Gene splicing is a fine-tuned process orchestrated by splice factors including RNA-binding motif 20 (RBM20), and their mutations are linked to the development of cardiac diseases. Here, we provide a step-by-step protocol to transfer RBM20-dependent splicing from rat to human. This protocol describes a PCR-based approach to adapt and quantify RBM20-dependent exon-expression of human target genes. We detail the primer design, the use of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) for RNA isolation, followed by quantification of splicing products.
Protocol
A quantitative RT-PCR protocol to adapt and quantify RBM20-dependent exon splicing of targets at the human locus

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SUMMARY
Gene splicing is a fine-tuned process orchestrated by splice factors including RNA-binding motif 20 (RBM20), and their mutations are linked to the development of cardiac diseases. Here, we provide a step-by-step protocol to transfer RBM20-dependent splicing from rat to human. This protocol describes a PCR-based approach to adapt and quantify RBM20-dependent exon-expression of human target genes. We detail the primer design, the use of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) for RNA isolation, followed by quantification of splicing products.

For complete details on the use and execution of this profile, please refer to Streckfuss-Bömeke et al. (2017).

BEFORE YOU BEGIN
Here we describe the RBM20 splice target gene Lim Domain Binding 3 (LDB3). However, we have also used this protocol for other RBM20 targets (e.g., Calcium-Calmodulin Dependent Protein Kinase II Delta (CAMKII); Sorbin and SH3 Domain Containing 1 (SORBS1)) as well as for newly identified RBM20 targets by Next Generation Sequencing. Furthermore, this protocol describes the specific steps for using human ventricular iPSC-CM with the RBM20 point mutation p.S635A. The protocol can also be used for patient-specific iPSC-CM harboring further cardiac disease-causing RBM20 mutations (e.g., p.R634W; Rebs et al., 2020), human myocardium or isolated human ventricular or atrial cardiomyocytes.

Note: Human iPSCs as well as human heart material have to be used according to the respective legal and ethical guidelines (RBM20-p.S635A-iPSC: Az. 21/1/11). iPSC and derivatives thereof are maintained in a humidified incubator at 37 °C and 5 % CO₂. All procedures are performed in Class II biosafety hoods located within laboratories rated at biosafety level 1 or 2 (depending on the virus status of iPSC lines and local regulations).

Cell culture of iPSC-CM and pellet preparation

© Timing: Differentiation of iPSC: 60–90 days; Pellet preparation: 15 min

In this protocol we use patient-specific iPSC from a dilated cardiomyopathy patient with a heterozygous RBM20 point mutation p.S635A (Streckfuss-Bömeke et al., 2017) in comparison with healthy control iPSC without RBM20 mutation (Borchert et al., 2017).
1. Cardiac differentiation of iPSC into iPSC-cardiomyocytes

Differentiate iPSC into predominantly ventricular iPSC-cardiomyocytes (iPSC-CM) by sequential manipulation of the Wnt signaling pathway. A detailed description of this protocol can be found in previous publications (Lian et al., 2013; Borchert et al., 2017; Cyganek et al., 2018). Use 60–90 days old iPSC-CM for analysis.

**Note:** This differentiation protocol produces mostly ventricular iPSC-CM (80% – 90%), with a minor population of atrial-CM (ca. 10%), a small proportion of pacemaker cells (1% – 5%), and a few non-CM. If desired, mainly atrial-CM can be produced with the addition of retinoic acid (Cyganek et al., 2018).

2. Pellet preparation

   a. Rinse the iPSC-CM with phosphate buffered saline (PBS). Add 1 mL PBS to the cells and use a cell scraper to detach the cells. Perform this step at room temperature (20 °C – 25 °C).
   b. Pipette scraped cells into a 1.5 mL tube and centrifuge for 1 min at 13.000 g at room temperature.
   c. Discard supernatant and snap freeze the pellet in liquid nitrogen. Store frozen pellets at −80 °C until usage.

**Primer design for RBM20 splice target LDB3**

© Timing: 1–2 h

RBM20 splice targets were first described in a RBM20 knockout rat model (Guo et al., 2012). It was reported that exon 5 in ldb3 is dependent on RBM20 splicing. This was verified by semi-quantitative PCR with primers spanning exon 3 to exon 9 and revealed two bands on an agarose gel (Guo et al., 2012). Based on this publication the primers used for rat are blasted against the human LDB3 locus to ascertain the location in the human sequence.

3. Blast the ldb3 primers for rat from the publication (Guo et al., 2012) against the human LDB3 sequence

   a. Use the Clustal Omega software to blast the rat ldb3 primers against the human sequence (LDB3: ENST00000623056.4). Allow for mismatches.
   b. Determine the location/exon in the human LDB3 gene (Figure 1).
c. Design primers to span the region of interest. In this case the forward primer targets exon 4 and the reverse primer exon 7 of human LDB3 (ENST00000429277.2) yielding a total of 766 bp if every exon/nucleotide is included (for LDB3: ENST00000623056.4). See key resources table for primer sequence details.

**KEY RESOURCES TABLE**

| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Critical commercial assays** | | |
| ReliaPrep RNA Tissue Miniprep Kit | Promega | Cat# Z6112 |
| QIAquick Gel Extraction Kit | Qiagen | Cat# 28706 |
| QuantiTect Reverse Transcription Kit | Qiagen | Cat# 205311 |
| **Experimental models: Cell lines** | | |
| iPSC-line | Borchert et al. (2017) | N/A |
| -healthy control line without RBM20 mutation | | |
| iPSC-line | Streckfuss-Bömeke, et al., 2017 | N/A |
| -line with RBM20 mutation p.S635A | | |
| **Oligonucleotides** | | |
| LDB3 exon 4–7 for semi-quantitative RT-PCR Fwd: CCCATTCCTCCACCA GAC | Microsynth | N/A |
| LDB3 exon 4–7 for semi-quantitative RT-PCR Rev: GAGACTGCAAGITTGGAGGAA | Microsynth | N/A |
| LDB3 exon4_5qPCR Fwd: TCAAAAGGTCCATTCCATC | Microsynth | N/A |
| LDB3 exon4_5qPCR Rev: CGGGAGAAGGCGAGGCTAAA | Microsynth | N/A |
| LDB3 expression exon3_4 (for normalization) qPCR Fwd: ACCTCGTG GTGGCCATTG | Microsynth | N/A |
| LDB3 expression exon3_4 (for normalization) qPCR Rev: GTGGAGATGGGAATGGGACG | Microsynth | N/A |
| **Software and algorithms** | | |
| Clustal Omega | EMBL-EBI | RRID:SCR_001591 https://www.ebi.ac.uk/Tools/msa/clustalo/ |
| NCBI primer blast | NCBI | RRID:SCR_003095 https://www.ncbi.nlm.nih.gov/tools/primer-blast/ |
| GraphPad Prism 8 | GraphPad Software, Inc. | RRID:SCR_002798 https://www.graphpad.com/scientific-software/prism/ |
| qPCR Cycler software | Bio-Rad | RRID:SCR_018064 |
| **Chemicals, peptides, and recombinant proteins** | | |
| SYBR Green IQ Supermix | Bio-Rad | Cat# 1708882 |
| Green GoTaq Buffer 5x | Promega | Cat# M7911 |
| Agarose | VWR | Cat# 35-1020 |
| Midori Green | Biozym | Cat# 617004 |
| DNA Page ruler 100 bp | Thermo Fisher Scientific | Cat# SM0241 |
| dNTP Mix | Bioline | Cat Bio-39029 |
| GoTaqG2 Polymerase | Promega | Cat# M784B |
| Isopropanol | Merck | Cat# 109634 |
| Tris base | CarlRoth | Cat# 5429.2 |
| Boric acid | Carl Roth | Cat# L6113 |
| EDTA0.5M pH=8.0 | AppliChem | Cat# A3145.0500 |
| Ethanol (99%) | Chemolysite | Cat# 2236.1000 |
| Nuclease-free water | Ambion | Cat# AM9937 |
| Dulbecco’s phosphate-buffered saline (PBS) | Thermo Fisher Scientific | Cat# 14190-094 |
| **Other** | | |
| Bio-Rad Sub-cell® GT Electrophoresis chamber | Bio-Rad | Cat# 1704402 |
| Cleaver Scientific nanoPAC-500 power supply | Cleaver Scientific | Cat# NANO PAC-500 |
| Bio-Rad Molecular Imager ChemiDoc™ XRS | Bio-Rad | Cat# 1708265 |

(Continued on next page)
MATERIALS AND EQUIPMENT

Alternatives: Any human iPSC-line or cardiac human tissue (harboring RBM20 mutations or not) can be used. Alternatively, other RNA/cDNA Kits suitable for iPSC-CM preparation can be used.

CRITICAL: Ethanol is a highly flammable liquid and vapor and causes severe eye irritation. Wear protective gloves and eye/face protection while handling and wash skin thoroughly after handling. If exposed or concerned: Get medical advice/attention. If inhaled: Move victim to fresh air and keep at rest in a position comfortable for breathing. If not breathing: Give artificial respiration. If on skin: Wash with plenty of soap and water then consult a physician. If in eyes: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: Get medical advice/attention. If swallowed: Immediately call a doctor/physician and rinse mouth. In case of fire: Use dry sand, dry chemical, or alcohol-resistant foam to extinguish. Store in a well-ventilated place and keep cool.

CRITICAL: Isopropanol is a highly flammable liquid and vapor. Causes serious eye irritation. When handling this substance: Ground and bond container and receiving equipment. Take actions to prevent static discharges. Keep away from heat, sparks, open flames and/or hot surfaces – No smoking. Use only in outdoors or in a well-ventilated area. Keep container tightly closed. Use explosion-proof equipment (electrical/ventilating/lighting/etc.). Use non-sparking tools. Wash parts of the body in contact with substance thoroughly after handling. Wear protective gloves and/or clothing, and eye and/or face protection. Avoid breathing the dust, fume, gas, mist, vapors or spray.

CRITICAL: Thioglycerol (added to the RNA Lysis Buffer, included in the ReliaPrep RNA Tissue Miniprep Kit) is harmful if swallowed. Toxic in contact with skin. Causes skin irritation. Causes serious eye irritation. May cause respiratory irritation. Avoid breathing dust/fume/gas/mist/vapors/spray. Wash hands thoroughly after handling. Wear protective gloves/protective clothing/eye protection/face protection. If swallowed: Call a poison center or doctor/physician if you feel unwell. If on skin: Wash with plenty of soap and water. If inhaled: Move victim to fresh air and keep at rest in a position comfortable for breathing. If in eyes: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If skin irritation occurs, get medical advice/attention. If eye irritation persists, get medical advice/attention. Take off immediately all contaminated clothing and wash it before reuse. Take off contaminated clothing and wash it before reuse.

CRITICAL: Guanidinium thiocyanate (ingredient of the RNA Lysis Buffer (ReliaPrep RNA Tissue Miniprep Kit)) is harmful if swallowed or if inhaled. Causes severe skin burns and

| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Bio-Rad CFX Connect Real-Time System qPCR Cycler | Bio-Rad | Cat# 1855201 |
| Eppendorf Centrifuge 5810 R | Eppendorf | Cat# 5811000015 |
| UV-Transilluminator | BACHOFER GmbH | Cat# IL-350-M |
| Feather Disposable Scalpel No.22 | CellPath | Cat# CAF-0022-70A |
| Cell scraper | Sarstedt | Cat# 83.3951 |
| qPCR plates | Bio-Rad | Cat# HSP9601 |
| PCR tubes | Sarstedt | Cat# 72.991.002 |
| 1.5mL tubes | Eppendorf | Cat# 0030 120.086 |
eye damage. Do not breathe dusts or mists. Wash thoroughly after handling. Do not eat, drink or smoke when using this product. Use only in outdoors or in a well-ventilated area. Wear protective gloves/protective clothing/eye protection/face protection. If swallowed: Call a poison center/doctor if you feel unwell. If swallowed: Rinse mouth. Do NOT induce vomiting. If on skin (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower. If inhaled: Move person to fresh air and keep comfortable for breathing. If in eyes: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a poison center/doctor. Wash contaminated clothing before reuse. Store locked up. Dispose of contents/container in accordance with local/national/international regulations.

△ CRITICAL: Guanidinium hydrochloride (ingredient of the Column Wash Solution (ReliaPrep RNA Tissue Miniprep Kit)) is harmful if swallowed or if inhaled. Causes skin irritation. Causes serious eye irritation. Do not breathe dust. Wear protective gloves/eye protection. If on skin: Wash with plenty of water. If in eyes: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

△ CRITICAL: UV-light at the wavelengths necessary to detect the DNA-Bands may cause skin and eye irritation, prolonged or repeated exposure has been linked to various diseases such as different types of skin cancer and the formation of cataracts. Limit access to areas where UV sources are used. Post warning signs at the entrance to labs or other work areas using UV sources. Wear protective eyewear and gloves. Cover arms and neck and limit exposure time. Never look directly at the beam. Use a manual or electronic shutter to close the beam when the source is not in use.

△ CRITICAL: Tris Base causes skin irritation. Causes serious eye irritation. May cause respiratory irritation. Avoid breathing dust/fume/gas/mist/vapors/spray. Wear protective gloves/protective clothing/eye protection/face protection. If in eyes: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing. If inhaled: If breathing is difficult, Move victim to fresh air and keep at rest in a position comfortable for breathing.

△ CRITICAL: Boric Acid has reproductive toxicity. Do not breathe dust/fume/gas/mist/vapors/spray. Wear protective gloves/protective clothing/eye protection/face protection. If exposed or concerned: Get medical advice/attention.

△ CRITICAL: EDTA is considered hazardous. Causes serious eye irritation, harmful if inhaled, and may cause damage to organs through prolonged or repeated exposure. Wear gloves and eye protection when handling. Use in well-ventilated area. Do not breathe dust/fume/Gas/mist/vapors/spray.

△ CRITICAL: In the final 1×TBE buffer the chemicals are diluted sufficiently as to make the above mentioned precautions unnecessary.

- 1×TBE buffer

| Reagent                  | Amount       |
|--------------------------|--------------|
| Tris Base                | 10.8 g       |
| Boric Acid               | 5.5 g        |
| EDTA0.5M pH=8            | 4 mL         |
| ddH₂O                    | Up to 1 L    |

Store at room temperature (20°C–25°C) for up to one month.
STEP-BY-STEP METHOD DETAILS
RNA isolation, cDNA transcription and PCR of LDB3

© Timing: 3–4 h

RNA isolation and cDNA synthesis (including the gDNA digestion) is followed by semi-quantitative PCR for LDB3.

1. RNA-isolation of 60–90 days old iPSC-CM
   For RNA isolation, use the ReliaPrep RNA Tissue Kit. Follow the manufacturer’s instructions for “Protocol for Isolation of RNA from Fibrous Tissue” (see page 3 and 4, ReliaPrep RNA Tissue Miniprep System FB145.pdf) except for the DNAse I digestion step. Perform the following steps:
   a. Dissolve the cell pellet in 500 μL lysis buffer by pipetting.
   b. Add 500 μL dilution buffer and vortex for 10 s.
   c. Centrifuge 3 min at 10.000 g.
   d. Transfer supernatant to a new 1.5 mL tube and add 340 μL isopropanol.
   e. Mix by vortexing for 10 s and transfer the solution onto the QiaAmp column.
   f. Wash the column twice with 500 μL washing buffer (centrifuge 1 min at 16.000 g and discard flow-through).
   g. Place column into a new 1.5 mL tube and add 40–50 μL water directly onto the membrane and incubate 5 min at room temperature.
   h. Centrifuge 1 min at 16.000 g and discard the column.
   i. Determine RNA concentration with a Nanodrop. Dilute the RNA with water if the concentration is higher than 200 ng/μL.

2. gDNA digestion and cDNA synthesis.
   a. Dilute 100 ng RNA in 13 μL water.
   b. Add 2 μL gDNA Removal Mix and incubate 2 min at 45°C.
   c. Add 5 μL RT mix (consisting of 4 μL RT mix and 1 μL RT enzyme).
   d. Incubate with the following program for cDNA synthesis
      i. 3 min at 25°C
      ii. 10 min at 45°C
      iii. 5 min at 85°C

   Note: The RT enzyme, gDNA Removal, and RT mix are provided by the QuantiNova RT Kit. Here, the SensoquestLabcycler was used, but any programmable thermocycler can be employed for this step.

3. PCR of LDB3
   a. Prepare 25 μL PCR reaction mix:

   | Reagent                              | Amount   |
   |--------------------------------------|----------|
   | cDNA (5 ng/μL)                       | 2 μL     |
   | green buffer (stock 5x)              | 5 μL     |
   | forward and reverse primer (stock 10 μM) | 1 μL     |
   | dNTP mix (stock 10 mM)               | 1.6 μL   |
   | Taq polymerase                       | 0.1 μL   |
   | ddH₂O                                | 14.3 μL  |
b. Run PCR.

### PCR cycling conditions for LDB3

| Steps            | Temperature | Time  | Cycles |
|------------------|-------------|-------|--------|
| Initial Denaturation | 95°C        | 3 min | 1      |
| Denaturation     | 95°C        | 30 s  | 40     |
| Annealing        | 56°C        | 30 s  |        |
| Extension        | 72°C        | 1 min |        |
| Final extension  | 72°C        | 10 min| 1      |
| Hold             | 4°C         |       | Forever|

**Note:** Since the PCR product is expected to be 766 bp (if every exon/nucleotide is included), the extension time is 1 min (1000 bp/min for Taq polymerase).

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**Gel electrophoresis and gel extraction of suitable PCR products**

@ **Timing:** 3–4 h

Run the PCR products on an agarose gel to visualize the different splice products of LDB3.

4. Gel electrophoresis
a. Following the PCR, separate the products by agarose gel electrophoresis.
   - Dissolve 1.5 g of agarose in 100 mL 1x TBE buffer (1.5% agarose gel) and boil in a microwave until the solution is clear. Add 9 µL of Midori Green to the solution and pour it into a gel casting chamber. Leave the gel to cool and harden at room temperature for 30–45 min.
   
   **Note:** The percentage of agarose in the gel is dependent on the expected size of the PCR bands.

b. Place the gel onto the tray of the electrophoresis chamber and fill the chamber with 1x TBE buffer fully covering the gel. Load the gel with 20 µL of the samples and a DNA page ruler. Connect the chamber to the power supply and perform the electrophoresis at 120 V for 90 min.

5. Gel extraction with QIAquick Gel Extraction Kit

Remove the gel from the casting chamber and place it onto the Bio-Rad Molecular Imager ChemiDoc™ XR5 (or any other Imager capable of taking images at UV-lighting). In the case of LDB3, two bands at approximately 700 bp and 250 bp become visible (Figure 2). Place the gel onto a UV-transilluminator and excise the bands under UV-lighting and transfer them into 2 mL tubes. Extract the PCR products using the QIAquick Gel Extraction Kit. Follow the manufacturer’s instructions (see https://www.qiagen.com/us/resources/resourcedetail?id=a72e2c07-7816-436f-b920-98a0ede5159a&lang=en) and elute the DNA from the QIAquick column with 25 µL nuclease-free water.

**Note:** Additional purification steps for better sequencing results are included in the instructions of the QIAquick Gel Extraction Kit.

 hü **Pause point:** Bands cut out from the gel can be stored at −20 °C for indefinite periods.

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**Sanger sequencing of PCR products and blast analysis**

@ **Timing:** 24 h

The PCR products are sequenced to determine which exons are included.
6. Sanger sequencing of the extracted DNA

Send 20 µL from the extracted DNA for Sanger sequencing analysis with the LDB3-exon 4 forward primer to Microsynth (Göttingen).

*Note:* The DNA concentration can range between 10–100 ng/µL with the Microsynth service provider.

*Alternatives:* Any other sequencing provider can be used.

7. Blast of the sequencing data

For this, the Clustal Omega webpage is used (Clustal Omega < Multiple Sequence Alignment <EMBL-EBI). Select “DNA” in the first line and subsequently paste the nucleotide sequence of human LDB3 locus as the first query in the sequences window. Use a “space” to paste the nucleotide sequence obtained from Sanger sequencing of the PCR bands underneath the first query. Use “>” before each query. Press “Submit”.

a. Blast the longer sequence (upper band from the gel) against the human LDB3 sequence and align at LDB3 exon 4, 5, 6 and 7 (Figure 2).

b. Blast the shorter sequence (lower band from the gel) against the human LDB3 sequence and align with LDB3 exon 4, 5, 6 and 7. In this transcript exon 5 is not included (Figure 2).

**△ CRITICAL:** Check, if the sequencing results match with the observation of the PCR-bands on the agarose gel. Here, the bands are approximately 400 bp apart. Exon 5 has a described length of 368 bp, which corresponds to the observation on the agarose gel (see Figure 2).

8. Design qPCR primers for expression of specific LDB3 exons

Design primers for qPCR to quantify mRNA transcripts including exon 5 (spanning exons 4 and 5) and primers against constitutive exons (spanning exons 3 and 4) to determine whole LDB3 expression for normalization (see key resources table for details of primer sequences).

*Note:* For all primer designs, the NCBI Primer-BLAST webpage is used (Primer designing tool https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

**Q-PCR of LDB3**

© Timing: 2–3 h

The RBM20-dependent exon 5 in LDB3 is quantified using qPCR and normalized against a constitutively expressed exon of LDB3.

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**Figure 2. DNA bands after PCR amplification of LDB3 using primer “LDB3 exon 4–7” for semi-quantitative RT-PCR (see key resources table) on an agarose gel.**

The two bands were excised from the gel, and the DNA bands were purified for Sanger sequencing. Sequencing results in combination with Clustalomega-based blasting demonstrated exons 4, 5, 6, 7 in the upper band and exons 4, 6, 7 in the lower band.
9. QPCR of LDB3
   a. Prepare 20 µL qPCR reaction mix containing the following: 1 µL (5 ng) cDNA, 10 µL SYBR Green mix (2X), 1 µL of each forward and reverse primer (stock 10 µM) and 8 µL of water
   b. Run qPCR.

### qPCR cycling conditions for LDB3

| Steps            | Temperature | Time  | Cycles |
|------------------|-------------|-------|--------|
| Initial denaturation | 95 °C       | 3 min | 1      |
| Denaturation      | 95 °C       | 10 s  | 40     |
| Annealing         | 60 °C       | 20 s  |        |
| Extension         | 72 °C       | 30 s  |        |
| Final denaturation | 95 °C       | 10 s  | 1      |
| Melt curve        | 60 °C – 95 °C (increment 0.5) | 5 s  | 1      |

### EXPECTED OUTCOMES

The iPSC-CM with the RBM20 mutation S635A has a higher value of LDB3-exon 5 (mean 0.01, Table 1) than the control-CM with RBM20-wt (mean 0.007, Table 1). This means that cells with the RBM20 mutation p.S635A show a tendency for more LDB3 transcripts that include exon 5 (Figure 3). Vice versa, the control-iPSC-CM have more LDB3 transcripts without exon 5 (Figure 3), which underscores the physiological role of RBM20 in the exclusion of exon 5 in LDB3 splicing in healthy cardiomyocytes/heart.

This protocol is designed to verify and investigate RBM20-dependent splice targets from previous publications into the human model system by using standard PCR-based techniques.

**Note:** The values are not derived as technical replicates from the same sample, but from different independent cardiac differentiations from the patient-specific or control iPSC-line.

### QUANTIFICATION AND STATISTICAL ANALYSIS

© Timing: 2–3 h

1. **Quantification of the qPCR data.** Here, the Cq-value for the LDB3 exon 4/5 is normalized against the expression level (Exon 3/4) of the LDB3 gene itself (instead of using a housekeeping gene).

   **Note:** A housekeeping gene is also analyzed to evaluate the quality of the RT reaction. For this, we use GAPDH. Typical Cq-values for 60–90 days old iPSC-CM are 18–21 for GAPDH.

### LIMITATIONS

In this set of experiments, we use 60–90 days old iPSC-CM. These cells represent a fetal/neonatal phenotype. Therefore, the expression pattern of splice isoforms may differ if adult cardiomyocytes (e.g., from heart biopsies) are used in this assay.

To increase the performance of the statistical analysis, a higher n number of at least 6 different cardiac differentiations should be assessed.

### TROUBLESHOOTING

**Problem 1**

No or additional bands are visible on the gel (see steps 4 and 5) **Gel electrophoresis**
Table 1. Cq-values for the quantification of LDB3-exon 5 inclusion

| iPSC-CM            | Cq value LDB3_3/4 expression | Cq value LDB3_Exon4/5 | Delta Cq | $2^{-\Delta Cq}$ |
|--------------------|------------------------------|-----------------------|----------|------------------|
| RBM20_S635A_1      | 30.5                         | 23.9                  | 30.5-23.9 = 6.6 | $2^{-6.6} = 0.0105$ |
| RBM20_S635A_2      | 28.8                         | 22.2                  | 28.8-22.2 = 6.58 | $2^{-6.58} = 0.0104$ |
| Control1_RBM20-wt  | 33.5                         | 25.9                  | 33.5-25.9 = 7.6 | $2^{-7.6} = 0.0050$ |
| Control2_RBM20-wt  | 30.3                         | 23.4                  | 30.3-23.4 = 6.89 | $2^{-6.89} = 0.0084$ |
| Control3_RBM20-wt  | 35.1                         | 28.3                  | 35.1-28.3 = 6.89 | $2^{-6.98} = 0.0079$ |

Potential solution

Should there be either no band or additional bands visible in the gel after the electrophoresis, perform a gradient-PCR and/or calculate the annealing temperature of your primer using the nearest neighbor method.

Problem 2

This protocol is used to establish another RBM20-splice target (e.g., SORBS1 or CACNA1C) but there are only one or more than two bands on the agarose-gel (see steps 4 and 5). Gel electrophoresis

Potential solution

If an unexpected number of bands are obtained for another RBM20-splice target, these bands should nevertheless be cut out and sequenced. With this, it can be verified if the primers bound to the desired gene and what exons are included. Here, two points have to be considered: Firstly, even if the targets described from mouse or rat models are considered homologous to human, it is not guaranteed that isoform expression in humans is equivalent to the animal model. Secondly, the maturation time point of the iPSC-CM might not be optimal. Therefore, this assay should be repeated with 30 days, 60 days and 90 days-old iPSC-CM.

Problem 3

No/unclear sequencing results (see step 6) as exemplified in Figure 4. Sanger sequencing of the extracted DNA

Potential solution

In case of unclear sequencing results, extend the running time of the electrophoresis until the loading dye has reached the lower end of the gel to achieve clearer separation of the PCR products. Alternatively, the agarose concentration of the gel can be increased to 2% to achieve better separation of the DNA bands. Also, perform the additional purification steps described in the instructions of the QIAquick Gel Extraction Kit, which includes extra washing steps with the washing buffer.

Try to keep the time the agarose gel is exposed to UV-light as short as possible. Exposition for longer periods of time leads to double-strand breaks in your PCR-product and subsequently lowers the quality of the sequencing results.

Problem 4

The cq values from the qPCR analysis vary significantly for the housekeeping gene among the samples (see steps 9 and 10). QPCR of LDB3

e.g.,

Sample a: cq-GAPDH: 18.3

Sample b: cq-GAPDH: 25.8

Sample c: cq-GAPDH: 18.4
Potential solution
In the example mentioned above the highcq value of GAPDH in sample b indicates that the PCR run
and/or RT reaction was unsuccessful. First, the PCR run should be repeated and if the result is
unchanged, the RT reaction for this sample has to be repeated. RNA purity and concentration should
be analyzed again.

Problem 5
Thecq values for LDB3_exon3/4 are negative or higher than 36, but the GAPDH value is comparable
to other samples (see steps 9 and 10). QPCR of LDB3

Potential solution
If thecq value for LDB3 is negative or higher than 36 (which is also interpreted as negative) indicates
that the RT reaction or qPCR run was unsuccessful. However, if a cq-value for the housekeeping
gene GAPDH is comparable to the other samples, the quality of the iPSC-CM differentiation batch
has to be questioned. Cardiac differentiation of iPSC does not yield 100% cardiomyocytes and the
quality of every differentiation batch has to be monitored. Since thecq value varies on many factors
like the RNA amount, RT method and/or PCR cycler used, no standard values for cardiac marker
genes are available to test the quality of a cardiac differentiation on the mRNA level. In general,
the quality of an iPSC-CM batch needs to be assessed via immunofluorescence staining or flow
cytometry with general cardiac markers like troponin C or myosin-light chain. We discard any
iPSC-CM differentiation with a cardiac-specific marker expression lower than 85%. In addition, the
amount of ventricular and atrial iPSC-CM should be analyzed for each cardiac differentiation exper-
iment via immunofluorescence by using antibodies against the ventricular marker MLC2v and the
atrial marker MLC2a.
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact. Prof. Dr. Katrin Streckfuss-Bömeke; katrin.streckfuss-boemeke@uni-wuerzburg.de

Materials availability
This study did not generate new unique reagents.

Data and code availability
The published article includes all [datasets/code] generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS
S.R. drafted the manuscript and conceived the protocol. T.B. contributed to manuscript writing. K.S.B. supervised the experimental studies, edited the manuscript, and provided funding support.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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