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Identification of homologous recombination events in mouse embryonic stem cells using Southern blotting and PCR

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1 **TITLE:**
2 Identification of Homologous Recombination Events in Mouse Embryonic Stem Cells Using
3 Southern Blotting and Polymerase Chain Reaction
4
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24 **KEYWORDS**
25 Homologous recombination, gene targeting, Southern blotting, PCR, mouse, embryonic stem
26 cells, probe, primer, genetic replacement, knockout/knock-in, Myh9 gene, genomic DNA
27
28 **SUMMARY:**
29 Here, we present a detailed protocol for identifying homologous recombination events that
30 occurred in mouse embryonic stem cells using Southern blotting and/or PCR. This method is
31 exemplified by the generation of nonmuscle myosin II genetic replacement mouse models using
32 traditional embryonic stem cell-based homologous recombination-mediated targeting
33 technology.
34
35 **ABSTRACT:**
36 Relative to the issues of off-target effects and the difficulty of inserting a long DNA fragment in
37 the application of designer nucleases for genome editing, embryonic stem (ES) cell-based gene-
38 targeting technology does not have these shortcomings and is widely used to modify
39 animal/mouse genome ranging from large deletions/insertions to single nucleotide substitutions.
40 Notably, identifying the relatively few homologous recombination (HR) events necessary to
41 obtain desired ES clones is a key step, which demands accurate and reliable methods. Southern
42 blotting and/or conventional PCR are often utilized for this purpose. Here, we describe the
43 detailed procedures of using those two methods to identify HR events that occurred in mouse ES
44 cells in which the endogenous Myh9 gene is intended to be disrupted and replaced by cDNAs

45 encoding other nonmuscle myosin heavy chain IIs (NMHC IIs). The whole procedure of Southern
46 blotting includes the construction of targeting vector(s), electroporation, drug selection, the
47 expansion and storage of ES cells/clones, the preparation, digestion, and blotting of genomic DNA
48 (gDNA), the hybridization and washing of probe(s), and a final step of autoradiography on the X-
49 ray films. PCR can be performed directly with prepared and diluted gDNA. To obtain ideal results,
50 the probes and restriction enzyme (RE) cutting sites for Southern blotting and the primers for
51 PCR should be carefully planned. Though the execution of Southern blotting is time-consuming
52 and labor-intensive and PCR results have false positives, the correct identification by Southern
53 blotting and the rapid screening by PCR allow the sole or combined application of these methods
54 described in this paper to be widely used and consulted by most labs in the identification of
55 genotypes of ES cells and genetically modified animals.
56
57 **INTRODUCTION:**
58 The technology of gene targeting by HR in murine ES cells provides a powerful tool for dissecting
59 the cellular consequences of specific genetic mutations[1,2]. The importance and significance of
60 this technology are reflected in its recognition by the 2007 Nobel Prize in Physiology or
61 Medicine[3,4]; meanwhile, it represents the advent of the modern era of gene engineering[5]. Gene
62 targeting through HR can be utilized to engineer virtually any alteration ranging from point
63 mutations to large chromosomal rearrangements in the genome of mouse ES cells[6,7]. It is well
64 known that, before the emergence of so-called genome editing tools, the generation of a gene
65 knockout mouse required the application of gene-targeting technology in ES cells[8-10]. During the
66 past two decades, more than 5,000 gene-targeted mice were produced by this approach for
67 modeling human diseases or studying gene functions[11]. A genome-wide knockout effort has been
68 established for distributing gene-targeting vectors, targeted ES cell clones, and live mice to the
69 scientific community[2,12-15]. Undoubtedly, ES cell-based HR-mediated gene-targeting technology
70 has greatly advanced our understanding of the functions of genes played in physiological or
71 pathological context.
72
73 Because HR is a relatively infrequent event in mammalian cells[16,17], the important and next step
74 following gene targeting in murine ES cells is to analyze numerous ES colonies for identifying a
75 few clones with mutations resulting from HR with the targeting vector[18]. The gold methods for
76 identifying HR events include Southern blotting and PCR[19,20]. The advantages of the approaches
77 include that Southern blotting can identify correctly targeted ES clones and allows researchers to
78 analyze the structure of the gene-targeted event, such as a verification of a single copy insertion
79 of the construct, while a PCR-based strategy permits more rapid screening for HR events[21,22].
80 Though these methods have drawbacks, such as that they are time-consuming and can have false
81 positives, the combinational usage of them is widely accepted and applied by most labs in
82 identifying HR events, particular in ES cells, for generating genetically modified animals.
83
84 Three isoforms of nonmuscle myosin II (NM II) in mammals, each consisting of two identical
85 NMHC IIs which are encoded by three different genes (named Myh9, Myh10, and Myh14) and
86 two pairs of light chains, are referred to as NM II-A, II-B, and II-C[23]. Previous studies have indicated
87 that at least the isoforms of NM II-A and II-B are essential for mouse development because the
88 *in vivo* ablation of these isoforms results in embryonic lethality[24-26]. To circumvent this problem

and obtain novel insights into the isoform-specific functions of NM II-A and II-B in the later stages of mouse development, a genetic replacement strategy using ES cell-based HR-mediated gene-targeting technology was adopted to generate a series of mouse models[27]. In the course of identifying the desired ES clones, both Southern blotting and PCR methods were utilized, and these proved to be efficient and reliable[27,28].

This paper intends to provide a detailed description of Southern blotting and PCR, including the design of targeting vector(s), probe(s), and primers, and the execution of experiments, as well as the analysis of results exemplified by identifying HR event occurrence in ES cells for creating genetic replacement NM II mouse models and representative data. The protocols of these two methods presented here can also be adopted for identifying the genotypes of genetically modified cells or animals.

PROTOCOL:

1. Design of Targeting Construct(s), Probes for Southern Blot, and Primers for PCR

1.1. Select the first coding exon (exon 2) of the Myh9 gene for disruption or insertion in the application of knockout/knock-in reported here.

1.2. Retrieve the 5-kb upstream and 5-kb downstream DNA sequences surrounding the Myh9 exon 2 from the **genome.ucsc.edu** website.

1.3. Analyze restriction digestion patterns of enzymes (REs) with 1 - 2 cuts in this 10-kb region by using pDRAW software to determine suitable RE(s) to digest the genomic DNA for Southern blotting.

Note: Dra I meets this requirement and is selected for the purpose.

1.4. Select a 4-kb fragment immediate upstream of the Myh9 exon 2 as the left homology arm (LHA) and a 1.7-kb fragment immediate downstream sequence as the right homology arm (RHA); choose a 1-kb fragment 5’ upstream of the LHA as the left probe (LP) for Southern blotting and a 1.2-kb fragment 3’ downstream of the RHA as the right probe (RP), based on the above analysis.

1.5. Use a primer3 program to design the forward and reverse primers for amplifying those four DNA fragments by PCR. Design a pair of primers with the forward primer resided near the 3’ terminal of a selection marker neomyocin resistance gene (P1) and the reverse primer located just outside the RHA (P2).

Note: This primer pair will be used for identifying targeted ES clones by PCR[29].

1.6. Find a 129Sv BAC clone covering mouse Myh9 gene locus by visiting the **bacpac.chori.org** website (note: isogenic DNA is preferred). Isolate BAC DNA using a kit suitable for purifying large pieces of DNA following the instructions provided by the manufacturer.

Note: Purified BAC DNA will be used as the template for PCR amplification.

1.7. Draw a schematic representation of the targeting constructs, probes, and primers to summarize this information.

2. Generation of Targeting Construct(s) and Probes for Southern Blot, and the Preparation of Primers for PCR

2.1. Order the PCR primers described above and dissolve them into a concentration of 20 µM.

2.2. Amplify the homology arms and probes by PCR in a reaction solution including 1-µL forward and 1-µL reverse primers, 1 µL of BAC DNA (50 ng) as the template, 5 µL of buffer for Pfu and 1 µL of Pfu ultra as the DNA polymerase, and H_2O up to 50 µL. Perform the PCR in a PCR machine under the following conditions: 95 °C for 3 min; 95 °C for 30 s; 60 °C for 30 s; 72 °C and 1 kb/min; 30 cycles; finally, 72 °C for 10 min.

2.3. Purify PCR products using a PCR cleanup kit according to the protocol provided by the manufacturer and elute the DNA fragments with 40 µL of H_2O. Digest the purified PCR products with predesigned REs in a reaction solution containing 5 µL of 10x buffer for REs, 2.5 µL of RE1 and 2.5 µL of RE2, and the eluted DNA, at 37 °C for 2 h. Run a 1% DNA gel to separate target DNA bands, excise the gel containing the target DNA under the UV light, purify the target DNA fragments from the gel using a DNA gel extraction kit according to the protocol provided by the manufacturer, and elute the DNA fragments with 40 µL of H_2O.

2.4. Clone the homology arms and replacement expression cassette(s) into the mpNTKV-LoxP vector according to the order of the amplified and purified RHA, LHA, and replacement expression cassette(s) released from other vectors to obtain the final targeting construct(s). Clone DNA fragments of the amplified and purified probes into the T-easy vector.

2.5. Confirm the nucleotide sequences of all cloned DNA fragments by sequencing[27,28].

3. Preparation of Targeting Construct(s), the Electroporation of ES Cells, and the Amplification of ES Clones

3.1. Prepare each targeting construct using a plasmid maxiprep kit according to the protocol provided by the manufacturer. Linearize each construct plasmid by digesting it with Not I in a 400-µL reaction including 40 µL of 10x buffer for Not I, 10 µL of Not I, 100 µg of DNA, and H_2O up to 400 µL, at 37 °C overnight.

3.2. Purify the linearized targeting construct(s).

3.2.1. Extract the digested reaction solution 1x with an equal volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) and centrifuge at a force of 2,000 x *g* for 10 min.

177
178 3.2.2. Transfer the supernatant to a new 1.5-mL tube, precipitate the DNA using 2.5x ethanol and
179 0.1x 3M sodium acetate (pH 5.2) (volume ratio), and centrifuge at a force of 2,000 x *g* for 10 min.
180
181 3.2.3. Remove the supernatant, wash the DNA pellet 1x with 1 mL of 75% ethanol, and centrifuge
182 at a force of 2,000 x *g* for 5 min.
183
184 3.2.4. Remove the supernatant and air-dry the DNA pellet for 5 min.
185
186 3.2.5. Dissolve the linearized DNA pellet in sterile Tris-EDTA (TE) buffer at a final concentration
187 of 1 µg/µL.
188
189 3.3. Mix 50 µg of each linearized targeting construct with 0.5 x 10^7 ES cells. Perform the
190 electroporation at 320 V and 250 µF. Plate the electroporated ES cells onto dishes with neo-
191 resistant MEF feeders.
192
193 3.4. After 24 h, switch to ES cell medium with 400 µg/mL G418 and 200 µM ganciclovir and
194 continue to culture for 4 - 5 d with a daily medium change. Pick up drug-resistant ES clones into
195 48-well plates.
196
197 Note: Normally, four 48-well plates are used per construct.
198
199 3.5. Duplicate the 48-well plates.
200
201 Note: One set of the plates is cryopreserved, and the other set is used for genomic DNA
202 preparation.
203
204 **4. Preparation of Genomic DNAs and the Digestion with Restriction Enzyme(s)**
205
206 4.1. Prepare gDNAs from ES cells using a commercial kit (genomic DNA purification kit) with
207 minor modifications.
208
209 4.1.1. Remove media from the ES cell culture and add 500 µL of nuclei lysis solution, including
210 RNaseA, directly to the wells to lyse the cells.
211
212 Note: The cell lysate can be stored at -80 °C or treated immediately.
213
214 4.1.2. Pipet up and down several times to lyse the cells fully and transfer them to a clean 1.5-mL
215 tube.
216
217 4.1.3. Add one-third of the volume of the protein precipitation solution to the 1.5-mL tube,
218 vortex vigorously for 20 s, chill the samples on ice for 5 min, and then, centrifuge at a force of
219 2,000 x *g* for 5 min.
220

221 Transfer the supernatant to another clean 1.5-mL tube containing an equal volume of
222 isopropanol; gently mix the solution. (Note that white thread-like strands can be seen at this
223 moment.) Centrifuge at a force of 2,000 x *g* for 1 min; then, discard the supernatant.
224
225 4.1.4. Wash the gDNA pellet with 1 mL of 70% ethanol at room temperature, centrifuge at a force
226 of 2,000 x *g* for 1 min, aspirate the supernatant carefully, and then, air-dry the gDNA pellet for 3
227 min.
228
229 4.1.5. Dissolve the gDNAs with 100 µL of DNA rehydration solution and, then, incubate at 65 °C
230 for 1 h or at 4 °C overnight.
231
232 4.1.6. Store the gDNAs at 2 - 8 °C.
233
234 4.2. Digest the gDNAs with predesigned RE Dra I. Set up a 30-µL digestion reaction by mixing 3 µL
235 of 10x buffer for Dra I, 3 µL of Dra I, 10 µg of gDNAs/sample, and H_2O up to 30 µL, and incubate
236 at 37 °C overnight.
237
238 4.3. Check the completeness of the digestion by DNA gel, analyzing 5 µL of the digested reaction,
239 and then, add the 3 µL of 10x DNA-loading buffer for the subsequent step.
240
241 **5. Southern Blotting and PCR Identification**
242
243 **5.1. Southern blotting screening**
244
245 5.1.1. Separate the digested gDNAs by electrophoresis and transfer to a membrane.
246
247 5.1.1.1. Prepare a 1% agarose electrophoresis gel with ethidium bromide (EB), load the samples
248 from step 4.3 and a 1-kb ladder, and run the gel with a low voltage (30 - 40 V) overnight.
249
250 5.1.1.2. Take out the gel and take a picture with a DNA gel-imaging system after electrophoresis.
251 Check whether the digested and separated gDNAs display a smear-like image.
252
253 5.1.1.3. Soak the gel in a tray with 0.2 N HCl solution and shake it gently for 20 min at room
254 temperature.
255
256 5.1.1.4. Transfer the gel to DNA-denaturing solution and shake it gently for 20 min at room
257 temperature.
258
259 5.1.1.5. Switch the gel into DNA-neutralizing solution and shake it gently for 20 min at room
260 temperature.
261
262 Note: The gel is prone to breakage after this step, so it must be handled carefully.
263

264 5.1.1.6. Use the rapid downward transfer system to transfer the DNAs from the gel to the
265 membrane. Assemble the TurboBlotter and blotting stack according to the instructions provided
266 by the manufacturer.
267
268 Note: 10x or 20x saline-sodium citrate (SSC) solution is used as a transfer buffer. In general, 3 h
269 of transfer is enough to transfer 95% of gDNAs from gel to membrane; however, a longer time of
270 transfer is innocuous.
271
272 5.1.1.7. Take out the membrane and wash it with 2x SSC for 1 min, absorb the liquid with tissues,
273 and then cross-link the DNA with the membrane using a UV crosslinker.
274
275 Note: The membrane can be stored at 4 °C for one week.
276
277 5.1.2. Label the DNA probes with radioactivity.
278
279 5.1.2.1. Purify the probe plasmids using a miniprep kit according to the protocol provided by the
280 manufacturer.
281
282 5.1.2.2. Release the DNA fragments of the probes from the plasmid vector by EcoR I digestion in
283 a reaction solution including 5 µL of buffer for EcoR I, 2 µL of EcoR I enzyme, 20 µg of plasmid
284 DNA, and H_2O up to 50 µL, for 2 h.
285
286 5.1.2.3. Run a 1% DNA gel for separating the probe DNA fragments from the vector and purify
287 the DNA fragments of the probes with a DNA gel extraction kit according to the protocol provided
288 by the manufacturer.
289
290 5.1.2.4. Using 1 µL of DNA solution, measure the DNA concentration of the probe DNA fragments
291 with a spectrophotometer at a wavelength of 260/280 nm.
292
293 5.1.2.5. Prepare 40-ng of probe DNAs in a 1.5-mL tube with 45 µL of TE buffer, boil for 3 min, spin
294 briefly, and then, place the tube(s) on ice for 2 min.
295
296 5.1.2.6. Add the heat-denatured probe DNAs to the tube containing ready-to-go DNA-labeling
297 beads (-dCTP), pipet up and down to mix, add 5 µL of [α^{32}P]dCTP, and then, incubate at 37 °C for
298 15 min.
299
300 5.1.2.7. Purify the labeled probes by using G-50 microcolumns according to the instructions
301 provided by the manufacturer and, then, measure the radioactivity by a scintillation counter
302 (optional).
303
304 5.1.3. Hybridize the membrane(s) with the labeled probes.
305
306 5.1.3.1. Prehybridize the membrane.
307

308 5.1.3.1.1. Prewarm the hybridization solution at 42 °C for 30 min. Mix 20 mL of prewarmed
309 hybridization solution with 200 µg of boiled salmon sperm DNA in a 50-mL tube.
310
311 5.1.3.1.2. Place the membrane into the hybridization tube. Add the mixed prehybridization
312 solution to the hybridization tube. Place it into the hybridization oven (set rolling and the
313 temperature at 42 °C) and let the prehybridization proceed for 30 min.
314
315 5.1.3.2. Hybridize the membrane with the labeled probe(s).
316
317 5.1.3.2.1. Take out the hybridization tube and pour the prehybridization solution into a 50-mL
318 tube; add the denatured probe (heated at 100 °C for 3 min) from step 5.1.2.7 to this tube and
319 mix gently.
320
321 Note: Reduce any inducing bubbles.
322
323 5.1.3.2.2. Return the mixed solution to the hybridization tube and perform the hybridization at
324 42 °C overnight.
325
326 5.1.4. Wash the membrane(s) to remove nonhybridized probes.
327
328 5.1.4.1. Place the membrane(s) into a tray with 1x SSC + 0.1% SDS and shake gently at 55 - 60 °C
329 for 10 min.
330
331 5.1.4.2. Transfer the membrane(s) to a tray with 0.5x SSC + 0.1% SDS and shake gently at 55 -
332 60 °C for 10 min.
333
334 5.1.4.3. Check the radioactivity on the membrane(s) by using a portable Geiger counter to decide
335 whether a third washing is required.
336
337 5.1.5. Expose the radioactivity on the membrane to X-ray films.
338
339 5.1.5.1. Remove the liquid from the washed membrane(s).
340
341 5.1.5.2. Enfold the membrane(s) with plastic wrap and fix it/them in the exposure cassette.
342
343 5.1.5.3. Expose the membrane to two sheets of X-ray film in a dark room.
344
345 5.1.5.4. Place the exposure cassette at -80 °C overnight or longer.
346
347 5.1.6. Develop the films to visualize the results. Evaluate whether a corresponding ES clone is the
348 desired one with the targeted recombination or not, according to the sizes of the DNA bands
349 detected by the probes.
350

351 5.1.7. Rehybridize the same membrane by another probe after stripping off the used probe
352 according to the following procedure: take out the used membrane, wash it 1x with clean H_2O,
353 and then, incubate it in striping solution (55% formamide, 2% SSPE, 1% SDS, H_2O) at 65 °C with
354 gentle shaking for 1 - 2 h.
355
356 **5.2. PCR identification**
357
358 5.2.1. Perform PCR identification of the desired ES clones in a 50-µL reaction solution including 5
359 µL of 10x PCR buffer, 2 µL of 50 mM $MgSO_4$, 1 µL of 10 mM dNTP, 1 µL of 20 µM forward primer,
360 1 µL of 20 µM reverse primer, 1 µL of high-fidelity platinum Taq, gDNAs (~100 ng), and H_2O up to
361 50 µL.
362
363 5.2.2. Use the following PCR reaction conditions: an initial denaturation at 94 °C for 3 min, 30
364 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and an extension at 68 °C for
365 132 s, and a final step of 68 °C for 10 min.
366
367 5.2.3. Analyze the PCR products by electrophoresis in 1.0% agarose.
368
369 5.2.4. Clone the PCR fragments with their expected size into the T-easy vector and sequence to
370 confirm the presence of a partial sequence of the target vector.
371
372 **REPRESENTATIVE RESULTS:**
373 In this paper, a detailed protocol of Southern blotting and PCR is described, which is utilized to
374 identify HR events that occurred in mouse ES cells for the generation of NM II genetic
375 replacement mouse models, using ES cells-based HR-mediated targeting technology. Though
376 Southern blotting and PCR, as well as traditional gene-targeting technology, have been widely
377 used for several decades, the successful application of them needs to be planned carefully. At
378 least these aspects are required to be considered: the length of the long and short arms, the
379 positions and length of the probes, the suitable REs for cutting the genomic DNAs, and the
380 primers for PCR, as summarized in **Figure 1**, which is helpful for subsequent analysis. As an
381 important step of Southern blotting, the prepared and digested genomic DNAs are required to
382 be separated on DNA gel for the detection by the probe. Because genomic DNAs are cut into a
383 lot of fragments with different lengths, they display a smear-like status on the DNA gel,
384 suggesting a complete digestion of the genomic DNAs, as indicated in **Figure 2**. As a final step of
385 Southern blotting, the signals of a radioactivity-labeled probe hybridizing with a target DNA
386 fragment are shown on the film, which reflect the occurrence of HR events in the ES clones,
387 thereby indicating whether an ES clone is the desired one. According to the predesign in this
388 study, ES clones with mutated allele have two distinct size bands, while wild-type ES clones only
389 have one band, suggesting the desired ES clones are heterozygous (**Figure 3**). Relative to the
390 procedure and results of Southern blotting, the operation and results of PCR are simple and direct.
391 Following the PCR reaction, the PCR products can be analyzed on the DNA gel. If the PCR bands
392 are specific and sequencing the cloned PCR products confirms the presence of a partial sequence
393 of target vector such as a neo-resistance gene, as well as genomic regions that are just outside
394 of the homology arm, the occurrence of HR events can be expected and verified (**Figure 4**).

395
396 **FIGURE LEGENDS:**
397
398 **Figure 1: Targeting constructs.** This is a schematic demonstrating the generation of multiple
399 targeting constructs. The wild-type (WT) Myh9 gene allele, gene-targeting vector, replacement
400 exogenous expression cassette(s), and the resultant mutated allele(s), as well as the probes (LP,
401 RP) for Southern blot and the primers (P1, P2) for PCR, are shown and described previously[27]. An
402 arrow on exon 2 indicates the translational initiation site. Following the successful occurrence of
403 HR, the replacement expression cassette and the neomycin resistance gene (Neo^r) are inserted
404 just 5' of the initiating ATG codon. Therefore, the endogenous Myh9 allele is disrupted and the
405 knocked-in gene(s) is/are expressed in the mutant cells and mice.
406
407 **Figure 2: Digested genomic DNAs with Dra I.** Genomic DNAs from ES clones targeted with the
408 construct replacing NMHC II-A with II-B are digested with Dra I and, then, separated on an
409 agarose gel by electrophoresis. A smear-like digested gDNA is observed. C1 - C8 depict individual
410 ES clones. A complete digestion of gDNA produces a lot of DNA fragments with a different length,
411 thereby displaying a smear-like image. This result also reflects the good quality of prepared
412 gDNAs and the completeness of the digestion.
413
414 **Figure 3: Representative results of Southern blotting.** These panels show a Southern blotting
415 screening of the genomic DNAs from ES clones targeted with the construct of replacing NMHC II-
416 A with II-AB, using the left and right probes. The mutated allele shows a 12.1-kb or 6-kb band
417 when the left probe or right probe is used, respectively, while the WT shows a 9.7-kb band. M =
418 marker; PC1 - PC5 = positive clones; NC = negative clone. The sizes of the Southern blotting bands
419 are also indicated. All procedures of Southern blotting are strictly carried out and the specificity
420 of the probes is good enough; there should be no nonspecific bands expect for the expected
421 bands.
422
423 **Figure 4: Representative results of PCR.** This panel shows the PCR identification of the genomic
424 DNAs from ES clones targeted with the construct of replacing NMHC II-A with II-BA using the
425 primer pair P1 + P2. The mutated allele yields a 2.1-kb band, while the WT allele yields no band.
426 M = marker; PC1 - PC3 = positive clones; NC = negative clone. The size of the PCR band is also
427 indicated. Since the primers are designed to only detect the mutated allele, the appearance of a
428 single and expected band reflects the specificity of the primers and the high quality of the
429 prepared gDNAs.
430
431 **DISCUSSION:**
432 Currently, designer nucleases for genome editing still cannot replace ES cell-based gene-targeting
433 technology due to its issues of off-target effects, and difficulty in inserting a long DNA
434 fragment[30,31]. As the golden methods for identifying HR events that occurred in mouse ES cells,
435 this report provides a detailed protocol of Southern blotting and PCR for the field. We validated
436 the reliability of these methods by analyzing individual clones from mouse ES cells targeted with
437 a series of constructs. The desired ES clones identified by these methods had been successfully
438 used to generate corresponding mouse models[27].

439
440 Though other techniques for the screening of targeted ES clones have been described[19,32], the
441 methods of Southern blotting and PCR cannot be completely replaced by those established
442 thereafter[32], because these initial techniques have a longer applied history and are widely
443 accepted and confirmed by the scientific society, performed by most biological labs, and are the
444 origin of other technologies. Importantly, the good performance of Southern blotting and PCR in
445 the identification of HR events is well exemplified in previous work[29]. The results from Southern
446 blotting indicate several unique features: among the randomly screened ES clones, over 90% of
447 them are desired ones, no nonspecific bands are detected, and the HR occurred preferentially on
448 one allele of the Myh9 gene. Meanwhile, the data from PCR, together with sequencing, confirm
449 that the occurrence of HR events is site-specific and match well with those from Southern blotting.
450
451 According to our practice, several factors should be considered when Southern blotting and PCR
452 are used to identify HR events in ES cells, thereby obtaining good and expected results. The first
453 one is the length of the homology arms; in general, increasing the homology arm length will
454 enhance the efficiency of HR[33]. However, this is not always the case. On the one hand, longer
455 arms increase the difficulty of manipulation; on the other hand, the length of the homology arms
456 (4 kb for the left arm and 1.7 kb for the right arm) reported here resulted in the highest HR
457 frequency obtained so far among similar experiments. Additionally, a reasonable length of
458 homology arms facilitates the identification by PCR. The second is the utilization of isogenic DNA
459 for preparing the homology arms and Southern blotting probes[34]. This can be satisfied by
460 ordering a BAC clone containing the region of the gene-of-interest or by using genomic DNA from
461 the cells intended to be targeted. The third is the selection of suitable REs for digesting genomic
462 DNAs. In general, one RE or the combination of two REs that cut the wild-type or mutant allele
463 only once or twice around the targeting region are preferred; furthermore, the resulting larger
464 DNA fragment should not exceed 15 kb and the size difference between the distinct DNA
465 fragments is over 2 kb. These requirements can facilitate the separation and identification of
466 expected bands by Southern blotting. The fourth is the length of the probes and the least
467 similarity with other sequences in the genome. Generally, the length of the probes is 500 – 1,000
468 bp. The similarity with other sequences in the genome can be analyzed with the NCBI BLAST
469 program. Furthermore, a software used to design the probes for Southern blotting has been
470 described[35]. The fifth factor to be considered is to use the conventional methods to prepare
471 genomic DNA for an enhancing yield. Genomic DNAs prepared from a confluent well of a 48-well
472 plate are generally enough for at least two rounds of Southern blotting analyses. As to designing
473 the primers for PCR, the best strategy is to use one primer present on the selection marker in
474 conjunction with a primer outside of the targeting arms. Additionally, sequencing the PCR
475 products is important for proving HR events[20,36]. Notably, PCR-based screening cannot
476 completely replace the information obtained through Southern blotting, while it can effectively
477 reduce the numbers of clones to be evaluated.
478
479 In conclusion, Southern blotting and PCR are well-demonstrated methods for screening ES clones
480 to identify HR-mediated gene-targeting events in ES cells. Though the detailed protocol described
481 here mainly focused on the screening of desired NM II genetic replacement ES clones, it can be
482 used for genotyping mice that are subsequently generated using the positive ES clones. It can be

easily adapted to the identification of HR events in other cell types, such as iPS cells or somatic cells.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Gao, G., McMahon, C., Chen, J., Rong, Y. A powerful method combining homologous recombination and site-specific recombination for targeted mutagenesis in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America.* **105** (37), 13999-14004 (2008).

2. Skarnes, W. *et al.* A conditional knockout resource for the genome-wide study of mouse gene function. *Nature.* **474** (7351), 337-342 (2011).

3. Vogel, G. Nobel Prizes. A knockout award in medicine. *Science.* **318** (5848), 178-179 (2007).

4. Salsman, J., Dellaire, G. Precision genome editing in the CRISPR era. *Biochemistry. Cell Biology.* **95** (2), 187-201 (2017).

5. Capecchi, M. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nature Reviews Genetics.* **6** (6), 507-512 (2005).

6. Van, d. W. L., Adams, D. J., Bradley, A. Tools for targeted manipulation of the mouse genome. *Physiological Genomics.* **11** (3), 133-164 (2002).

7. Glaser, S., Anastassiadis, K., Stewart, A. F. Current issues in mouse genome engineering. *Nature Genetics.* **37** (11), 1187 (2005).

8. Bradley, A., Evans, M., Kaufman, M. H., Robertson, E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature.* **309** (5965), 255-256 (1984).

9. Robertson, E., Bradley, A., Kuehn, M., Evans, M. Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. *Nature.* **323** (6087), 445-448 (1986).

10. Thomas, K. R., Capecchi, M. R. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell.* **51** (3), 503-512 (1987).

527 11. Skarnes, W. C. *et al.* A conditional knockout resource for the genome–wide study of mouse
528 gene function. *Nature.* **474** (7351), 337 (2011).
529
530 12. Collins, F. S., Rossant, J., Wurst, W. A mouse for all reasons. *Cell.* **128** (1), 9-13 (2007).
531
532 13. Poueymirou, W. T. *et al.* F0 generation mice fully derived from gene-targeted embryonic stem
533 cells allowing immediate phenotypic analyses. *Nature Biotechnology.* **25** (1), 91-99 (2007).
534
535 14. Pettitt, S. J. *et al.* Agouti C57BL/6N embryonic stem cells for mouse genetic resources. *Nature*
536 *Methods.* **6** (7), 493-495 (2009).
537
538 15. Gertsenstein, M. *et al.* Efficient Generation of Germ Line Transmitting Chimeras from
539 C57BL/6N ES Cells by Aggregation with Outbred Host Embryos. *PLoS One.* **5** (6), e11260 (2012).
540
541 16. Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A., Kucherlapati, R. S. Insertion of DNA
542 sequences into the human chromosomal |[beta]|-globin locus by homologous recombination.
543 *Nature.* **317** (6034), 230-234 (1985).
544
545 17. Deng, C., Capecchi, M. R. Reexamination of gene targeting frequency as a function of the
546 extent of homology between the targeting vector and the target locus. *Molecular & Cellular*
547 *Biology.* **12** (8), 3365 (1992).
548
549 18. Lay, J. M., Friishansen, L., Gillespie, P. J., Samuelson, L. C. Rapid confirmation of gene targeting
550 in embryonic stem cells using two long-range PCR techniques. *Transgenic Research.* **7** (2), 135-
551 140 (1998).
552
553 19. Langerak, P., Nygren, A. O. H., Schouten, J. P., Jacobs, H. Rapid and quantitative detection of
554 homologous and non-homologous recombination events using three oligonucleotide MLPA.
555 *Nucleic Acids Research.* **33** (22), e188 (2005).
556
557 20. Gómezrodríguez, J. *et al.* Advantages of q-PCR as a method of screening for gene targeting in
558 mammalian cells using conventional and whole BAC-based constructs. *Nucleic Acids Research.* **36**
559 (18), e117-e117 (2008).
560
561 21. Kim, H. S., Smithies, O. Recombinant fragment assay for gene targeting based on the
562 polymerase chain reaction. *Nucleic Acids Research.* **16** (18), 8887-8903 (1988).
563
564 22. Joyner, A. L., Skarnes, W. C., Rossant, J. Production of a mutation in mouse En-2 gene by
565 homologous recombination in embryonic stem cells. *Nature.* **338** (6211), 153-156 (1989).
566
567 23. Ma, X., Adelstein, R. S. The role of vertebrate nonmuscle Myosin II in development and human
568 disease. *Bioarchitecture.* **4** (3), 88-102 (2014).
569

570 24. Malonek, D. Relationships between the dynamics of cortical blood flow, oxygenation, and
571 volume changes following sensory stimulation. *Proceedings of the National Academy of Sciences*
572 *of the United States of America.* **94** (1997).
573
574 25. Takeda, K., Kishi, H., Ma, X., Yu, Z. X., Adelstein, R. S. Ablation and mutation of nonmuscle
575 myosin heavy chain II-B results in a defect in cardiac myocyte cytokinesis. *Circulation Research.*
576 **93** (4), 330-337 (2003).
577
578 26. Conti, M. A., Evenram, S., Liu, C., Yamada, K. M., Adelstein, R. S. Defects in cell adhesion and
579 the visceral endoderm following ablation of nonmuscle myosin heavy chain II-A in mice. *Journal*
580 *of Biological Chemistry.* **279** (40), 41263-41266 (2004).
581
582 27. Wang, A. *et al.* Nonmuscle myosin II isoform and domain specificity during early mouse
583 development. *Proceedings of the National Academy of Sciences of the United States of America.*
584 **107** (33), 14645-14650 (2010).
585
586 28. Zhang, Y. *et al.* Mouse models of MYH9-related disease: mutations in nonmuscle myosin II-A.
587 *Blood.* **119** (1), 238 (2012).
588
589 29. Liu, T. *et al.* Identification and characterization of MYH9 locus for high efficient gene knock-
590 in and stable expression in mouse embryonic stem cells. *PLoS One.* **13** (2), e0192641 (2018).
591
592 30. Saito, S., Adachi, N. Advances in the Development of Gene-Targeting Vectors to Increase the
593 Efficiency of Genetic Modification. *Biological & Pharmaceutical Bulletin.* **39** (1), 25-32 (2016).
594
595 31. Langerak, P., Nygren, A., Schouten, J., Jacobs, H. Rapid and quantitative detection of
596 homologous and non-homologous recombination events using three oligonucleotide MLPA.
597 *Nucleic Acids Research.* **33** (22), e188 (2005).
598
599 32. Martin, S. L. *et al.* A single amino acid substitution in ORF1 dramatically decreases L1
600 retrotransposition and provides insight into nucleic acid chaperone activity. *Nucleic Acids*
601 *Research.* **36** (18), 5845-5854 (2008).
602
603 33. Kamisugi, Y., Cuming, A. C., Cove, D. J. Parameters determining the efficiency of gene
604 targeting in the moss Physcomitrella patens. *Nucleic Acids Research.* **33** (19), e173 (2005).
605
606 34. Luo, Y., Bolund, L., Sørensen, C. B. Pig gene knockout by rAAV-mediated homologous
607 recombination: comparison of BRCA1 gene knockout efficiency in Yucatan and Göttingen
608 fibroblasts with slightly different target sequences. *Transgenic Research.* **21** (3), 671-676 (2012).
609
610 35. Croning, M. D., Fricker, D. G., Komiyama, N. H., Grant, S. G. Automated design of genomic
611 Southern blot probes. *BMC Genomics.* **11** (1), 74 (2010).
612

613 36. Zimmer, A., Gruss, P. Production of chimaeric mice containing embryonic stem (ES) cells
614 carrying a homoeobox Hox 1.1 allele mutated by homologous recombination. *Nature.* **338** (6211),
615 150-153 (1989).
616

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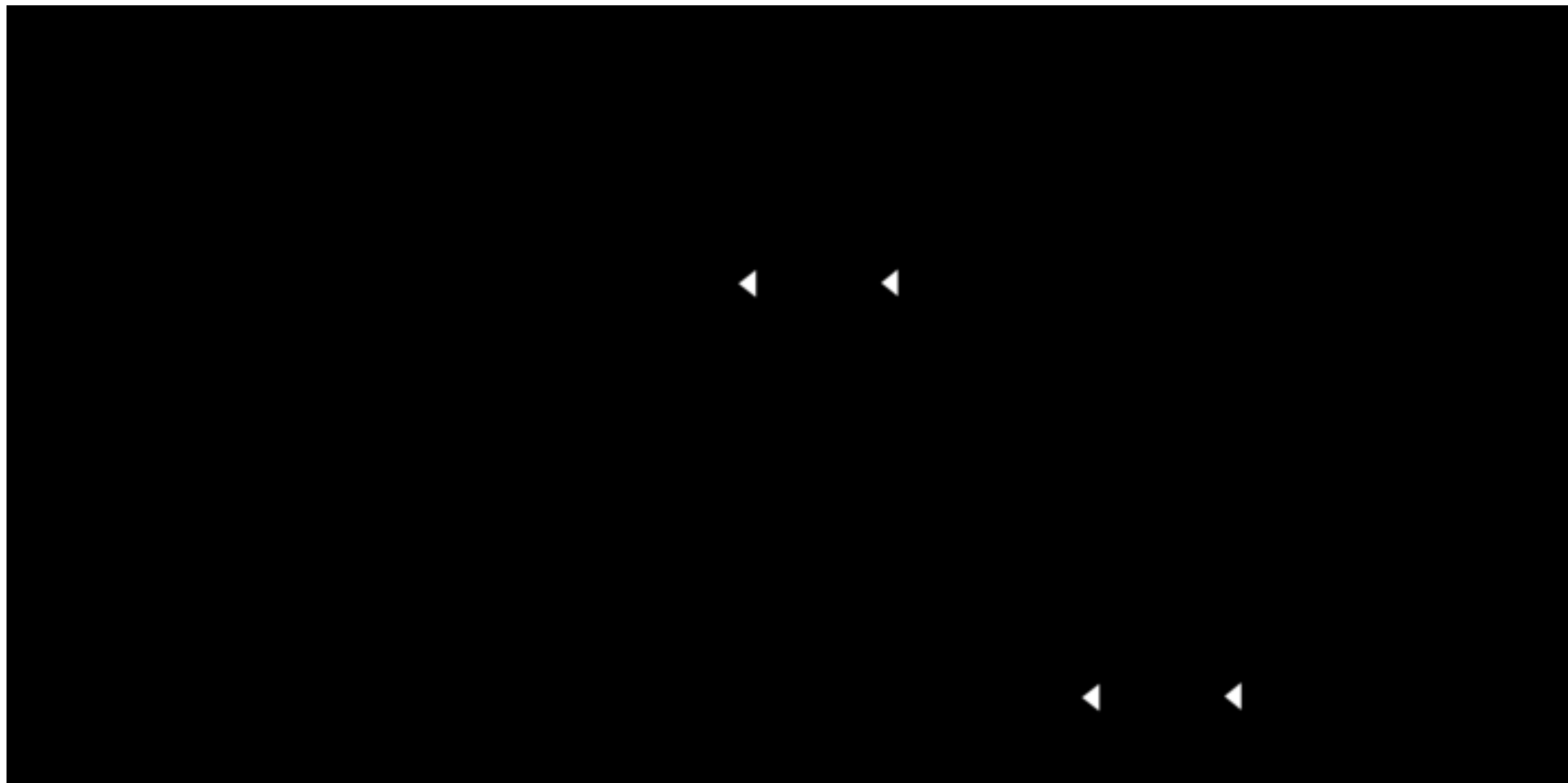

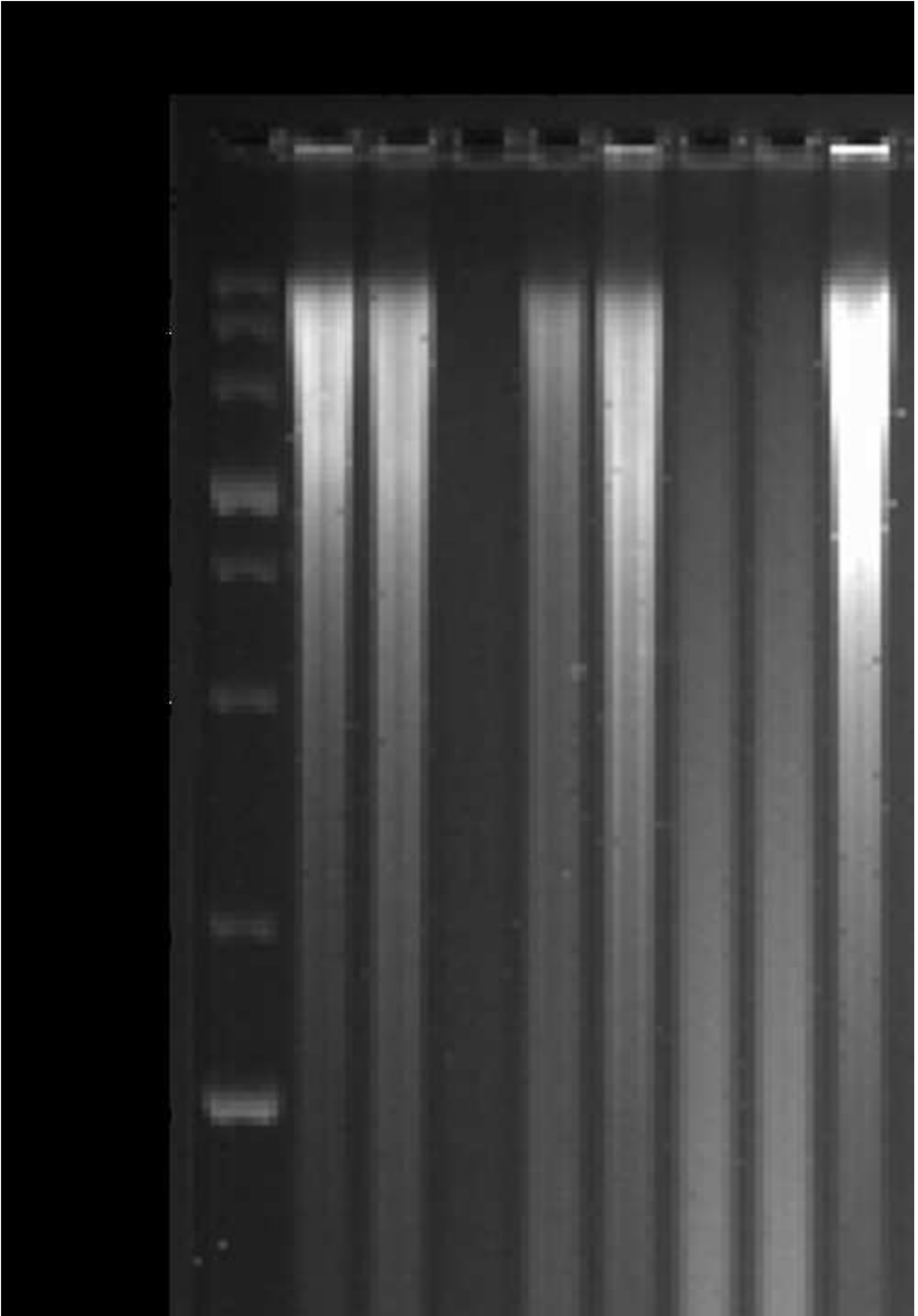

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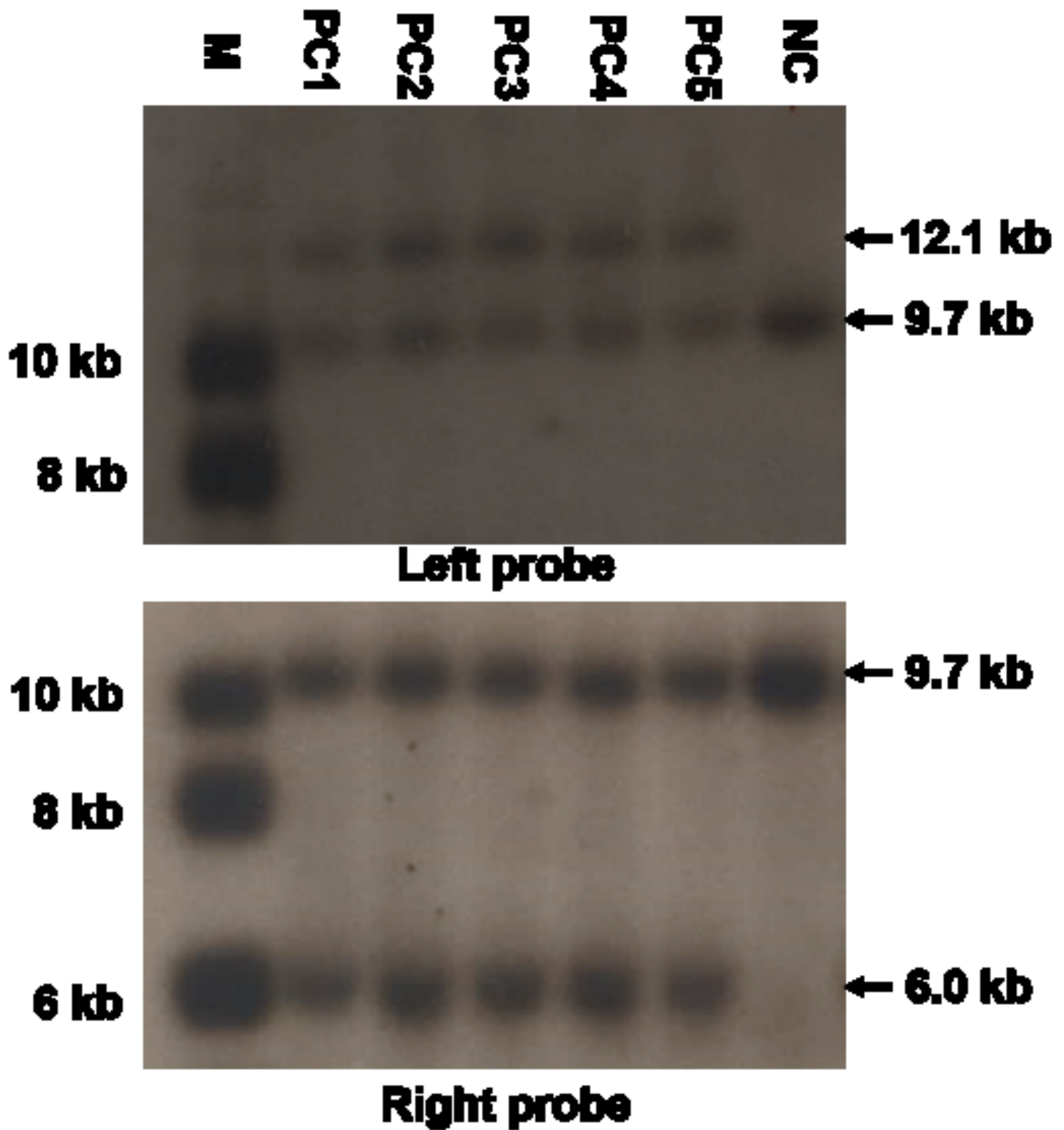

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G418	Thermo Fisher
Salmon Sperm DNA Solution	Thermo Fisher
Platinu *Taq* DNA Polymerase High Fidelity	Thermo Fisher
Not I	Thermo Scientific
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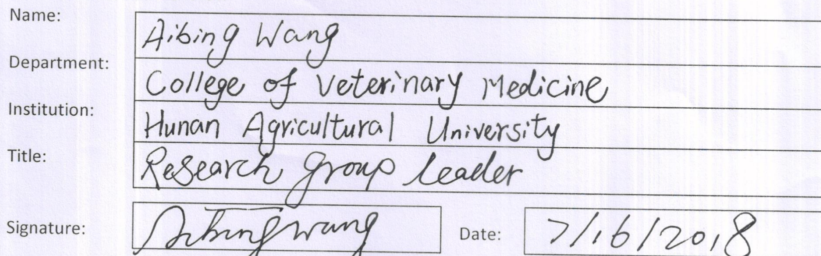

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