.8	P1
17		489	40.62	125.8	
18		523	14.46	41.8	
19		582	34.71	90.4	
20		683	647.42	1,436.4	S
21	▶	10,380	75.00	10.9	Upper Marker
22		12,010	0.00	0.0	



Supplemental Protocols

Supplemental Protocol 1:
Generation of DNA fragments by PCR assembly of pooled oligos

Each lab will have their own preference for how to produce genes of interest that can be further processed by Authenticase for error correction. If you desire to take a "DIY" approach and choose to design oligos on your own followed by PCR to amplify the gene of interest, the following protocol may be helpful. Steps 1 and 2 are suggestions to generate PCR fragments less than 1 kb from your oligonucleotide design (each ≤ 60-mer) (Figure 6).

1. Convert gene of interest into oligonucleotides less than 60 nt.

1.1. Encode gene of interest in DNA manipulation software and break up into oligos of 60 nt or less.

1.2. Order oligos from your preferred vendor or synthesize them in-house.

1.3. Adjust each oligo to 10 µM to facilitate downstream processes.

2. Prepare gene-specific oligo pool.

2.1. Transfer 5 µl of each oligo (10 µM) to a low bind microcentrifuge tube to form a pool of oligos encoding the gene of interest. Add nuclease-free water to a final volume of 500 µl (100 fmol/µl).

REAGENT	REACTION	FINAL RXN CONC.
Oligo 1.1 (10 µM stock)	5 µl	
Oligo 1.2 (10 µM stock)	5 µl	
…		
Oligo 1.x (10 µM stock)	5 µl	
Nuclease-free water	to 500 µl	100 nM of each oligo

2.2. Setup PCR reaction:

REAGENT	REACTION	FINAL RXN CONC.
Q5 Hot Start High-Fidelity 2X Master Mix	25 µl	1X
10 µM Forward Primer	2.5 µl	0.5 µM
10 µM Reverse Primer	2.5 µl	0.5 µM
Template DNA (pooled oligos from 2.1)	5 µl	500 fmol of each oligo
Nuclease-free water	to 50 µl	

2.3. Setup PCR reaction conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	2 minutes	
Denaturation	98°C	10 seconds	36
Annealing	60–64°C*	10 seconds	
Extension (for 500–700 bp)	72°C	40 seconds	
Final Extension	72°C	5 minutes	
Hold	4–10°C		

* Please visit tmcalculator.neb.com to determine correct annealing temperature.

Supplemental Protocol 2:
Using colony PCR to identify positive clones

1. Identify colonies.

1.1. Identify colonies containing full length of gene of interest without overnight culturing, purification of plasmid DNA, and restriction digest or Sanger sequencing. Pick up 6 colonies from the outgrowth plate, replica plate onto appropriate selective plate, and transfer remaining colony into individual PCR tubes containing the following components:

REAGENT	REACTION	FINAL RXN CONC.
One*Taq* Hot Start Quick-Load 2X Master Mix	12.5 µl	1X
10 µM Forward Primer	0.5 µl	0.2 µM
10 µM Reverse Primer	0.5 µl	0.2 µM
Nuclease-free water	11.5 µl	
Colony	1 colony	

2. Setup PCR reaction conditions:

2.1. PCR reaction conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	95°C	5 minutes	
Denaturation	95°C	15 seconds	33
Annealing	55–64°C*	15 seconds	
Extension (for 500–1,000 bp)	72°C	30–60 seconds	
Final Extension	72°C	5 minutes	
Hold	4°C		

* Please visit tmcalculator.neb.com to determine correct annealing temperature.

3. Identify positive clones.

3.1. Examine the size of PCR amplicons (5–10 µl) on an agarose gel. Select 4 amplicons with the anticipated size of gene of interest to proceed.

3.2. Clean up these reactions using Exo-CIP Rapid PCR Cleanup Kit (NEB #E1050) or go back to the replica plate (after overnight incubation) to start cultures for miniprep DNA preparation (e.g., Monarch PCR & DNA Cleanup Kit (5 µg – NEB #T1030)).

3.3. Submit Exo-CIP treated PCR reactions or plasmid DNA for Sanger sequencing with appropriate primers.

Ordering Information

NEB #	PRODUCT	SIZE
M0689S/L	Authenticase	25/125 reactions

COMPANION PRODUCTS

NEB #	PRODUCT
M0494	Q5 Hot Start High-Fidelity Master 2X Mix
M0568	Thermolabile Exonuclease I
M0525	Quick CIP
T1030	Monarch PCR & DNA Cleanup Kit (5 µg)
E5520	NEBuilder HiFi DNA Assembly Cloning Kit
N3234	PCR Marker
M0486	One*Taq*® Quick-Load 2x Master Mix with Standard Buffer
B1500	Nuclease-free Water

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	8/23

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