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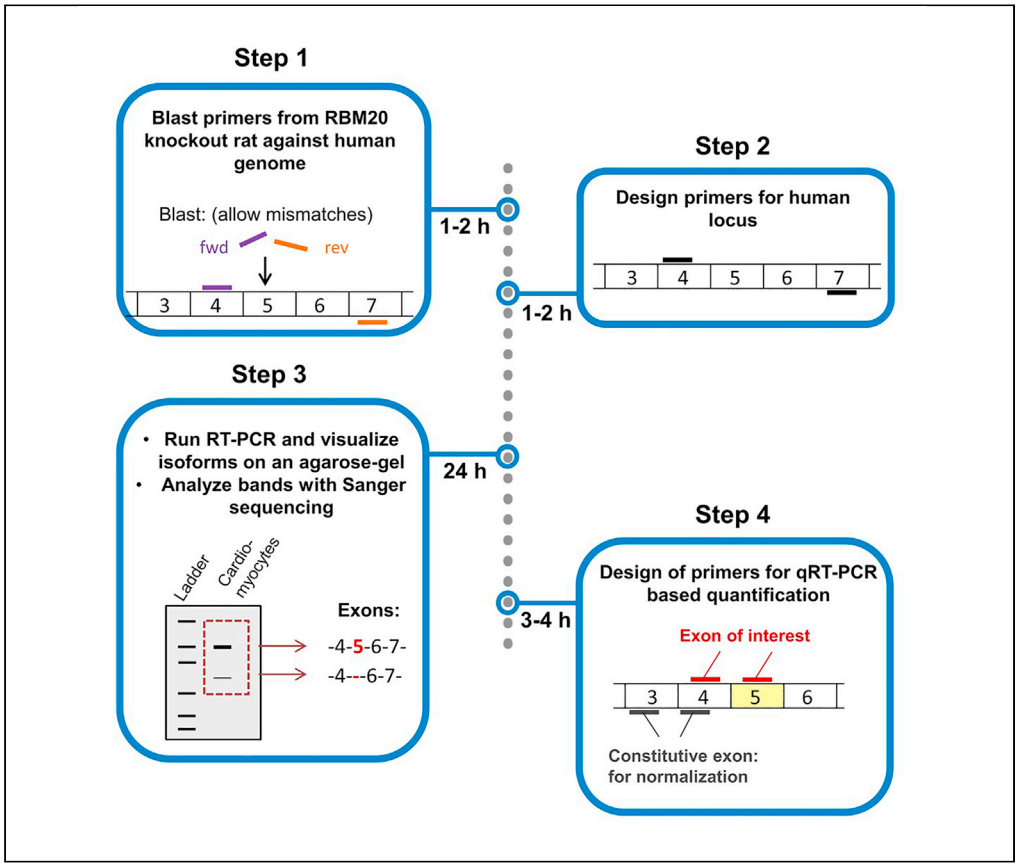
A quantitative RT-PCR protocol to adapt and quantify RBM20-dependent exon splicing of targets at the human locus



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.

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Highlights

Humanized primer usage to detect multiple splicing products by RT-PCR

Use of Sanger sequencing to annotate the exons included within a splicing product

Detailed description for primer design to quantify specific exon expression by qRT-PCR

Use of patient-specific iPSC-CM recapitulating RBM20-based dilated cardiomyopathy

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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 (*CAMKIIδ)*,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 % CO_2. 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).

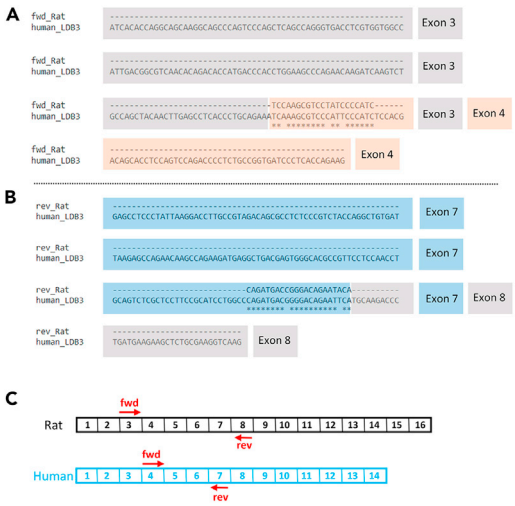

Figure 1. Blast of *ldb3* primers for rat against the human *LDB3* locus

(A) The *ldb3* forward primer for rat (Guo et al., 2012) was blasted against the human *LDB3* locus. The primer shows 3 mismatches compared to the sequence of the human exon 4.

(B) The *ldb3* reverse primer for rat (from Guo et al., 2012) was blasted against the human *LDB3* locus. The primer shows 2 mismatches compared to the sequence of the human exon 7. The color code in A and B is related to the human *LDB3* locus.

(C) Schematic summary of localization of primers for *ldb3* in rat (from Guo et al., 2012) in ENSRNOT0000083623.1 and the localization on the human locus in *LDB3* in ENST00000623056.4.

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
QuantiNova Reverse Transcription Kit	Qiagen	Cat# 205311
Experimental models: Cell lines		
iPSC-line -healthy control line without RBM20 mutation	Borchert et al. (2017)	N/A
iPSC-line -line with RBM20 mutation p.S635A	Streckfuss-Bömeke, et al., 2017	N/A
Oligonucleotides		
LDB3 exon 4–7 for semi-quantitative RT-PCR Fwd: CCCATTCCCATCTCCACGAC	Microsynth	N/A
LDB3 exon 4–7 for semi-quantitative RT-PCR Rev: GAGACTGCAGGTTGGAGGAA	Microsynth	N/A
LDB3exon4_5qPCR Fwd: TCAAAGCGTCCCATTCCCATC	Microsynth	N/A
LDB3exon4_5qPCR Rev: CGGGAGAAGGCAGGGCTAAA	Microsynth	N/A
LDB3 expression exon3_4 (for normalization) qPCR Fwd: ACCTCGTGGTGGCCATTG	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/
GrapdhPad 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 5×	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%)	Chemsolute	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# NANOPAC-500
Bio-Rad Molecular Imager ChemiDoc™ XRS	Bio-Rad	Cat# 1708265

(*Continued on next page*)

Continued

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

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. May cause drowsiness or dizziness. 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: Remove 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

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
ddH_2O	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 5×)	5 µL
forward and reverse primer (stock 10 µM)	1 µL
dNTP mix (stock 10 mM)	1.6 µL
Taq polymerase	0.1 µL
ddH_2O	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).

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 1×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 1×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™ XRS (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.

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

Sanger sequencing of PCR products and blast analysis

Timing: 24 h

The PCR products are sequenced to determine which exons are included.

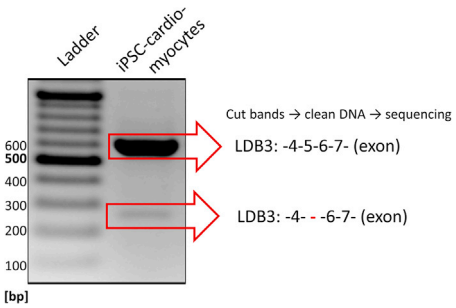

Figure 2. DNA bands after PCR amplification of *LDB3* using primer "LDB3 exon 4–7" for semi-quantitative RT-PCR (see key resources table) on an agarose gel

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.

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.

9. QPCR of LDB3
   a. Prepare 20 µL qPCR reaction mix containing the following: 1 µL (5 ng) cDNA, 10 µL SYBR Green mix (2×), 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*_Exon4/5	Cq value *LDB3*_3/4 expression	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 = 6.98	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

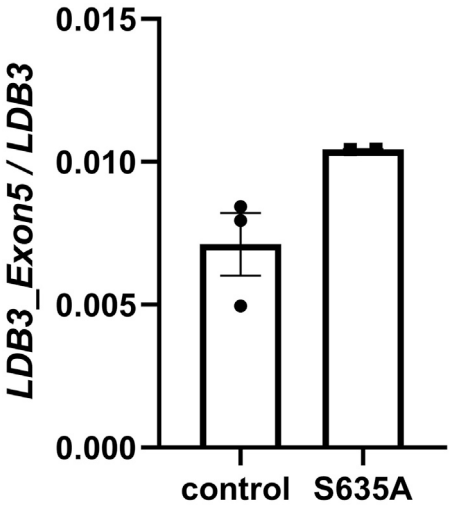

Figure 3. Relative Expression of *LDB3*-exon 5
Differential mis-splicing for exon 5 in *LDB3* in RBM20_S635AiPSC-CM. Every dot represents one differentiation experiment. Data is shown as bar graph with standard error (control).

Potential solution

In the example mentioned above the high cq 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

The cq 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 the cq 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 the cq 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 experiment via immunofluorescence by using antibodies against the ventricular marker MLC2v and the atrial marker MLC2a.

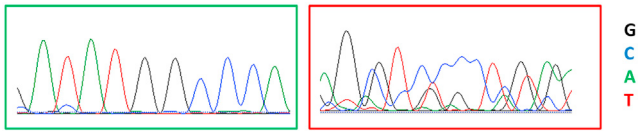

Figure 4. Example tracks of good and bad sequencing results
Left: Example of a good Sanger sequencing result. The peaks of the respective nucleotides are clearly separated. Right: Example of a bad Sanger sequencing result. Nucleotide peaks are super imposed, and no clear sequence can be retrieved.

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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