

List of supplemental data files:

**Supplemental Figure 1.** IRF5 expression is markedly elevated in UUO mouse renal tissues. (A)Gross anatomy of kidney in sham and UUO. (B)The renal parenchyma in the 7d after UUO group was significantly thinner compared to the sham-operated group. (C)Representative H&E and Masson staining images in sham-operated and 7d after UUO. Bars = 50µm. (D)Significant deposition of blue-stained, coarse collagen fibers and marked inflammatory cell infiltration were observed in the obstructed renal tissue. (E)Immunohistochemical images of α-SMA, Vimentin, and IRF5 in sham and UUO renal tissues. Typical images and quantification are included. Bars = 50 µm. (F)The α-SMA and Vimentin positive area in 7d after UUO was significantly higher than that in sham-operated. The number of IRF5 positive cells in the UUO group was significantly higher compared to sham-operated. (G)Renal Vimentin, Acta2, and Irf5 mRNA expression in the indicated mouse groups were measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Data are expressed as the mean ± SD. Comparisons between two groups were performed using an unpaired two-tailed Student's t-test. N = 3-8. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; \*\*\*\*P < 0.0001.

**Supplemental Figure 2.** Validation of IRF5 expression in the indicated groups were measured by Western blot. (A)Renal IRF5 protein expression in the WT mice and global Irf5 deletion mice were measured by Western blot. (B)Renal IRF5 protein expression in the Irf5fl/fl mice and myeloid-specific Irf5 deletion mice were measured by Western blot.(C)Cells IRF5 protein expression in the BMDMs from Irf5fl/fl mice and myeloid-specific Irf5 deletion mice were measured by Western blot.(D)Renal IRF5 protein expression in the control groups and IRF5 inhibitor treatment groups were measured by Western blot. Data are expressed as the mean± SD. Statistical comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post hoc test. N = 3. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001.

**Supplemental Figure 3.** Specificity validation of IRF5 staining. (A) Immunofluorescence images of anti-IRF5 antibodies ab181553 (abcam, Red) and ab21689 (abcam, Green) on kidney sections from WT and Irf5-/- mice after UUO. (B) Immunofluorescence images of anti-IRF5 antibodies ab181553 (abcam, Red) and ab21689 (abcam, Green) on kidney sections from patient with kidney fibrosis. Bars = 50 µm.

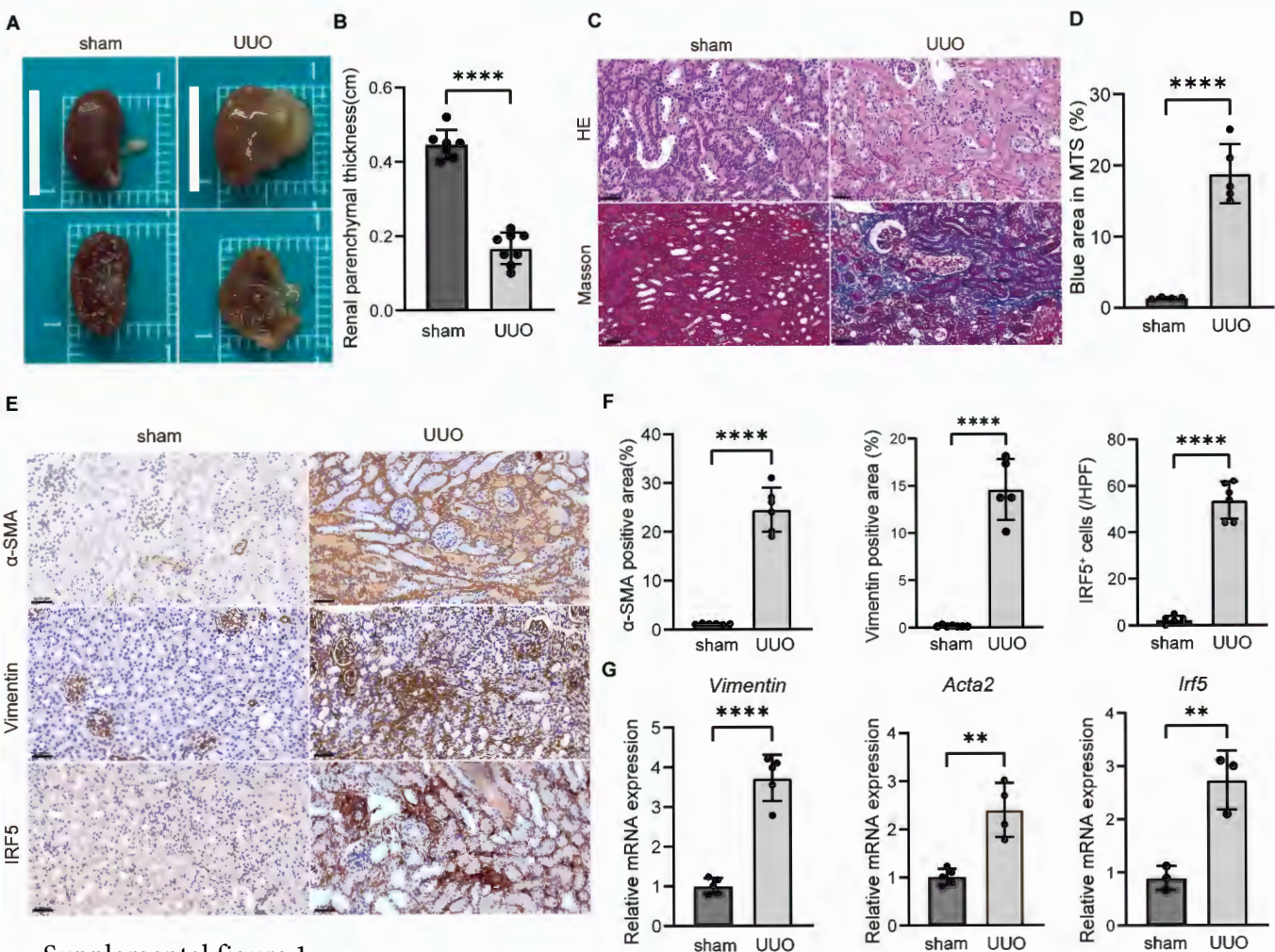
**Supplemental Figure 4.** Specificity validation of MMP9 staining. (A) Immunofluorescence images of anti-MMP9 antibodies 10375-2-AP (proteintech, Red) and MA5-15886 (Invitrogen, Green) on kidney sections from WT and Irf5-/- mice after UUO. Bars = 50 µm.

**Supplemental Certificate 1.**

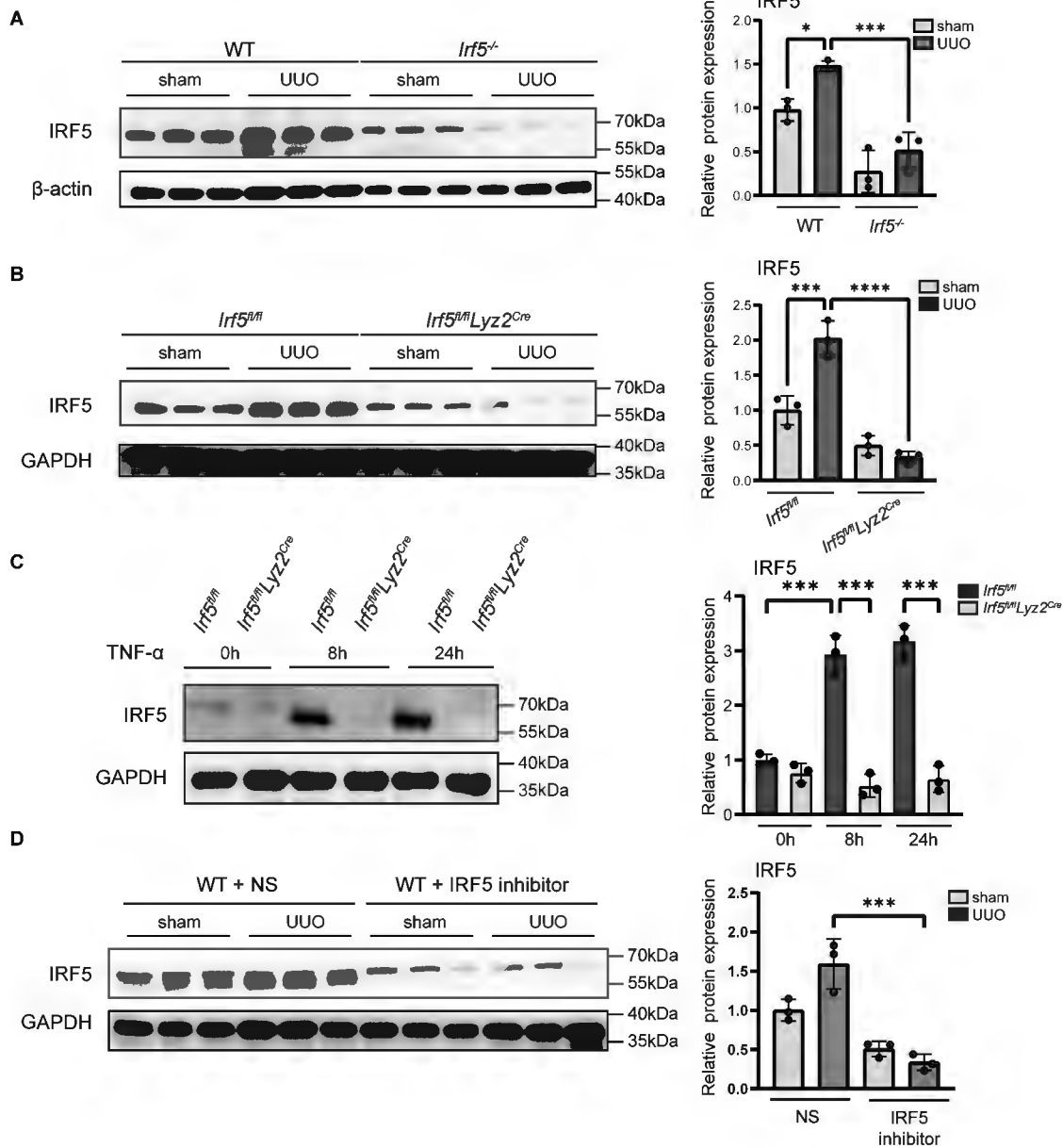
**Supplemental Certificate 2.**

**Supplemental Certificate 3.**

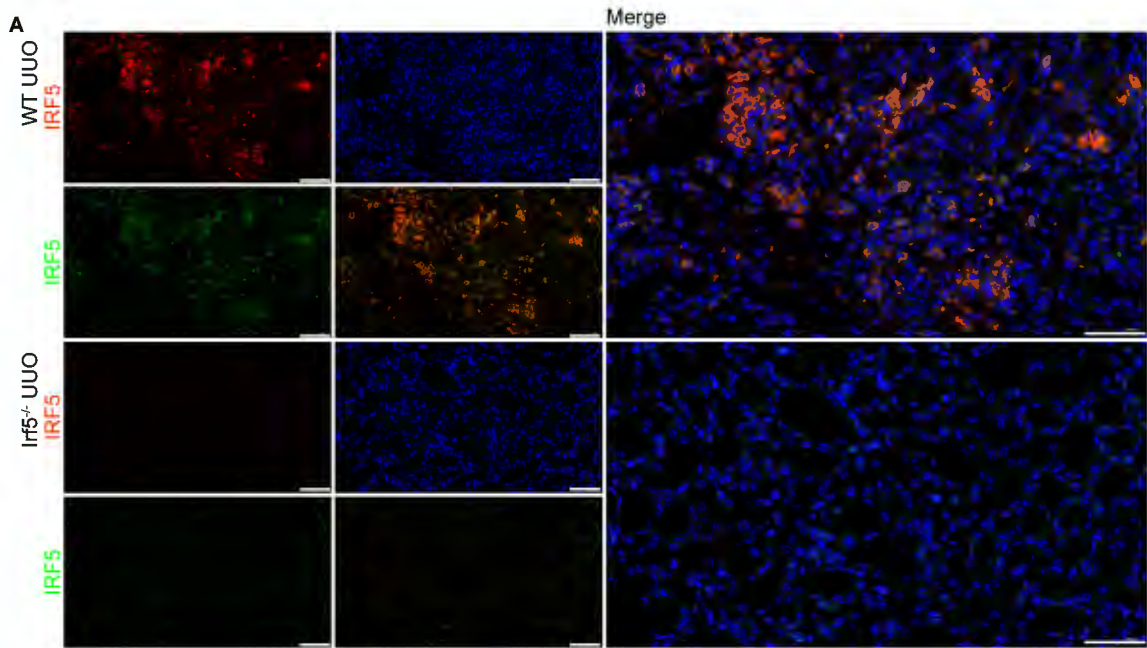
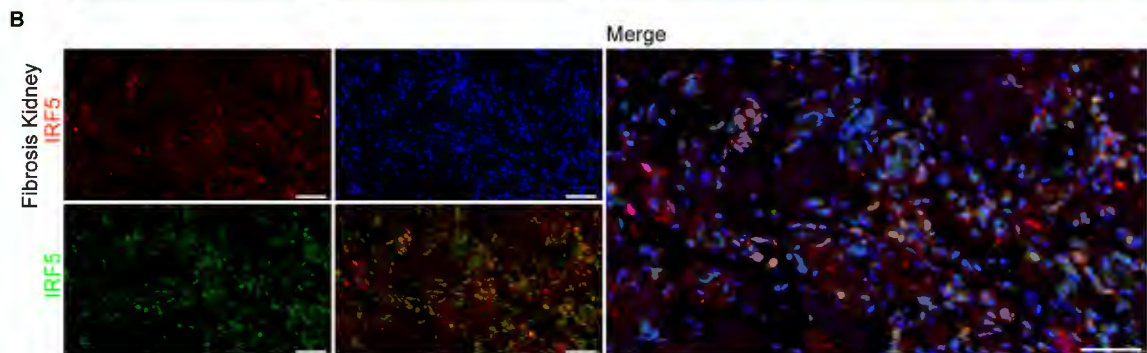
**Supplemental Certificate 4.**



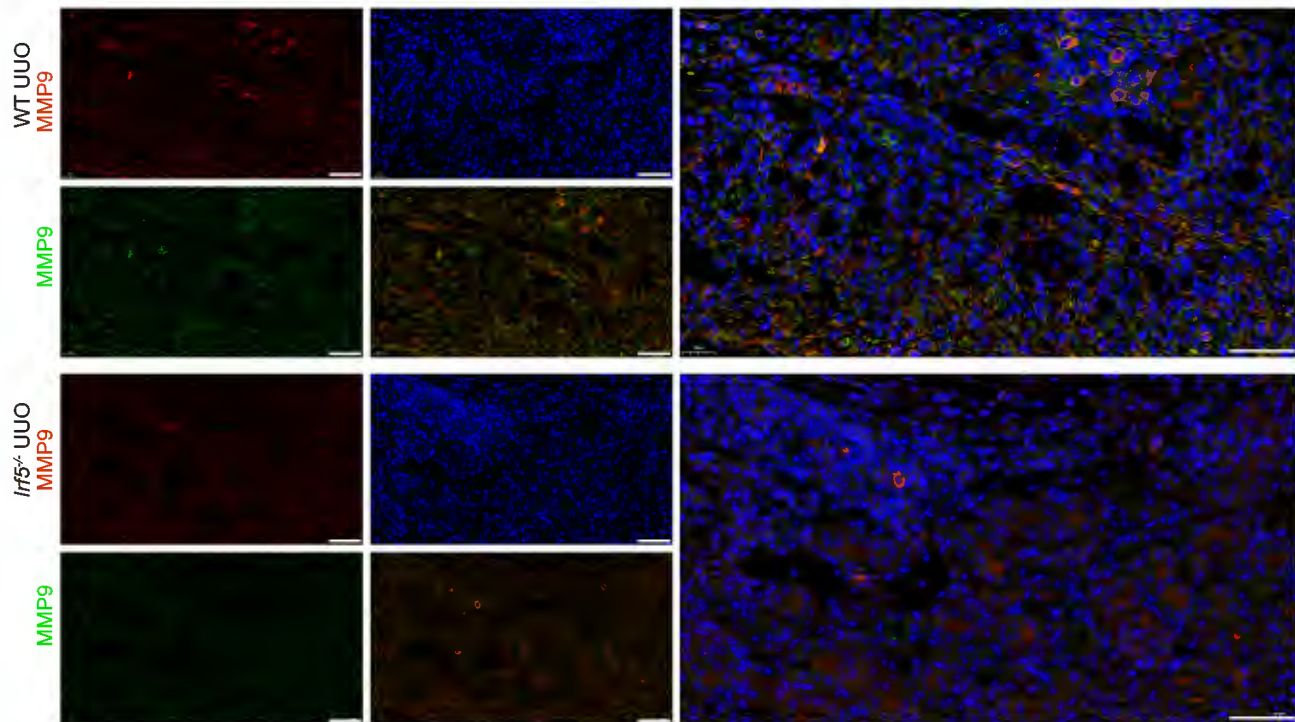
Supplemental figure 1.



Supplemental figure 2.



Supplemental figure 3.

**A**

Supplemental figure 4.

Supplemental Table 1. Background information on the kidney fibrosis patients  
and normal controls

Kidney fibrosis patients (n=30)

|                                        |            |                | Number(n) | Percentage(%) |
|----------------------------------------|------------|----------------|-----------|---------------|
| Age (years/Mean $\pm$ SD)              | <18        | 3.0 $\pm$ 1.2  | 4         | 13.3%         |
|                                        | $\geq$ 18  | 60.2 $\pm$ 8.7 | 26        | 86.7%         |
| Gender                                 | Male       |                | 17        | 56.7%         |
|                                        | Female     |                | 13        | 43.3%         |
| Reason of obstruction                  | UPJO*      |                | 4         | 13.3%         |
|                                        | Nephrolith |                | 20        | 66.7%         |
|                                        | Tumor      |                | 6         | 20%           |
| Degree of<br>inflammatory infiltration | Mild       |                | 8         | 26.7%         |
|                                        | Moderate   |                | 9         | 30%           |
|                                        | Severe     |                | 13        | 43.3%         |
| Degree of<br>kidney fibrosis           | Mild       |                | 6         | 20%           |
|                                        | Moderate   |                | 10        | 33.3%         |
|                                        | Severe     |                | 14        | 46.7%         |

\*UPJO: Ureteropelvic Junction Obstruction

Normal controls (n=12)

|                           |           |                 | Number(n) | Percentage(%) |
|---------------------------|-----------|-----------------|-----------|---------------|
| Age (years/Mean $\pm$ SD) | <18       | 2.8 $\pm$ 1.4   | 4         | 33.3%         |
|                           | $\geq$ 18 | 52.1 $\pm$ 16.3 | 8         | 66.7%         |
| Gender                    | Male      |                 | 5         | 41.7%         |
|                           | Female    |                 | 7         | 58.3%         |

Supplemental Table S9. The list of antibodies used for immunohistochemistry  
(IHC), immunofluorescence (IF) and western blot (WB)

| Antigen        | Company     |            | Host   | Reactivity   | Dilution   |           |           |
|----------------|-------------|------------|--------|--------------|------------|-----------|-----------|
| IRF5           | abcam       | ab181553   | Rabbit | Mouse, Human | IHC 1:500  | IF 1:1000 | WB 1:1000 |
| IRF5           | abcam       | ab21689    | Rabbit | Mouse, Human | IF 1:2000  | WB 1:5000 |           |
| IRF5           | proteintech | 3A4A10     | Mouse  | Mouse, Human | IF 1:500   |           |           |
| $\alpha$ -SMA  | eBioscience | ab2572996  | Mouse  | Mouse, Human | IHC 1:500  | IF 1:250  | WB 1:100  |
| Vimentin       | abcam       | ab92547    | Rabbit | Mouse, Human | IHC 1:500  | IF 1:500  | WB 1:1000 |
| CD68           | abcam       | ab283654   | Rabbit | Mouse        | IHC 1:100  | IF 1:50   |           |
| CD20           | abcam       | ab64088    | Rabbit | Mouse, Human | IHC 1:100  | IF 1:100  |           |
| CD3            | abcam       | ab16669    | Rabbit | Mouse, Human | IHC 1:500  | IF 1:200  |           |
| MMP9           | proteintech | 10375-2-AP | Rabbit | Mouse, Human | IHC 1:400  | IF 1:200  | WB 1:1000 |
| MMP9           | Invitrogen  | MA5-15886  | Mouse  | Mouse, Human | IF 1:1000  |           |           |
| GAPDH          | Invitrogen  | ab10977387 | Mouse  | Mouse        | WB 1:5000  |           |           |
| $\beta$ -actin | Invitrogen  | ab2745831  | Rabbit | Mouse        | WB 1:10000 |           |           |
| F4/80          | proteintech | 28463-1-AP | Rabbit | Mouse        | WB 1:3000  |           |           |
| TNF- $\alpha$  | proteintech | 60291-1-Ig | Mouse  | Mouse        | WB 1:1000  |           |           |

Supplemental Table S10. The list of primers for RT-qPCR

| Gene                          | Direction | Sequence                             |
|-------------------------------|-----------|--------------------------------------|
| <i>Actin</i>                  | Forward   | 5'-CAG CCT TCC TTC TTG GGT ATG-3'    |
|                               | Reverse   | 5'-GGC ATA GAG GTC TTT ACG GAT G-3'  |
| <i>Acta2</i>                  | Forward   | 5'-GTC CCA GAC ATC AGG GAG TAA-3'    |
|                               | Reverse   | 5'-TCG GAT ACT TCA GCG TCA GGA-3'    |
| <i>Il-1<math>\beta</math></i> | Forward   | 5'-CTG CAG CTG GAG AGT GTG G-3'      |
|                               | Reverse   | 5'-GGG GAA CTC TGC AGA CTC AA-3'     |
| <i>Il-6</i>                   | Forward   | 5'-GCC TTC TTG GGA CTG ATG CT-3'     |
|                               | Reverse   | 5'-GCC ATT GCA CAA CTC TTT TCT CA-3' |
| <i>Irf5</i>                   | Forward   | 5'-AGA GAC AGG GAA GTA CAC TGA AG-3' |
|                               | Reverse   | 5'-TGG AAG TCA CGG CTT TTG TTA AG-3' |
| <i>Tgf<math>\beta</math>1</i> | Forward   | 5'-CCA CCT GCA AGA CCA TCG AC-3'     |
|                               | Reverse   | 5'-CTG GCG AGC CTT AGT TTG GAC-3'    |
| <i>Tnfa</i>                   | Forward   | 5'-CAG GCG GTG CCT ATG TCT C-3'      |
|                               | Reverse   | 5'-CGA TCA CCC CGA AGT TCA GTA G-3'  |
| <i>Vimentin</i>               | Forward   | 5'-CAC TAG CCG CAG CCT CTA TTC-3'    |
|                               | Reverse   | 5'-GTC CAC CGA GTC TTG AAG CA-3'     |

## Irf5 基因敲除小鼠项目完成报告



## Irf5 基因敲除小鼠项目完成报告

项目编号：XM709673

项目类型：Cas9-KO

项目背景：C57BL/6J

客户姓名：蔡哲钧

客户单位：浙江大学

## 目录

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## 一、基因基本信息

敲除基因名称 (NCBI 号) : Irf5 (27056)

敲除基因 NCBI 网址链接 : <https://www.ncbi.nlm.nih.gov/gene/27056>

敲除基因名称 (MGI 号) : Irf5 (MGI:1350924)

敲除基因 Ensembl 网址链接 :

[http://uswest.ensembl.org/Mus\\_musculus/Gene/Summary?db=core;g=ENSMUSG000000029771;r=6:29526625-29541871](http://uswest.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG000000029771;r=6:29526625-29541871)

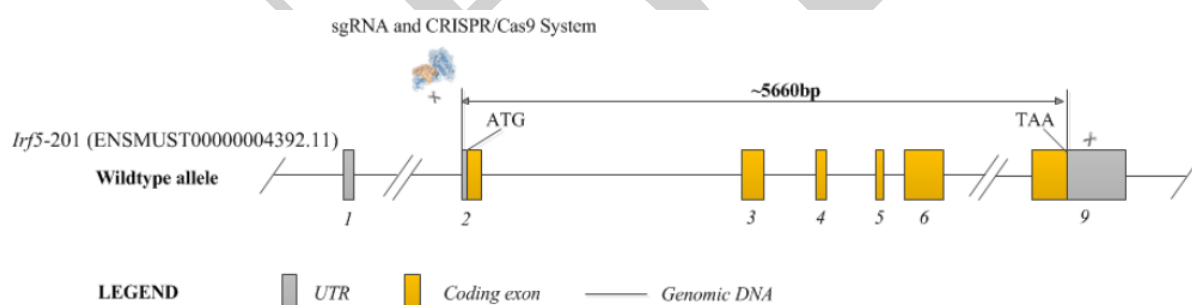
9771;r=6:29526625-29541871

方案针对的转录本 (Ensembl 号) : Irf5-201 (ENSMUST000000004392.11)

方案敲除的 exon : Exon2~Exon9

## 二、项目策略

### 2.1 项目策略图



### 2.2 项目策略概述

利用 CRISPR/Cas9 技术, 设计并体外转录 gRNA, 将 Cas9、gRNA 同时注射到小鼠的受精卵中。

Cas9 蛋白在 gRNA 引导下结合到靶位点进而造成 DNA 双链断裂, 从而实现靶位点碱基序列缺失, 最终实现及基因敲除。

### 三、gRNA 序列信息

| gRNA 名称 | gRNA 序列 ( 5' →3' )   | PAM |
|---------|----------------------|-----|
| gRNA1   | AAGGAAACTCCCGTGCTAGT | GGG |
| gRNA2   | CTTGCCCCAGACGCCTAGA  | AGG |
| gRNA3   | CGTAGACTGATATAGCAATG | TGG |
| gRNA4   | GGTGATTCTCTAATATGCC  | TGG |

### 四、F0 代阳性小鼠信息

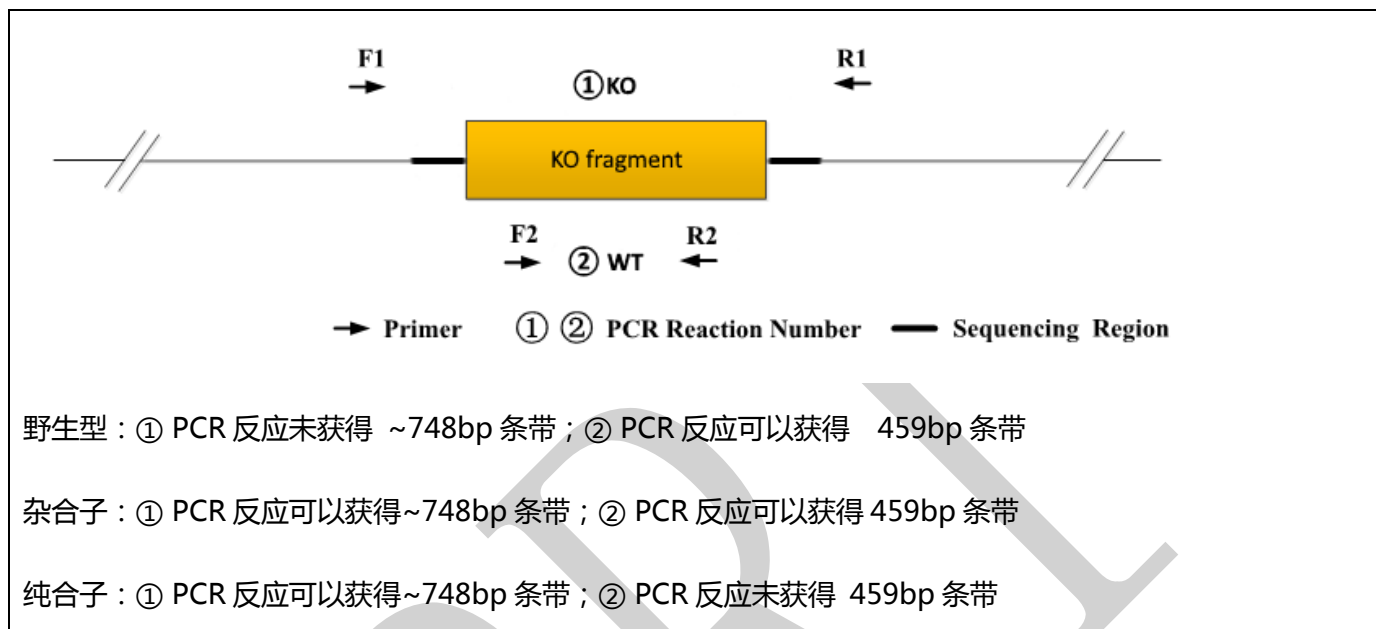
经受精卵显微注射，胚胎移植，获得 F0 小鼠。通过 PCR，测序确认，共获得如下的阳性 F0 代小鼠。

具体信息如下：

| ID | Gender | color | Gty | DOB      | Gen | Remark |
|----|--------|-------|-----|----------|-----|--------|
| 1  | ♂      | B     | 阳性  | 2018/5/7 | F0  | 配繁     |
| 4  | ♂      | B     | 阳性  | 2018/5/7 | F0  | 配繁     |
| 9  | ♀      | B     | 阳性  | 2018/5/7 | F0  | 配繁     |
| 12 | ♂      | B     | 阳性  | 2018/5/7 | F0  |        |
| 15 | ♂      | B     | 阳性  | 2018/5/7 | F0  |        |
| 17 | ♀      | B     | 阳性  | 2018/5/7 | F0  |        |
| 18 | ♀      | B     | 阳性  | 2018/5/7 | F0  | 配繁     |
| 19 | ♀      | B     | 阳性  | 2018/5/7 | F0  |        |

## 五、F1 代小鼠鉴定结果与小鼠信息

### 5.1 鉴定策略示意图



### 5.2 PCR 引物信息

| PCR 序号 | 引物名称 | 引物详细名称             | 引物序列                   | 产物大小            | 备注 |
|--------|------|--------------------|------------------------|-----------------|----|
| ①KO    | F1   | 709673-Irf5-ko-tF1 | CAGAGTCCTGTGAACGAACCTG | WT: 6816bp      | /  |
|        | R1   | 709673-Irf5-ko-tR1 | ACAACAGAAGCCACTGGCAC   | Targeted:~748bp |    |
| ②WT    | F2   | 709673-Irf5-wt-tF1 | TCTTTGTGCTAGGGATCAAGGC | WT: 459bp       | /  |
|        | R2   | 709673-Irf5-wt-tR1 | CAGGTTACAGTATGTGTGGGCC | Targeted: 0bp   |    |

### 5.3 F1 小鼠鉴定结果

| 鉴定结果                                                                                  |
|---------------------------------------------------------------------------------------|
| <p>根据 PCR 策略示意图，经 PCR 和测序确认，<b>22, 23, 28, 29</b> 号小鼠为阳性 F1 代小鼠。</p> <p>具体删除方式如下：</p> |

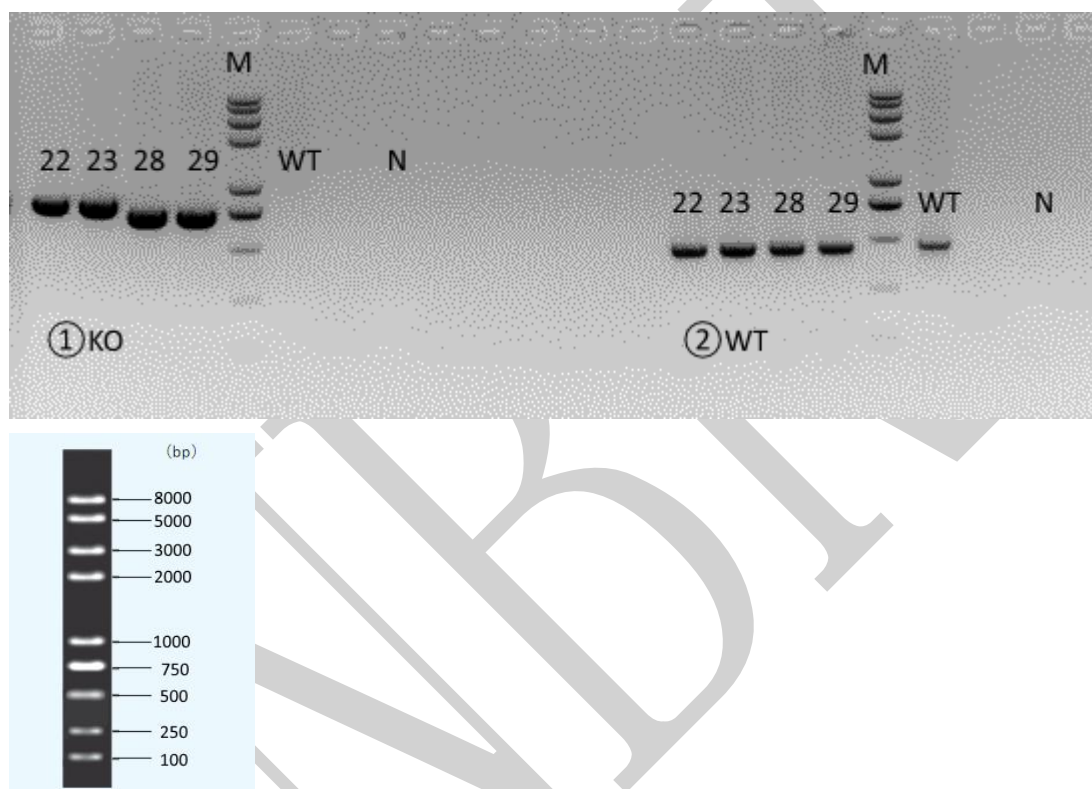
22# , 23# : -11bp,+2bp,-5973bp/wt

actcaagaatgggtggcccaca-----**11bp**-----**CC**cacgggagttccttctt//gccccagacgc-----**5973bp**-----ctgg  
ctggaagaaatgaagccagc

28# , 29# : -6068bp,+1bp/wt

gcactcaagaatgggtggcccacagca-----**6068bp**-----**G**gcctggctgggaagaaatgaagccagcagcc

电泳图如下：



注：数字为鼠尾号，WT 为 C57BL/6J 野生型，N 为 negative 空白对照，M 为 DNA Marker

#### 5.4 PCR 条件：

| PCR Reaction Component |                                                  |             |
|------------------------|--------------------------------------------------|-------------|
| Seg.                   | reaction component                               | Volume (μl) |
| 1                      | 2 × Taq Master Mix , Dye Plus , (Vazyme P112-03) | 12.5        |
| 2                      | ddH2O                                            | 9.5         |

|             |                       |      |       |
|-------------|-----------------------|------|-------|
| 3           | Primer A(10pmol/μl)   |      | 1     |
| 4           | Primer B(10pmol/μl)   |      | 1     |
| 5           | Template(≈100ng/μl)   |      | 1     |
| PCR program |                       |      |       |
| Seg.        | Temp.                 | Time | Cycle |
| 1           | 95°C                  | 5min | 20×   |
| 2           | 98°C                  | 30s  |       |
| 3           | 65°C ( -0.5°C/cycle ) | 30s  |       |
| 4           | 72°C                  | 45s  |       |
| 5           | 98°C                  | 30s  | 20×   |
| 6           | 55°C                  | 30s  |       |
| 7           | 72°C                  | 45s  |       |
| 8           | 72°C                  | 5min |       |
| 9           | 10°C                  | hold |       |

### 5.5 阳性 F1 代小鼠信息如下：

| ID | Gender | color | Gty                             | DOB       | Gen | F/M          |
|----|--------|-------|---------------------------------|-----------|-----|--------------|
| 22 | ♂      | B     | -11bp,+2bp,-5973bp/wt , 编码区完全删除 | 2018/7/16 | F1  | 4#/C57BL/6J  |
| 23 | ♂      | B     | -11bp,+2bp,-5973bp/wt , 编码区完全删除 | 2018/7/16 | F1  | 4#/C57BL/6J  |
| 28 | ♂      | B     | -6068bp,+1bp/wt , 编码区完全删除       | 2018/7/20 | F1  | C57BL/6J/18# |
| 29 | ♂      | B     | -6068bp,+1bp/wt , 编码区完全删除       | 2018/7/20 | F1  | C57BL/6J/18# |

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## B6.129P2-Lyz2<sup>tm1(cre)lfo</sup>/J

Stock No: 004781

Protocol 26499: Separated MCA Assay - Lyz2<sup><tm1(cre)lfo></sup>

Version 5.2

### Notes

Use Perfect Match® (Stratagene) added before Taq to delete non-specific bands.

The genotyping protocol(s) presented here have been optimized for reagents and conditions used by The Jackson Laboratory (JAX). To genotype animals, JAX recommends researchers validate the assay independently upon receipt of animals into their facility. Reaction cycling temperature and times may require additional optimization based on the specific genotyping reagents used.

### Expected Results

Mutant = ~700 bp

Heterozygote = ~700 bp and ~350 bp

Wild type = 350 bp

Separated by gel electrophoresis on a 1.5% agarose gel.

### JAX Protocol

#### Protocol Primers

| PRIMER   | 5' LABEL | SEQUENCE 5' → 3'            | 3' LABEL | PRIMER TYPE | REACTION | NOTE |
|----------|----------|-----------------------------|----------|-------------|----------|------|
| oIMR3066 |          | CCC AGA AAT GCC AGA TTA CG  |          | Mutant      | A        |      |
| oIMR3067 |          | CTT GGG CTG CCA GAA TTT CTC |          | Common      | A, B     |      |
| oIMR3068 |          | TTA CAG TCG GCC AGG CTG AC  |          | Wild type   | B        |      |

#### Reaction A

| COMPONENT            | FINAL CONCENTRATION |
|----------------------|---------------------|
| ddH <sub>2</sub> O   |                     |
| Kapa 2G HS buffer    | 1.30 X              |
| MgCl <sub>2</sub>    | 2.60 mM             |
| dNTPS-kapa           | 0.26 mM             |
| oIMR3066             | 0.50 uM             |
| oIMR3067             | 0.50 uM             |
| Glycerol             | 6.50 %              |
| Dye                  | 1.00 X              |
| Kapa 2G HS taq polym | 0.03 U/ul           |
| DNA                  |                     |

#### Cycling

| STEP | TEMP °C | TIME | NOTE                                       |
|------|---------|------|--------------------------------------------|
| 1    | 94.0    | --   |                                            |
| 2    | 94.0    | --   |                                            |
| 3    | 65.0    | --   | -0.5 C per cycle decrease                  |
| 4    | 68.0    | --   |                                            |
| 5    |         | --   | repeat steps 2-4 for 10 cycles (Touchdown) |
| 6    | 94.0    | --   |                                            |
| 7    | 60.0    | --   |                                            |
| 8    | 72.0    | --   |                                            |
| 9    |         | --   | repeat steps 6-8 for 28 cycles             |
| 10   | 72.0    | --   |                                            |
| 11   | 10.0    | --   | hold                                       |

JAX uses a very high speed Taq (~1000 bp/sec), use cycling times recommended for your reagents.

JAX uses a 'touchdown' cycling protocol and therefore has not calculated the optimal annealing temperature for each set of primers.

## Reaction B

| COMPONENT            | FINAL CONCENTRATION |
|----------------------|---------------------|
| ddH <sub>2</sub> O   |                     |
| Kapa 2G HS buffer    | 1.30 X              |
| MgCl <sub>2</sub>    | 2.60 mM             |
| dNTPS-kapa           | 0.26 mM             |
| oIMR3067             | 0.50 uM             |
| oIMR3068             | 0.50 uM             |
| Glycerol             | 6.50 %              |
| Dye                  | 1.00 X              |
| Kapa 2G HS taq polym | 0.03 U/ul           |
| DNA                  |                     |

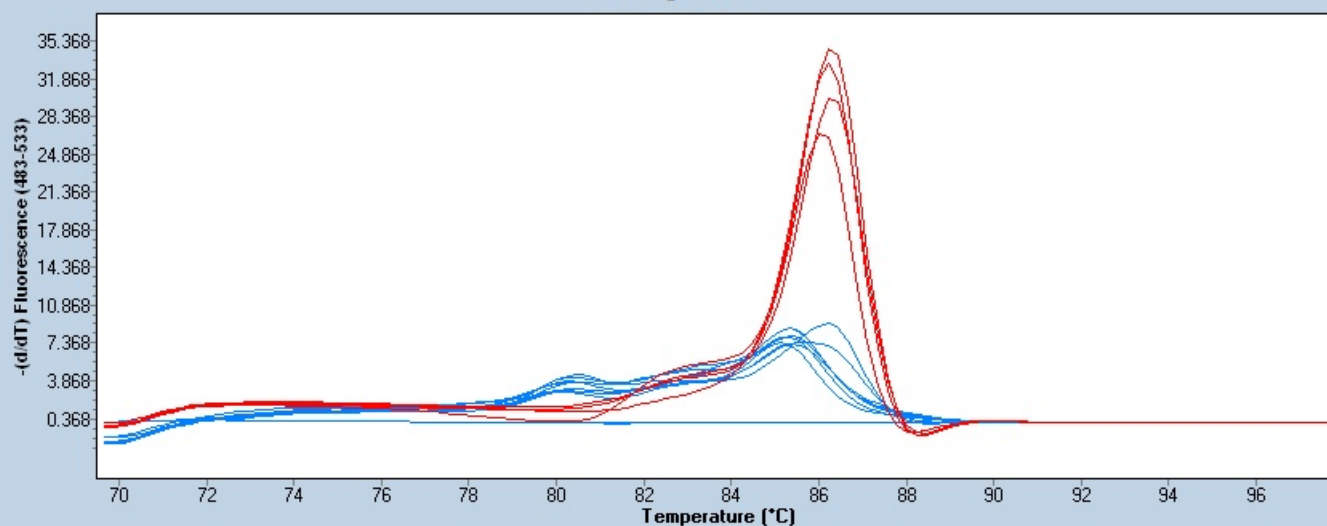
## Cycling

| STEP | TEMP °C | TIME | NOTE                                       |
|------|---------|------|--------------------------------------------|
| 1    | 94.0    | --   |                                            |
| 2    | 94.0    | --   |                                            |
| 3    | 65.0    | --   | -0.5 C per cycle decrease                  |
| 4    | 68.0    | --   |                                            |
| 5    |         | --   | repeat steps 2-4 for 10 cycles (Touchdown) |
| 6    | 94.0    | --   |                                            |
| 7    | 60.0    | --   |                                            |
| 8    | 72.0    | --   |                                            |
| 9    |         | --   | repeat steps 6-8 for 28 cycles             |
| 10   | 72.0    | --   |                                            |
| 11   | 10.0    | --   | hold                                       |

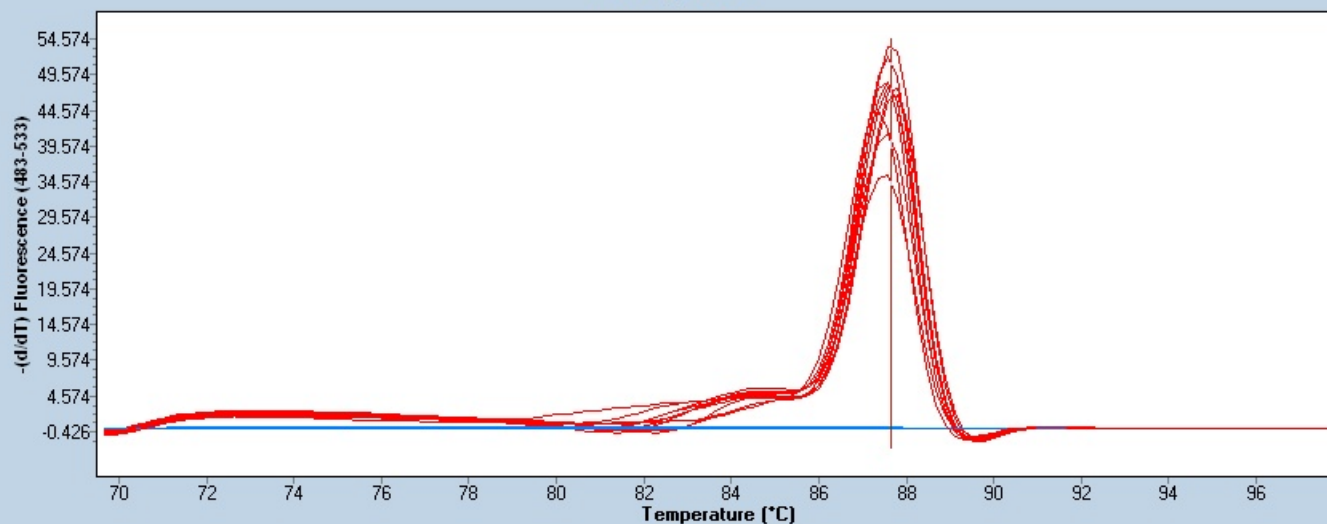
JAX uses a very high speed Taq (~1000 bp/sec), use cycling times recommended for your reagents.

JAX uses a 'touchdown' cycling protocol and therefore has not calculated the optimal annealing temperature for each set of primers.

### Melting Peaks



### Melting Peaks



## C57BL/6-*Irf5*<sup>tm1Ppr</sup>/J

Stock No: 017311

Protocol 36936: Separated PCR Assay - *Irf5*<tm1Ppr>-Cre recombined

Version 1.0

### Notes

The genotyping protocol(s) presented here have been optimized for reagents and conditions used by The Jackson Laboratory (JAX). To genotype animals, JAX recommends researchers validate the assay independently upon receipt of animals into their facility. Reaction cycling temperature and times may require additional optimization based on the specific genotyping reagents used.

### Expected Results

Mutant = ~300 bp

Heterozygote = 196 bp and ~300 bp

Wild type = 196 bp

### JAX Protocol

#### Protocol Primers

| PRIMER | 5' LABEL | SEQUENCE 5' → 3'           | 3' LABEL | PRIMER TYPE       | REACTION | NOTE |
|--------|----------|----------------------------|----------|-------------------|----------|------|
| 12932  |          | AGG GCC TGT CCA GAA TTA GG |          | Mutant Reverse    | B        |      |
| 50085  |          | CTT TGC CAT GAA CCA CTC AG |          | Wild type Forward | A        |      |
| 50086  |          | GAT GGT GTT GTC CCC ATC CT |          | Wild type Reverse | A        |      |
| 50087  |          | CCA CAG CAC CCA CTA GCA C  |          | Mutant Forward    | B        |      |

#### Reaction A

| COMPONENT            | FINAL CONCENTRATION |
|----------------------|---------------------|
| ddH <sub>2</sub> O   |                     |
| Kapa 2G HS buffer    | 1.30 X              |
| MgCl <sub>2</sub>    | 2.60 mM             |
| dNTPS-kapa           | 0.26 mM             |
| 50085                | 0.50 uM             |
| 50086                | 0.50 uM             |
| Glycerol             | 6.50 %              |
| Dye                  | 1.00 X              |
| Kapa 2G HS taq polym | 0.03 U/ul           |
| DNA                  |                     |

#### Cycling

| STEP | TEMP °C | TIME | NOTE                                       |
|------|---------|------|--------------------------------------------|
| 1    | 94.0    | --   |                                            |
| 2    | 94.0    | --   |                                            |
| 3    | 65.0    | --   | -0.5 C per cycle decrease                  |
| 4    | 68.0    | --   |                                            |
| 5    |         | --   | repeat steps 2-4 for 10 cycles (Touchdown) |
| 6    | 94.0    | --   |                                            |
| 7    | 60.0    | --   |                                            |
| 8    | 72.0    | --   |                                            |
| 9    |         | --   | repeat steps 6-8 for 28 cycles             |
| 10   | 72.0    | --   |                                            |
| 11   | 10.0    | --   | hold                                       |

JAX uses a very high speed Taq (~1000 bp/sec), use cycling times recommended for your reagents.

JAX uses a 'touchdown' cycling protocol and therefore has not calculated the optimal annealing temperature for each set of primers.

Reaction B

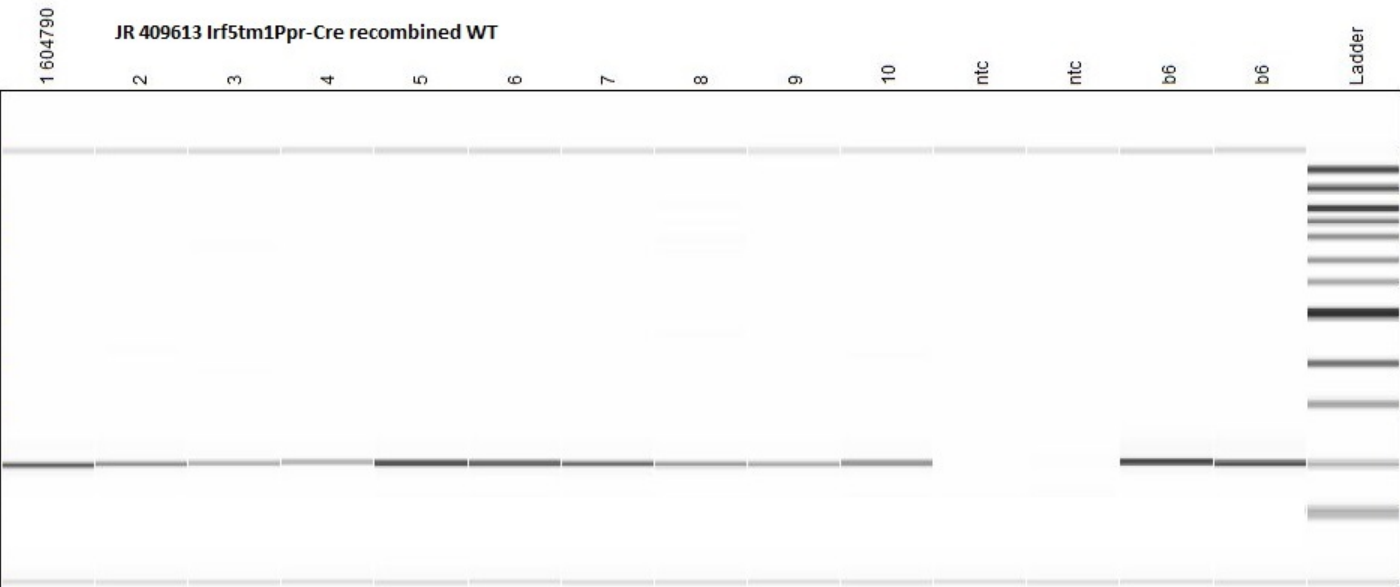
| COMPONENT            | FINAL CONCENTRATION |
|----------------------|---------------------|
| ddH2O                |                     |
| Kapa 2G HS buffer    | 1.30 X              |
| MgCl2                | 2.60 mM             |
| dNTPS-kapa           | 0.26 mM             |
| 12932                | 0.50 uM             |
| 50087                | 0.50 uM             |
| Glycerol             | 6.50 %              |
| Dye                  | 1.00 X              |
| Kapa 2G HS taq polym | 0.03 U/ul           |
| DNA                  |                     |

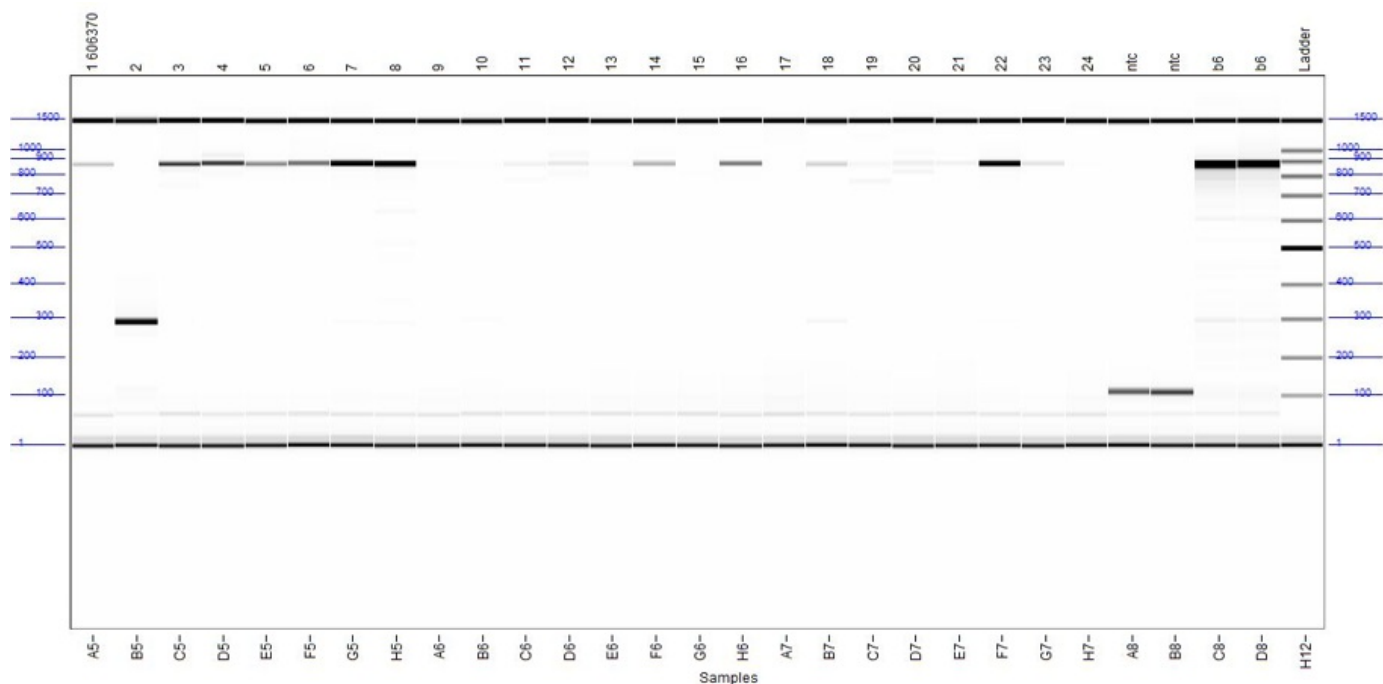
Cycling

| STEP | TEMP °C | TIME | NOTE                                       |
|------|---------|------|--------------------------------------------|
| 1    | 94.0    | --   |                                            |
| 2    | 94.0    | --   |                                            |
| 3    | 65.0    | --   | -0.5 C per cycle decrease                  |
| 4    | 68.0    | --   |                                            |
| 5    |         | --   | repeat steps 2-4 for 10 cycles (Touchdown) |
| 6    | 94.0    | --   |                                            |
| 7    | 60.0    | --   |                                            |
| 8    | 72.0    | --   |                                            |
| 9    |         | --   | repeat steps 6-8 for 28 cycles             |
| 10   | 72.0    | --   |                                            |
| 11   | 10.0    | --   | hold                                       |

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JAX uses a 'touchdown' cycling protocol and therefore has not calculated the optimal annealing temperature for each set of primers.





Supplemental Certificate 4

