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2010

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Chapter

6

Gene modification in embryonic stem cells by single-stranded DNA oligonucleotides

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Methods Mol Biol. (2009) **530**, 79-99.

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Oligonucleotide-mediated gene targeting is an attractive alternative for current procedures to subtly modify the genome of mouse embryonic stem (ES) cells. However, oligonucleotide-directed substitution, insertion or deletion of a single or a few nucleotides was hampered by DNA mismatch repair (MMR). We have developed strategies to circumvent this problem based on findings that the central MMR protein MSH2 acts in two different mismatch recognition complexes: MSH2/MSH6, which mainly recognizes base substitutions and MSH2/MSH3, which has more affinity for larger loops. We found that oligonucleotide-mediated base substitution could effectively be obtained upon transient suppression of MSH2 protein level, while base insertions were effective in ES cells deficient for MSH3. This method allows substitution of any codon of interest in the genome.

1. INTRODUCTION

Knockout mice are invaluable for the study of gene function and have provided useful models for human genetic disorders. Thus far, the vast majority of mutant mice has been generated by "gene disruption" through homologous recombination in embryonic stem (ES) cells [1]. However, for more accurate mimicking of human disease and in view of the modular nature of many proteins that are composed of separate domains, each responsible for specific interactions and functions, there is an urgent need for "subtle" mutants that affect only one function of a protein but leave others intact.

Current procedures for subtle modification of the ES cell genome are costly and time-consuming. Briefly, in a two-step protocol, homologous recombination is used to introduce the subtle mutation into the gene of interest concomitantly with a dominantly/negatively selectable marker gene, the latter subsequently being removed via intra-chromosomal homologous or Cre/lox-mediated site-specific recombination [2,3].
An alternative approach to introduce subtle gene modifications may be the use of synthetic single-stranded DNA oligonucleotides (ssODNs). Since this procedure has been proven successful in the yeast *Saccharomyces cerevisiae* [4] and in human cells [5], extensive efforts have been made to establish reliable protocols for ssODN-directed gene modification that can eventually be applied in human gene therapy. Thus far, these procedures made use of chemically-modified ssODNs, chimeric RNA/DNA oligonucleotides or triple-helix-forming oligonucleotides [6-9], all containing phosphorothioate linkages or 2'-*O*-methyl-RNA residues to protect oligonucleotides from intracellular nucleolytic degradation [10]. Several reports have addressed the relevance of transcription [11,12], DNA replication [13], homologous recombination [14] and DNA damage repair [15,16], however, the mechanism of transfer of genetic information from the oligonucleotide to the target remains largely elusive.

Several years ago, we have demonstrated the feasibility of subtle gene modification in ES cells by non-chemically-modified ssODNs [17]. However, we found that "oligo-targeting" was strongly suppressed by DNA mismatch repair (MMR) activity. As a readout for oligo-targeting, we used inactive neomycin resistance (*neo*) genes carrying either a mutation in the start codon (AAG)

or a 2 bp frameshift mutation (extra GT) disrupting the open reading frame. Single copies of these reporters had been inserted into the *Rosa26* locus of wild-type and *Msh2*-deficient (*Msh2*$^{-/-}$) ES cells. The latter cells lack the mismatch recognition protein MSH2 and are completely devoid of MMR activity [18]. We found that ssODNs of ±35 residues substituting, deleting or inserting 1- or 2-nt could restore *neo* activity (giving G418-resistance) in *Msh2*$^{-/-}$ ES cells with a frequency of 1-7 per 10^5 cells [17]. In wild-type cells, MSH2 activity suppressed single-nucleotide substitution 150-fold and single-nucleotide insertion over 700-fold. Thus, oligo-targeting was only effective in the absence of MMR [17], an observation that has been confirmed in *Escherichia coli* [19]. Unfortunately, constitutive MMR deficiency leads to numerous inadvertent mutations, hampering the general application of ssODN-directed gene modification.

To circumvent this problem we have exploited previous findings that MSH2 acts in two different mismatch recognition complexes, MSH2/MSH6 and MSH2/MSH3 [20]. MSH2/MSH6 mainly recognizes base substitutions and small loops of one or two nucleotides, while the MSH2/MSH3 complex has more affinity for larger loops of unpaired bases.

Gene knockout [21]. By comparing the efficacy of a large series of insertion ssODNs, we found that 4 nt insertions are predominantly recognized by the MSH2/MSH3 dimer. Occasionally, a 4 nt insertion was suppressed by MSH2/MSH6 activity. We found a simple rule for minimizing recognition by MSH2/MSH6 and hence achieving effective 4 nt insertions in *Msh3*$^{-/-}$ cells: the 4 nt insertion must not be capable of forming base pairs with the complementary strand and should be placed between G or C residues. As *Msh3*$^{-/-}$ cells do not show an overt mutator phenotype and *Msh3*-deficient mice do not develop cancer, these cells may become the routine target cells for ssODN-mediated *gene disruption*. *E.g.*, we have used *Msh3*$^{-/-}$ ES cells to generate TAAA insertions in the Fanconi anemia *Fancf* gene and the Roberts syndrome gene *Esco2*.

Codon substitution [22]. Base substitutions were recognized by both heterodimers. We therefore developed a procedure to transiently suppress the level of MSH2 in wild-type cells by RNA interference. By expression of a short-hairpin RNA sequence against *Msh2* from a transiently transfected pSUPER vector (pS-MSH2), we achieved 10-fold reduction of MSH2 level for approximately three days after which MSH2 level raised back to wild-type. During this period cells were permissive for ssODN-mediated substitution of 4 nt reaching frequencies of 60-80% of the levels in *Msh2*$^{-/-}$ cells. Simple nucleotide substitutions were still largely suppressed as was spontaneous mutagenesis. These results indicate that upon reduction of MSH2 protein level by RNA interference residual MMR activity persists. This activity largely suppresses spontaneous mutagenesis, but is permissive for 4 nt substitutions, allowing the replacement of virtually every codon in the genome. We recently found that also transient suppression of the MMR gene *Mlh1* using vector pS-MLH1 renders cells permissive for oligo-targeting. We have successfully applied the MSH2 or MLH1 knockdown strategy to substitute codons in *Rb*, *Msh2* and *p53*.

Mouse mutants [22]. RB(N750F) ES cells were used to generate the first mutant mouse line that was created via ssODN-mediated gene targeting. Similarly, we generated mouse lines from *Fancf*^{TAAA} and *Esco2*^{TAAA} mutant ES cells, demonstrating the general applicability of ssODN-mediated gene modification for the generation of mutant mouse lines.

2. MATERIALS

ES cells are routinely cultured on irradiated primary mouse embryonic fibroblasts (MEFs). However, during transfections and drug selections, cells are cultured on gelatin-coated plates in buffalo rat liver (BRL)-conditioned medium. This is because MEFs or other feeder layers decrease the transfection efficiency of the ES cells by taking up a large proportion of the transfected DNA. In addition, feeder cells may not tolerate the high concentrations of selective drugs that are used. Protocols for the preparation of MEF feeder layers and culturing of ES cells are given in reference [23], while the use of BRL-conditioned medium is described in reference [24]. This chapter provides protocols for ssODN-mediated gene targeting and subsequent subcloning of mutant ES cells. Figure 1 shows a flow diagram of the procedure. Fetal bovine serum (FBS) used for ES cell culture must not contain any components that promote differentiation and/or inhibit proliferation, enabling ES cells to retain their totipotent state to generate germ line chimeras.

2.1. Media and Solutions

1. CM (complete medium): 500 mL Glasgow Minimal Essential Medium (GMEM) (GIBCO/Invitrogen, 21710) supplemented with 10% fetal bovine serum (FBS, HyClone), 1 mM sodium pyruvate (100 mM stock, GIBCO/Invitrogen, 11360), 1X non-essential amino acids (100X stock, GIBCO/Invitrogen, 11140).
2. CM+β+LIF (leukaemia inhibitory factor): 100 mL CM, 0.1 mL β-mercaptoethanol (1000x), 1 mL LIF (100X).
3. β-Mercaptoethanol (1000x, 0.1M): 0.1 mL 2-mercaptoethanol (14.2 M, Merck, 1.15433) and 14.1 mL water. Sterilize by filtration through 0.22 µm Millex-GV filter. Store at 4°C for up to one month. Working concentration: 0.1 mM.
4. LIF (100X): dissolve 1 mL ESGRO® (10^7 Units, Chemicon International, ESG1107) in 99 mL CM. Working concentration: 10^3 U/mL. Store at 4°C.
5. BRL medium: CM conditioned on a monolayer of buffalo rat liver (BRL-3A) cells, filtered through a 0.2 µm filter unit (Nalgene, 450-0020).
6. BRL+β+LIF (60% medium): 100 mL CM, 150 mL BRL medium, 1.5 mL L-glutamine 200 mM (GIBCO/Invitrogen, 25030), 0.25 mL β-mercaptoethanol (1000X), 2.5 mL LIF (100x).
7. Distilled water: 500 mL bottles (GIBCO/Invitrogen, 15230).
8. 1% Gelatin (10X): 5 g (w/v) gelatin (Sigma, G1890) in 500 mL water. Gently heat in microwave oven (do not boil). Sterilize through a 0.2 µm filter unit (Nalgene, 450-0020) while still warm and store aliquots at 4°C. Before use, dilute to 0.1% with water and coat tissue culture dishes for 30 min at RT.
9. PBS (phosphate-buffered saline): Dulbecco's PBS (1X) without calcium and magnesium, 500 mL bottles (GIBCO/Invitrogen, 14190).

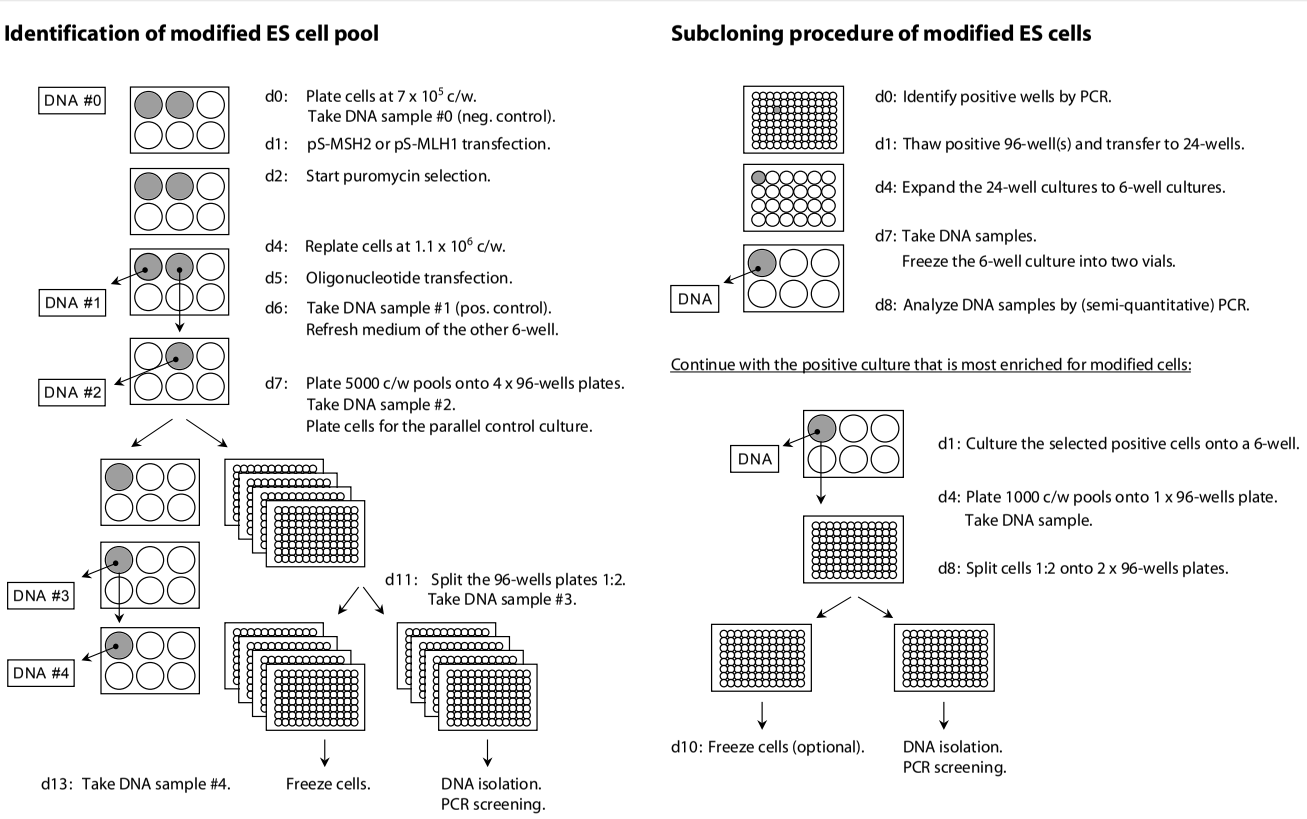

Figure 1 Flow diagram of oligo-targeting procedure and subcloning of modified cells.

10. Trypsin/EDTA stock (TVP): 500 mL PBS supplemented with 5 mL Trypsin 2.5% (GIBCO/Invitrogen, 15090), 12.5 mL 40 mM ethylene diamine tetraacetic acid (EDTA), 5 mL chicken serum (GIBCO/Invitrogen, 16110). Sterilize by filtration through a 0.2 µm filter unit (Nalgene, 450-0020) and store aliquots at -20°C.
11. 2X TVP: add 0.1 mL Trypsin 2.5% to 9.9 mL TVP. Store at -20°C.
12. 10X TVP: add 0.9 mL Trypsin 2.5% to 9.1 mL TVP. Store at -20°C.
13. Puromycin (100X): dissolve 20 mg (w/v) puromycin (Sigma, P7255) in 10 mL CM. Sterilize through a 0.22 µm filter and store aliquots at -20°C.
14. Targeting oligonucleotide (ssODN): unmodified 40-mer synthetic DNA oligonucleotides, deprotected and desalted (Sigma-Aldrich), reconstituted in sterile PBS at 1 µg/µl.
15. TransFast Transfection Reagent (Promega, E2431): add 400 µl nuclease-free water per vial, vortex vigorously for 10 s and store at -20°C for 16-24 h before use.
16. Lysis Buffer: 100 mM Tris pH 8.0, 5.0 mM EDTA, 0.2% (w/v) sodium dodecyl sulphate (SDS), 200 mM NaCl, 200 µg/mL (freshly added) Proteinase K (Merck, 24568.0100).
17. DirectPCR Lysis Reagent for cells (Viagen Biotech, 302-C).
18. $T_{10}E_{0.1}$ buffer: 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0).
19. PCR primers: 100 µM in nuclease-free water.
20. Taq DNA polymerase: 5 U/µL.
21. 10 mM dNTP mix: dilute each dNTP (dGTP, dATP, dCTP, dTTP) in nuclease-free water to 10 mM and store aliquots at -20°C.
22. 50X TAE: Dissolve 242 g Tris-base, 16.81 g EDTA, 20.5 g sodium acetate in 1 liter distilled water. Adjust to pH 7.9 with acetic acid.
23. Agarose gels: melt 3% (w/v) multi purpose agarose (Roche) in 0.5X TAE by gently boiling in a microwave oven. Cool until hand warm, add ethidium bromide and pour into prepared gel former. Run gels at 80-100 V.

2.2. General Equipment

1. Tissue culture plates:
   a. 96-well microwell plates, flat bottom, 0.3 cm^2 (Costar, 3596).
   b. 24-well microwell plates, 1.8 cm^2 (Costar, 3527).
   c. 6-well microwell plates, 10 cm^2 (Costar, 3506).
   d. 10 cm TC Petri dishes (Falcon, 353003).
2. Sterile 1, 2, 5, 10 and 25 mL plastic pipettes.
3. Sterile 10, 20, 200 and 1,000 µL filter tips.
4. Sterile 15 and 50 mL tubes.
5. 1.8 mL CryoTube vials with external thread (Nunc, 375418).
6. 12-well multichannel pipette.
7. VACUBOY hand operator (INTEGRA Biosciences, 155 500) with 8-channel plastic aspiration adapter for disposable tips with ejector (INTEGRA Biosciences, 155 520).
8. 50 mL disposable polystyrene reagent reservoirs (Costar, 4871).

9. 96-tube freezing boxes (Greiner, 975561) with 1.3 mL polypropylene freezing tubes with attached strip caps, 8.5 x 44 mm (Greiner, 102261).
10. Inverted microscope for routine morphological analysis of ES cells.
11. CASY 1 cell counter (Schärfe System, model TT or DT).
12. Humidified tissue culture incubator maintained at 37°C and 5% CO_2.
13. Tissue culture laminar flow cabinet.
14. Water bath: 37°C.
15. Freezers: -20°C and -80°C.
16. Liquid nitrogen container.
17. DNA thermal cycler (MJ Research, PTC-200).
18. Electrophoresis equipment (Bio-Rad).

3. METHODS

3.1. Design of Targeting Oligonucleotide

1. Typical targeting ssODNs consist of ~40 nucleotides. The center of the ssODN contains 3-4 bases that comprise the desired genetic alteration (substitution or insertion of nucleotides); the remainder is identical to the gene of interest (*see* examples in **Figure 2B**).
2. The 5' and 3' homologous arms are 18 nt in length.
3. Successful gene targeting experiments require optimal sequence identity between the targeting ssODN and the genomic DNA sequence. For ssODN design, ideally use genomic DNA sequence information derived from the same mouse strain as the ES cells (in our case: mouse strain 129Ola). However, this information is often not available.
4. Upon alignment of the targeting ssODN with its complementary genomic sequence, mismatches will be formed. If possible, avoid creating mismatches that are well recognized by the mismatch repair system, such as G/T mismatches.
5. By creating 4 nt substitutions, virtually every codon in the mouse genome can be replaced based on the redundancy of the genetic code. However, a different codon usage of the novel codons may affect expression of the target gene. Try to choose codons with high codon usage (*see* www.kazusa.or.jp/codon).
6. Oligonucleotides can be designed in the *sense* or *anti-sense* orientation, *i.e.*, complementary or identical to the transcribed strand of the target gene (*see* **Note 1**).
7. Substitution ssODNs: choose an alteration that affects four adjacent bases (MMMM; M indicates mutation), three adjacent bases (MMM), or non-adjacent bases like MxMM (**Figure 2B**).
8. Insertion ssODNs: the 4 nt insertion must not be capable of forming base pairs with the complementary strand and should be placed between G or C residues (**Figure 2B**).

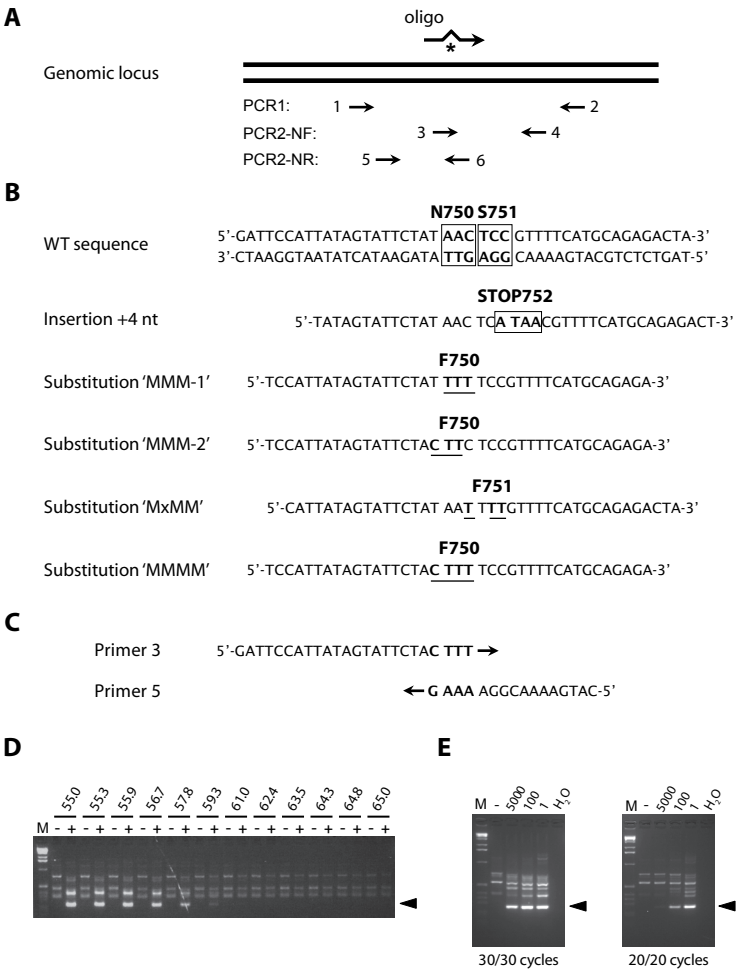

Figure 2 Targeting ssODN and PCR primer design.
*(**A**) Schematic representation of PCR-based detection of modified cells. A single-stranded DNA oligonucleotide (ssODN) is designed to introduce a genomic modification in any given gene. Primer pair 1/2 is used to pre-amplify a DNA fragment surrounding the modification. This fragment is the template in PCR2 using nested primer pairs 3/4 or 5/6 of which primers 3 and 6 are specific for the mutation. Asterisk indicates modification. Arrows indicate the location and direction of PCR primers. NF, nested forward; NR, nested reverse. (**B**) Genomic DNA sequence and possible targeting ssODNs to introduce a stop codon (Insertion +4 nt), a substitution of codon 750 (Substitution MMM-1, MMM-2, MMMM), or codon 751 (Substitution MxMM). M indicates altered nucleotide. (**C**) Sequence of mutation-specific primers 3 and 6 for detection of substitution 'MMMM'. (**D**) Gradient PCR to optimize the annealing temperature of PCR2. Genomic DNA from untransfected ES cells (DNA #0) is used as negative control (-), whereas sample DNA #1 is used as positive control (+). Temperatures are shown above the lanes. In this example, annealing at 56°C is optimal for screening. Arrowhead indicates mutation-specific band. Lane M, molecular mass standards. (**E**) Semi-quantitative PCR analysis of positive cell cultures from subsequent screening rounds to show enrichment for modified cells. PCR amplification of genomic DNA from 5,000, 100 and 1 cells/well cultures using 30 cycles (left panel) or 20 cycles (right panel) in both PCR1 and PCR2. Arrowhead indicates mutation-specific band. Lane M, molecular mass standards. (-), Genomic DNA from untransfected ES cells.*

3.2. Generation of Codon Substitutions in Wild-Type ES Cells

*3.2.1. Knockdown of Msh2 or Mlh1 in Wild-Type ES Cells (see **Note 2**)*

Day –4

1. Gelatinize one well of a 6-well plate.
2. Thaw irradiated feeder MEFs in a 37°C water bath and resuspend in 10 mL CM.
3. Spin for 5 min at 300*g*, remove the supernatant and carefully resuspend the MEFs in CM by gentle pipetting.
4. Plate MEFs onto one 6-well (10 cm^2) plate. Feeder layer cells should be prepared at least 1-2 days before ES culture.

Day –3

1. Thaw ES cells in a 37°C water bath.
2. Spin the cells in 10 mL CM for 5 min at 300*g*, remove the supernatant and resuspend the cells in 3 mL CM+β+LIF.
3. Plate ES cells onto one 6-well plate with MEFs at a density of approximately 2 x 10^6 cells per well.

Day 0

1. Wash the cells with PBS and trypsinize with 0.5 mL 10X TVP for 5 min at 37°C (*see* **Note 3**). Add 0.5 mL BRL+β+LIF (60% medium) and resuspend by pipetting.
2. Count the number of cells.
3. Plate cells onto two gelatin-coated 6-well plates at a density of 7 x 10^5 per well in 3 mL BRL+β+LIF (60% medium) (*see* **Note 4**).
4. Take 1 x 10^6 cells for DNA isolation (DNA #0, negative control for PCR) (*see* **Section 3.4.3**).
5. Dissolve the pS-MSH2 or pS-MLH1 vector in sterile PBS at 1 µg/µL. Store overnight at 4°C.
6. Thaw one vial of TransFast Transfection Reagent and add 400 µL nuclease-free water. Vortex vigorously and store at -20°C.

Day 1

1. Vortex TransFast Transfection Reagent before use.
2. For each well, add 3 µg of pS-MSH2 *or* pS-MLH1 vector and 27 µL of TransFast to 1.4 mL CM without FBS. Vortex the mixture and leave for 10-15 min at RT.
3. Wash the cells with PBS. Vortex the mixture again and add to the cells. Incubate for 75 min at 37°C/5% CO_2.
4. Add 4 mL BRL+β+LIF (60% medium) and incubate overnight at 37°C/5% CO_2.

Day 2

1. Prepare two gelatin-coated 6-well plates with 3 mL BRL+β+LIF (60% medium) + 40 µL puromycin (100X).
2. Trypsinize transfected ES cells and transfer 2 x 1 mL of cell suspension (*i.e.*, all cells) to two 6-well plates with selective medium (final concentration of puromycin is 20 µg/mL).
3. Incubate for 2 days.

3.2.2. Transfection with Targeting ssODN

Day 4

1. Wash the cells in the two 6-well plates twice with PBS and trypsinize.
2. Count the number of surviving cells (*see* **Note 5**).
3. Replate the ES cells onto two gelatin-coated 6-well plates at 1.1 x 10^6 cells/well in 3 mL BRL+β+LIF (60% medium) without puromycin (*see* **Note 6**).
4. Dissolve the targeting ssODN in sterile PBS at 1 µg/µL. Store overnight at -20°C.

Day 5

1. To transfect the two 6-well plates with targeting ssODN, add for each well 3 µg of targeting ssODN and 27 µL of TransFast (vortex before use) to 1.4 mL CM without FBS. Vortex the mixture and leave for 10-15 min at RT.
3. Wash the cells with PBS. Vortex the mixture again and add to the cells. Incubate for 75 min at 37°C/5% CO_2.
4. Add 4 mL BRL+β+LIF (60% medium) and incubate overnight at 37°C/5% CO_2.

Day 6

1. Trypsinize and count cells of one 6-well plate (*see* **Note 7**).
2. Take 1 x 10^6 cells for DNA isolation (*see* **Section 3.4.3**). This sample (= DNA #1) will be used as a positive control for PCR screening (*see* **Note 8**). Discard the remainder of the cells.
3. Refresh the medium of the other 6-well plate with BRL+β+LIF (60% medium).
4. Prepare 12 x 96-well plates with MEFs and 100 µL CM+β+LIF medium. Use a multichannel pipette when handling 96-well plates.

Day 7

1. Trypsinize and count ES cells.
2. Add 2 x 10^6 cells to 40 mL CM+β+LIF medium. Transfer 100 µL portions of cell suspension to 4 x 96-well plates with MEFs and 100 µL CM+β+LIF medium (5,000 cells/well).
3. Plate 1 x 10^6 cells onto one 6-well plate with MEFs. These cells are cultivated in parallel to the 96-well plates in order to monitor the degradation of unincorporated ssODNs.
4. Take 1 x 10^6 cells for DNA isolation (= DNA #2) (*see* **Section 3.4.3**).

Day 10

1. Refresh the medium of the 4 x 96-well plates with 5,000 cells/well.
2. Count the number of colonies in each 96-well to determine the plating efficiency (should be 500-800 colonies).
3. From the parallel 6-well plate with control cells, take 1 x 10^6 cells for DNA isolation (= DNA #3) (*see* **Section 3.4.3**) and transfer 1 x 10^6 cells to a fresh 6-well plate with MEFs.

Day 11

1. When ES colonies have reached ~90% confluency, split the 5,000 cells/well cultures into duplicates. Trypsinize only two plates at the same time to reduce the stress of trypsinization.
2. Wash the wells with 100 µL PBS and trypsinize with 25 µL 10X TVP for 5 min at 37°C/5% CO_2.
3. Add 175 µL CM+β+LIF and resuspend.
4. Transfer two 100 µL portions of cell suspension to two new 96-well plates with MEFs and 100 µL CM+β+LIF medium.
5. Label the side of each plate so that lids and bases of the plates are both identifiable: four master plates for freezing the cells and four duplicates for DNA isolation. This minimizes confusion during freezing and PCR analysis.

Day 12

1. Refresh the medium of the 8 x 96-well plates with 200 µL CM+β+LIF medium.

3.2.3. Freezing of 96-Well Plates

Day 13

1. Prepare CM (without LIF) with 11.43% dimethylsulfoxide (DMSO) at 4°C.
2. Wash cells of the master plates with PBS and trypsinize with 25 µL 10X TVP for 5 min at 37°C/5% CO_2. When freezing 4 x 96-well plates, trypsinize and process only two plates at the same time to reduce the stress of trypsinization and DMSO.
3. Add 175 µL CM plus 11.43% DMSO and resuspend carefully (final concentration: 10% DMSO).
4. Transfer 200 µL of trypsinized cells to individual tubes of a 96-tube freezing box (Greiner, 975561). Store cells in freezing boxes on ice until all plates have been prepared for freezing (*see* **Note 9**).
5. Freeze the cells by a rate controlled cooling program and store them in liquid nitrogen. Alternatively, wrap the freezing boxes in paper towels and place them overnight at -80°C. Transfer to liquid nitrogen the next day.
6. Isolate DNA from the four duplicate plates (*see* **Section 3.4.1** or **3.4.2**).
7. From the parallel 6-well plate with control cells, take 1 x 10^6 cells for DNA isolation (= DNA #4) (*see* **Section 3.4.3**). Discard the remainder of the cells.

Day 14

1. Identify wells containing mutated cells in the 4 x 96-well DNA plates by PCR1 and PCR2 (*see* **Section 3.4**).

3.3. Generation of Gene Knockouts in Msh3-Deficient ES Cells

1. Plate thawed *Msh3*-deficient ES cells onto MEFs in 3 mL CM+β+LIF at a density of approximately 2 x 10^6 cells per well. Cultivate the cells to (semi)confluency for 3 days.
2. Wash cells with PBS and trypsinize with 0.5 mL 10X TVP for 5 min at 37°C/5% CO_2. Add 0.5 mL BRL+β+LIF (60% medium) and

resuspend by pipetting. Count the number of cells.

3. Replate the ES cells onto two gelatin-coated 6-well plates at 9 x 10^5 cells/well in 3 mL BRL+β+LIF (60% medium).
4. Take 1 x 10^6 cells for DNA isolation (DNA #0, negative control for PCR) (*see* **Section 3.4.3**).
5. Dissolve the targeting ssODN in sterile PBS at 1 µg/µL. Store overnight at -20°C.
6. The following day, proceed with transfection of the targeting ssODN as described above (*see* **Section 3.2.2**, Day 5).

3.4. Identification of Positive Clones by PCR Analysis

3.4.1. Genomic DNA Isolation from 96-Well Plates (Method 1)

1. Remove culture medium by inverting the 96-well plate. Drain the remaining fluid on a tissue. Optional: cells can be stored dry in a 96-well plate at -20°C.
2. Add 90 µL DirectPCR Lysis Reagent (Viagen Biotech, 302-C) supplemented with 300 µg/mL Proteinase K to wells.
3. Carefully seal the 96-well plates with adhesive covers to prevent evaporation and incubate at least 2 h at 55°C.
4. Heat inactivate for 45 min at 85°C. Lysates are now ready for use in PCR. Mix lysates well by pipetting up and down several times.
5. Store lysates at -20°C (before use in PCR, thaw for 15 min at 85°C).

3.4.2. Genomic DNA Isolation from 96-Well Plates (Method 2)

1. Add 50 µL lysis buffer with 200 µg/mL Proteinase K to dry wells.
2. Carefully seal the 96-well plate and incubate for at least 2 h at 55°C.
3. Add 100 µL of 100% ethanol to the lysates. Seal the plate with silicone sealing film (Bio-Rad, MSA-5001) or adhesive covers that are ethanol-resistant to prevent detachment of the cover and subsequent cross-contamination of the samples. Mix well by carefully inverting the plate at least five times until white precipitates are visible.
4. Centrifuge plates at 3,000*g* for 30 min at RT.
5. Remove supernatant by inverting the plate.
6. Add 100 µL of 70% ethanol.
   Centrifuge at 3,000*g* for 15 min at RT.
7. Remove supernatant by inverting the plate. Briefly air-dry the DNA precipitates.
8. Add 100 µL $T_{10}E_{0.1}$ and seal plates with adhesive covers to prevent evaporation.
9. Incubate overnight at 55°C.
10. Store lysates at 4°C.

3.4.3. Genomic DNA Isolation from Cell Pellets

1. Spin 1 x 10^6 cells cells in an Eppendorf centrifuge at 1,000*g* for 3 min and remove supernatant.
2. Add 100 µL lysis buffer with 200 µg/mL Proteinase K.
3. Incubate for at least 2 h at 55°C.
4. Add 200 µL of 100% ethanol to the lysates and mix well.
5. Centrifuge at 22,000*g* for 30 min at RT.
6. Remove supernatant.
7. Add 200 µL of 70% ethanol.
   Centrifuge at 22,000*g* for 15 min at RT.
8. Remove supernatant. Briefly air-dry the DNA precipitates.
9. Add 200 µL $T_{10}E_{0.1}$.
10. Incubate overnight at 55°C.
11. Store lysates at 4°C.

3.4.4. PCR Primer Design

For detection of targeted ES cells, primers 1 and 2 are used to amplify a fragment that is subjected to a nested mutation-specific PCR using primers 3/4 or primers 5/6 (**Figure 2A**). Primers 3 and 6 are specific for the planned modification, having 3' nucleotides that are complementary to the desired alteration (*e.g.* **Figure 2C** shows primers for identification of the mutation introduced by ssODN MMMM). Due to improper annealing of primer 3 or 6, no product should be formed with unmodified DNA.

1. For PCR primer design, use genomic DNA sequences that are derived from the same mouse strain as the ES cells. However, this is not always possible.
2. PCR1: Design primers that form a 600-800 bp product comprising the altered genomic sequence. The melting temperature (T_m) of the primers should be 60-65°C (*see* www.sigma-genosys.com/calc/DNACalc.asp).
3. PCR2-nested forward (NF): The mutation-specific forward primer (primer 3) should be complementary to the desired genetic alteration. *Important*: the T_m of this primer must be 51-53°C. The T_m of the reverse primer (primer 4) must be 60-65°C. PCR2-NF with primers 3/4 should result in amplification of a 200-300 bp product.
4. PCR2-nested reverse (NR): Similar to PCR2-NF, but now the reverse primer (primer 6) is mutation-specific with a T_m of 51-53°C. The T_m of the forward primer (primer 5) must be 60-65°C. PCR2-NR with primers 5/6 should result in amplification of a 200-300 bp product.

3.4.5. PCR Optimization

1. Use temperature gradients to optimize the annealing temperatures of PCR1 and PCR2 (**Figure 2D**) in order to obtain mutation-specificity. Conditions will depend on the primers and thermal cyclers used.
2. For optimization of PCR1, genomic DNA derived from untrans-

fected ES cells is used as template. Choose the annealing temperature that gives a strong specific signal and no background signal. Annealing is usually performed at 60-65°C.

3. For optimization of PCR2, use sample DNA #1 (*see* **Note 8**) as positive control and genomic DNA derived from untransfected ES cells as negative control. Both control samples are first subjected to PCR1, followed by PCR2 at different annealing temperatures. *Important*: The annealing temperature of PCR2 must be approximately 5°C higher than the T_m of the mutation-specific primer to ensure mutation specificity. Choose the annealing temperature that gives a strong specific signal in the positive control and no signal in the negative control (**Figure 2D**). Annealing is usually performed at 56-58°C.

3.4.6. PCR Reactions

1. Prepare a PCR1 master mix for 440 reactions (4 x 96-well plates) or a smaller quantity as required containing: 7,260 µL nuclease-free water, 1,100 µL 10X PCR buffer containing 15 mM $MgCl_2$, 55 µL 100 µM forward primer, 55 µL 100 µM reverse primer, 220 µL 10 mM dNTPs and 110 µL Taq polymerase (5U/µL) to a total volume of 8,800 µL.
2. Label 96-well Thermowell PCR plates (Corning, 6511, Model M) and add 20 µL of PCR1 master mix to each well (use a multichannel pipette).
3. In each plate, add 5 µL of sample DNA #1 to well A1 as positive control.
4. Add 5 µL genomic DNA to each well (do not add genomic DNA to well A1).
5. Add two drops of mineral oil (Sigma, M8410) to each well to prevent evaporation.
6. Transfer plates to the PCR machine and amplify for 30 cycles using an initial denaturation step of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60-65°C for 1 min, elongation at 72°C for 1 min 30 s, and a final elongation step of 72°C for 10 min.
7. Prepare a PCR2 master mix for 440 reactions (4 x 96-well plates) or a smaller quantity as required containing: 9,020 µL nuclease-free water, 1,100 µL 10X PCR buffer containing 15 mM $MgCl_2$, 55 µL 100 µM nested forward primer, 55 µL 100 µM nested reverse primer, 220 µL 10 mM dNTPs and 110 µL Taq polymerase (5U/µL) to a total volume of 10,560 µL.
8. Label new PCR plates and add 24 µL of PCR2 master mix to each well.
9. Add 1.2 µL of PCR1 product to each well and add two drops of mineral oil.
10. Transfer plates to the PCR machine and amplify for 30 cycles using an initial denaturation step of 94°C for 5 min, followed by

30 cycles of denaturation at 94°C for 30 s, annealing at 56-58°C for 1 min, elongation at 72°C for 1 min, and a final elongation step of 72°C for 10 min (*see* **Note 10**).

11. Add 5 µL loading buffer to the PCR reactions and separate 15 µL of PCR2 product by electrophoresis on a 3% agarose gel in 0.5X TAE containing ethidium bromide at 80-100 V (*see* **Note 11**).

3.5. Subcloning of Single Mutant ES Cell Clones

3.5.1. Thawing Procedure

1. Cut the tube with the desired clone out of the freezing box and thaw at RT.
2. Transfer the cells to a sterile 15 mL Falcon tube containing 5 mL CM+β+LIF.
3. Centrifuge at 300*g* for 5 min.
4. Resuspend the cells in 1 mL CM+β+LIF and transfer to a 24-well plate with MEFs. Culture the cells to (semi)confluency for 3 days.
5. Expand the 24-well cell culture to a 6-well culture with MEFs. Culture the cells to (semi)confluency for 3 days.

3.5.2. Subcloning Procedure

1. Trypsinize and count ES cells.
2. Take 1 x 10^6 cells for DNA isolation (DNA 5000-2) (*see* **Section 3.4.3**) and freeze the cells from the 6-well culture into 4 vials.
3. Analyze DNA samples by PCR to assess whether the culture still contains modified cells after freezing (*see* **Section 3.4.6**). Select the well that gives the strongest PCR signal, *i.e.*, that is most enriched for the modified ES cells.
4. Thaw and plate cells from the selected well onto one 6-well plate with MEFs. Culture the cells to (semi)confluency for 3 days.
5. Trypsinize and count ES cells.
6. Take 1 x 10^6 cells for DNA isolation (DNA 5000-3) (*see* **Section 3.4.3**) to assess the recovery of the modified cells after expansion.
7. Plate 1 x 10^5 cells onto 1 x 96-well plate with MEFs (1,000 cells/well) for the next screening round. Discard the remainder of the cells.
8. Culture the cells to (semi)confluency for 3 days. Count the number of colonies in each 96-well to determine the plating efficiency (should be 100-500 colonies). Subclones may need to be passaged to a fresh 96-well plate before splitting to reach (semi)confluency.
9. Split the 1,000 cells/well cultures into two duplicate 96-well plates.
10. One plate, the master plate, is cultured to semiconfluency (2-3 days) and processed for freezing (*see* **Section 3.2.3** and **Note 12**).
11. The duplicate plate is cultured as confluent as possible for DNA

isolation (*see* **Section 3.4.1** or **3.4.2**).

12. Identify wells containing modified cells by PCR.
13. Thaw the master plate and culture all positive wells on 24-wells with MEFs.
14. Expand the 24-well cultures to 6-well cultures on MEFs. Culture to (semi)confluency for 3 days. Take 1 x 10^6 cells for DNA isolation and freeze the remainder of the cells.
15. Analyze the DNA by semi-quantitative PCR (*i.e.*, perform PCR1 and PCR2 both with 20 cycles of amplification instead of 30 cycles) and determine which sample gives the strongest signal (*see also* **Note 13** and **Figure 2E**).
16. Repeat the procedure as described above from step 4.
    After the 1,000 cells/well subclones, use the following screening rounds (*see* **Note 14**):
    a. Plate 1 x 10^4 cells onto 1 x 96-well plate with MEFs (100 cells/well) (*see* **Note 15**).
    b. Plate 1,000 cells onto 1 x 96-well plate with MEFs (10 cells/well) and, in parallel, plate 6000 cells in 48 mL of BRL+β+LIF (60% medium) onto six gelatin-coated 10 cm TC Petri dishes for colony picking to obtain single-cell colonies (*see* **Note 16**). Refeed the cells on the 10 cm dishes every 2-4 days. After 6-8 days, individual colonies can be seen. Proceed with **Section 3.5.3**.

*3.5.3. Culturing of Single-Cell Colonies (see **Note 17**)*

1. Prepare a 96-well plate (V-bottom, Costar, 3894) with 15 µL of PBS per well using the multichannel pipette.
2. Use a pipette with sterile 10 µL tips (limit the volume to 2 µL) to scrape off individual colonies and transfer them to the PBS in the 96-well plate. Pick a series of 192-384 colonies (2-4 96-well plates) (*see also* **Note 16**).
3. Add 15 µL of 2X TVP to each well and incubate for 5 min at 37°C/5% CO_2.
4. Add 70 µL of CM+β+LIF medium and resuspend.
5. Transfer trypsinized colonies (100 µL) to a 96-well plate with MEFs and 100 µL CM+β+LIF medium. Refresh the medium the next day.
6. Culture the cells to semiconfluency (2-3 days).
7. Wash the cells with 100 µL PBS and trypsinize with 25 µL 10X TVP for 5 min at 37°C/5% CO_2.
8. Add 175 µL CM+β+LIF and resuspend.
9. Transfer two 100 µL portions of cell suspension to two new 96-well plates with MEFs and 100 µL CM+β+LIF medium.
10. One plate is cultured to semiconfluency (2-3 days) and processed for freezing (*see* **Section 3.2.3**).
11. The duplicate plate is cultured as confluent as possible for DNA isolation (*see* **Section 3.4.1** or **3.4.2** and **Note 12**).

4. NOTES

1. If the targeting procedure did not succeed with an ssODN in the "sense" orientation, the targeting is usually effective when repeated with the complementary ssODN, that is, in the "anti-sense" orientation. PCR primers and conditions for identification of the modified cells remain unchanged.
2. Knockdown of MSH2 generally allows substitution of 4 nt. Knockdown of MLH1 renders cells permissive to smaller substitutions as well (3 nt) and the success rate is generally larger. However, knockdown of MLH1 may lead to a higher spontaneous mutation frequency.
3. 10X TVP is used for trypsinization of ES cells cultivated on MEF feeders, whereas TVP is used for trypsinizing ES cells grown on gelatin-coated dishes.
4. Transfection efficiency of the ES cells should be approximately 25% for plasmids. This can be measured by transfecting GFP or β-galactosidase (LacZ) expression vectors followed by FACs analysis or staining for LacZ activity, respectively. For the ES cell lines we used, the optimal cell density was approximately 1.2×10^6 cells per 6-well plate on the day of transfection which was achieved by plating the cells at 7×10^5 cells per 6-well plate the day before.
5. If the number of cells that are puromycin-resistant after selection for 2 days is less than 7×10^5, transfections were not efficient. Apparently, the cells are in bad shape and it is useless to continue. We usually count between 7×10^5 and 1.5×10^6 cells per 6-well plate.
6. After puromycin selection, the plating efficiency of the ES cells is somewhat decreased and more variable. To deal with this phenomenon, the cells are plated at 1.1×10^6 cells/well rather than 7×10^5 cells/well.
7. The day after transfection, there should approximately be $2\text{-}3 \times 10^6$ cells per 6-well plate based on the predicted growth rate of a healthy culture. When cells have been growing much slower or much faster, transfection efficiencies will be sub-optimal. In that case, do not proceed but rather repeat the whole procedure with a fresh batch of ES cells.
8. Targeting ssODNs that were transfected into the ES cells may lead to the appearance of false positives in the PCR analysis. Non-degraded targeting ssODNs can participate as PCR primers in PCR1 resulting in amplification of a "false" mutation-specific product. This product is subsequently amplified in PCR2. Usually, targeting ssODNs are degraded after two weeks of culturing, which we monitor by taking DNA samples (DNA #1, #2, #3, and #4) from a parallel cell culture and subjecting these samples to both PCR1 and PCR2. Sample DNA #1 should always give a mutation-specific product and can actually be used as positive control for optimization of PCR2 (*see* **Section 3.4.5**). Usually, the mutation-specific product is already decreased or gone in sample DNA #2 or DNA #3. This indicates that the targeting ssODN has sufficiently been degraded and is not likely to amplify false positive products when screening the 96-well plates.

9. The following procedures can be used as alternative for the freezing boxes. Resuspend trypsinized cells in CM with DMSO and leave the cell suspensions in the 96-well culture plate. Wrap the plate in paper towels. Place it in a small plastic box at -20°C for several hours and then at -80°C (problem: many cells are lost from the culture). Alternatively, keep the ES cells in culture by passaging them to fresh 96-well plates with MEFs.
10. Screen the 96-well plates only with one of the nested PCRs: either use PCR2-NF or PCR2-NR. Select the nested primer pair that gives the most robust signal during PCR optimization. After identification of positive subclones, the other primer pair can be used for confirmation of the screening results.
11. False-positives are rare but can be excluded from the analysis by pre-treating the genomic DNA samples with Exonuclease I (ExoI), which degrades single-stranded DNA. Add 5 µL genomic DNA, 1 µL 10X ExoI buffer, 1 µL Exonuclease I (20 U/µL, NEB, M0293S) and 3 µL of nuclease-free water to a total volume of 10 µL. Incubate for 5 h at 37°C and heat-inactivate for 20 min at 80°C. Use 10 µL of ExoI-treated DNA as template in PCR1 in a 50 µL reaction. For PCR2, use 3 µL of PCR1 product as template in a 25 µL reaction.
12. Alternatively, isolate DNA from the duplicate 96-well plate when the cells in the master 96-well plate are only ~50% confluent. Refresh the medium of the master plate and culture the cells for another day, while performing the PCR analysis on the duplicate DNA plate. Transfer the selected positive cells from the master plate to a fresh 24-well with MEFs and proceed with the subcloning procedure without freezing the cells.
13. Semi-quantitative PCR is used to monitor the enrichment of modified cells during each screening round. Make sure that all DNA samples are dissolved at equal concentrations: the same number of cells into an equal amount of $T_{10}E_{0.1}$. Then, perform both PCR1 and PCR2 with only 20 cycles of amplification. Select the positive cell culture that shows a much stronger signal, that is, is strongly enriched for the modified cells compared to the original positive cell culture from the previous screening round.
14. If ~50% of the 1,000 cells/well plate gives a positive PCR signal, this indicates that the original 5,000 cells/well pool is already strongly enriched for modified cells. Rather than expanding the positive cells from the 1,000 cells/well plate, continue with the 5,000 cells/well positive culture and plate the cells now at a density of 100 cells/well.
15. When proceeding from the 1,000 cells/well to the 100 cells/well screening round, the number of positive wells usually is 5-20%. However, many of these positive wells are not enriched compared to the 1,000 cells/well positive culture. Therefore, it is necessary to expand a large number of positive wells (24 clones if possible) to identify the most enriched pool by semi-quantitative PCR. If none of the 100 cells/well positives is enriched,

reseed the cells from the 1,000 cells/well clone again at 100 cells/well in order to identify an enriched subclone.

16. The 10 cells/well plates are cultured in parallel to the cells for colony picking. The number of positive wells in the 10 cells/well culture can be used to estimate the number of colonies that need to be picked. When all picked colonies are negative, plate the 100 cells/well culture again for colony picking and pick more colonies based on the results of the 10 cells/well plate. Since most of the 10 cells/well cultures are hardly enriched, it is not useful to continue the subcloning with these cells, rather start from the 100 cells/well culture again.
17. After identification of a positive picked colony, another round of subcloning is necessary because colonies may be derived from multiple cells. Plate cells from the selected colony on 10 cm TC Petri dishes. Pick 24 individual colonies and perform PCR analysis. In case of clonality, all colonies should be positive in the PCR. Use one of these latter colonies for expansion and freeze an appropriate number of vials. After establishment of a pure clonal cell line, verify the presence of the desired modification by sequencing. Isolate total RNA and prepare cDNA by reverse transcription using primers spanning the mutation. Clone the resulting PCR fragment in pGEM®-T Easy vector (Promega, A1360). Sequence multiple clones since both wild type and modified alleles should be present.

ACKNOWLEDGEMENTS

We acknowledge financial support for our work on ssODN-mediated gene modification from the Dutch Cancer Society (NKI 2000-2233) and the Netherlands Genomics Initiative (050-71-007 and 050-71-051).

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