Chapter 14

Evaluation of Exon Skipping and Dystrophin Restoration in In Vitro Models of Duchenne Muscular Dystrophy

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Abstract

Several exon skipping antisense oligonucleotides (eteplirsen, golodirsen, viltolarsen, and casimersen) have been approved for the treatment of Duchenne muscular dystrophy, but many more are in development targeting an array of different *DMD* exons. Preclinical screening of the new oligonucleotide sequences is routinely performed using patient-derived cell cultures, and evaluation of their efficacy may be performed at RNA and/or protein level. While several methods to assess exon skipping and dystrophin expression in cell culture have been developed, the choice of methodology often depends on the availability of specific research equipment.

In this chapter, we describe and indicate the relevant bibliography of all the methods that may be used in this evaluation and describe in detail the protocols routinely followed at our institution, one to evaluate the efficacy of skipping at RNA level (nested PCR) and the other the restoration of protein expression (myoblot), which provide good results using equipment largely available to most research laboratories.

Key words Dystrophin, Antisense oligonucleotide, Duchenne muscular dystrophy, Eteplirsen, Myoblot, Exon skipping

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1 Introduction

Duchenne muscular dystrophy (DMD) is an X-linked inherited neuromuscular disorder caused by mutations in the dystrophin gene (*DMD*) and characterized by rapid progression of muscle weakness and loss of ambulation in early adolescence. Most *DMD* mutations are deletions that disrupt the open reading frame (ORF), preventing the synthesis of dystrophin protein. On the other hand, Becker's muscular dystrophy (BMD) is a milder neuromuscular disorder caused by in-frame mutations in the same gene. In this case, mutations do not disrupt the ORF and a shorter but semi-functional form of dystrophin is produced resulting in a milder form of disease [1, 2].

The reading-frame rule, exemplified in Becker patients, is the basis of the concept of exon skipping by antisense oligonucleotides (AONs) as a possible therapy for DMD. In this case, AONs are designed to attach to specific RNA sequences and disrupt the binding of the spliceosome, which in turn causes the skipping of a particular exon and the restoration of the expression of a truncated, but semi-functional, dystrophin [3]. This therapeutic approach could benefit around 83% of DMD patients, but due to the vast array of different *DMD* deletions and mutations causing DMD, to treat each subset of DMD patients, different exons would need to be targeted by specific AONs [4, 5]. Eteplirsen, golodirsen, viltolarsen, and casimersen are AONs that facilitate the skipping of *DMD* exons 51, 53, 53, and 45 respectively, and have been approved by the FDA (2016, 2019, 2020, and 2021) [6–10]. These drugs would only be applicable to 13% (eteplirsen) and 8% of DMD patients (golodirsen, viltolarsen, and casimersen). New AONs, skipping other exons, are being designed for the remaining deletions that may benefit from this approach [11–14].

The preclinical development of new exon-skipping AONs relies on the use of cell cultures from patients harboring specific deletions. It is common practice to design a panel of candidate AONs and evaluate their efficacy in culture before advancing to preclinical testing, and many methods are followed to evaluate them, at both RNA and protein levels, as represented in Fig. 1.

1.1 Evaluating Exon Skipping at RNA Level

When evaluating exon skipping at RNA level, several methods, both quantitative and semi-quantitative, are routinely used. A recent publication compared five different quantification techniques: quantitative real-time PCR (qPCR) [18], digital droplet PCR (ddPCR) [23], single PCR assessed with an Agilent bioanalyzer, and nested PCR with agarose gel image analysis [15–17]. They concluded that ddPCR was the most precise and less dispersed quantitative method for exon-skipping detection, while the other techniques may overestimate exon skipping and present high data variation.

The ddPCR protocol is based on the mix of the DNA sample with an oil–water media to divide the template in several thousand droplets. Each droplet contains none or one single template strand that would provide individual amplification reactions and absolute quantification. It is a highly reproducible and efficient technique as it does not depend on the PCR efficiency and may be adapted to quantify exon skipping using specific probes. Nevertheless, the technique requires highly specific equipment not available in most laboratories [23].

Quantitative real-time PCR for *DMD* transcripts uses custom probes to specifically detect skipped transcripts that are lately amplified for transcript quantification. Data are normalized with endogenous transcript controls but the requirement of a pre-amplification

Fig. 1 Methods that may be used to evaluate exon skipping in cell culture. Currently, the most accurate methods are digital droplet PCR (ddPCR) and capillary western immunoassay (Wes), but the equipment required to perform these experiments is not present in the majority of research laboratories. Advantages (green) and disadvantages (red) of each method are presented in this figure. Main bibliographical references for these methods are: RT-PCR followed by gel densitometry analysis [15, 16], single-round PCR followed by bioanalyzer [16, 17], qPCR [18], ddPCR [13], capillary western immunoassay (Wes) [19], Western blotting [20], myoblots [21]. Several attempts to compare these methods have been published by a consortium to evaluate dystrophin protein quantification methods [20] and another to compare methods to quantify exon skipping [17]. A recent meeting to find consensus among stakeholders on all dystrophin protein quantification methods described was also recently published [22]. Created with [Biorender.com](https://biorender.com)

step and specific probes for each of the deletions tested may decrease linearity and increase costs [18].

Most methods are semi-quantitative at best and provide an indication of efficacy that should be evaluated further by assessing the downstream consequences of exon skipping (dystrophin restoration). Our approach combines the study of exon skipping at RNA level by a rather simple nested PCR plus gel densitometry analysis method, with a quantitative evaluation of dystrophin protein by myoblots.

1.2 Evaluating Dystrophin Restoration in Cell Culture

Dystrophin protein quantification can be challenging due to its large size (3685 amino acids) and expression pattern, and it has been an extremely hot topic when evaluating the results of dystrophin restoration clinical trials [24]. A recent meeting of stakeholders interested in dystrophin quantification methodology highlighted the problems derived from the variability of dystrophin

Fig. 2 Workflow of an exon skipping evaluation experiment. *RNA evaluation*: a 6-well plate is seeded and transfected with two different AON concentrations. Cells are collected after 48 h for RNA extraction, reverse transcription (RT), and nested PCR analysis by densitometry. *Dystrophin protein quantification*: a 96-well plate is seeded, transfected, and allowed to differentiate for a week. Plates are fixed before being analyzed by myoblot. Created with [Biorender.com](https://biorender.com)

levels between different muscles and individuals as well as the lack of standardized controls [22]. When analyzing preclinical in vitro experiments, other points need to be considered: patient-derived cell cultures are difficult to expand and differentiate, and dystrophin expression is only detectable when cells are differentiated into myotubes. Western blotting analysis from cultured cells requires very large amounts of protein lysate that does not produce good quantitative results while using a lot of sample.

A novel advance in protein quantification is the capillary Western immunoassay (Wes), based on the use of capillary as separation modules for protein isolation followed by dystrophin detection by an immunoassay that allows accurate quantification. The Wes system is said to detect low protein concentrations, in a range of 0.125–1.25 μg [19, 22]. However, this equipment is not yet commonly found in most laboratories.

We detail in this chapter the quantitative method developed at our department: myoblots, a method based on the in-cell western blotting optimized for the quantification of muscle proteins in cell culture [21]. Myoblots are performed in 96-well plates seeded with patient-derived cells, which are allowed to differentiate. Signal is normalized by cell number, and the differentiation status of the cultures is assessed as a quality control of the experiment.

A summary of our strategy to evaluate exon skipping drugs in cell culture is shown in Fig. 2. In the example used in this chapter, we evaluated two concentrations of an AON skipping exon 51 on immortalized cell cultures [25] from a patient harboring a mutation that causes the deletion of exon 52 from the CNMD Biobank (London, UK).

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2 Materials

2.1 Cell Culture and Transfection

1. Standard tissue culture facilities.
2. Low background fluorescence and low evaporation 96-well plates with clear, flat bottom.
3. The AON used in the example was synthesized by Eurogentec, Belgium with the following sequence: 5'-[T*C*A*-A*G*G*-A*A*G*-A*T*G*-G*C*A*-T*T*T*-C*T]-3'; where * is a 2' MOE phosphorothioate linkage. AON stock at 100 μ M in distilled H₂O is stored at -80 °C.
4. SMMC: Skeletal Muscle Cell Growth Medium Complete. SMMC is supplemented with 5% Supplement Mix, 10% fetal bovine serum, 1% GlutaMax (dipeptide L-alanine-L-glutamine), 2% PenStrep, and 0.06% Gentamicine.
5. DMEM: Dulbecco's Modified Eagle Medium.
6. Opti-MEM Reduced Serum Media.
7. Differentiation medium: DMEM supplemented with 2% horse serum.
8. 0.05% Trypsin-EDTA.
9. Lipofectamine™ 2000 Transfection Reagent.

2.2 RT and Nested PCR Analysis

1. Access to thermocycler, electrophoresis equipment, and gel documentation system.
2. Nanodrop or other equipment to measure RNA concentration.
3. RNeasy Mini Kit.
4. SuperScript™ IV First-Strand Synthesis System (*see Note 1*).
5. PCR Master Mix including: 33.5 μ l RNase free-water, 5 μ l 10 $\times$ PCR Buffer, 3 μ l 50 mM MgCl₂, 1 μ l 10 mM dNTPs, 0.5 μ l Taq DNA Polymerase recombinant (5 U/ μ L), plus two sets of specific primers (10 μ M) described in Table 1 (*see Note 2*).
6. Agarose.
7. 1 $\times$ TAE buffer.
8. SYBR safe DNA gel stain.
9. 5 $\times$ PCR Loading Buffer.

Table 1
Primer sequences

Primers			Sequence	Length
RT	55R	Exon 55 reverse	AACTGCCAATGTCCTACAGGAT	22 bases
First PCR	48F	Exon 48 forward	AAAAGACCTTGGGCAGCTTG	20 bases
	54R	Exon 54 reverse	TGACTTGGCCCTGAAACTTC	20 bases
Second PCR	49F	Exon 49 forward	CCAGCCACTCAGCCAGTG	18 bases
	53R	Exon 53 reverse	CCTTCAGAACCGGAGGCAA	19 bases

Specific primer 55R was used in the RT reaction. Two different sets of primers targeting regions between exons 48F-54R (first PCR) and 49F-53R (second PCR) were used for the nested-PCR method

- 10. HyperLadder 100 bp.
- 11. Gel extraction kit to isolate PCR fragments for sequencing.

2.3 Myoblots

- 1. Access to an Odyssey CLx Scan (LI-COR Biosciences).
- 2. Orbital shaker.
- 3. Ice-cold methanol.
- 4. 1× PBS.
- 5. Permeabilization Buffer (PB): 1× PBS, Triton 0.1%.
- 6. Intercept® Blocking Buffer (LI-COR Biosciences).
- 7. Washing Buffer (WB): 1× PBS, Tween 0.1%.
- 8. Primary antibodies:
  - (a) MF20 antibody (Developmental Studies Hybridoma Bank (DSHB)).
  - (b) Mandys1, kindly provided by Prof. G Morris, The MDA Monoclonal Antibody Resource.
  - (c) Mandys106, kindly provided by Prof. G Morris, The MDA Monoclonal Antibody Resource.
  - (d) Dys1 (Leica Biosystems).
- 9. Secondary antibodies:
  - (a) Goat Anti-Mouse IgG H&L (Biotin, Abcam).
  - (b) IRDye 800CW Streptavidin (LI-COR Biosciences).
  - (c) IRDye 800CW Goat anti-Mouse IgG (LI-COR Biosciences).
  - (d) CellTag™ 700 Stain from (LI-COR Biosciences).

3 Methods

3.1 Cell Culture and Transfection

1. Cells are seeded in 6-well plates: 350,000 cells per well or 96-well plates: 7500 cells per well in SMMC:DMEM (1:1) (*see Note 3*).
2. The following day, when 80% confluence is reached, growth medium is replaced by differentiation medium (DM).
3. On day 3, AON transfection is performed as follows [18]. Some examples of the distribution of the experimental conditions in the 96-well plate are described in Fig. 3 (*see Note 4*).
4. Remove DM from wells.
5. Wash with PBS.
6. Add 50 μ l of Opti-MEM per well in the 96-well plate and 1 ml per well in the 6-well plate.
7. Incubate at 37 °C and 5% CO₂ while preparing the following steps.
8. Dilute the AON to 100 μ M in distilled H₂O.
9. Prepare mixes for transfection. Volumes in this example are calculated for a single well of a 6-well plate and two different antisense conditions, 100 nM and 300 nM, adjust the volumes for the number of replicates. In 96-well plates, adjust volumes for a final volume of 50 μ l per well.
10. The transfection is performed with Lipofectamine 2000® Reagent using 2 μ l per 1 ml of final transfection volume. In this particular example, volumes are calculated as: prepare one tube with 250 μ l of Opti-MEM and 1 μ l of AON at 100 μ M (for AON 100 nM condition), another with 250 μ l of Opti-MEM and 3 μ l of AON at 100 μ M (for AON 300 nM condition), a third tube with 250 μ l of Opti-MEM alone (no AON control), and a fourth tube with 750 μ l Opti-MEM and 6 μ l of lipofectamine.
11. Add 250 μ l of the lipofectamine mix to each AON tube and incubate all three tubes (100 nM, 300 nM, and lipofectamine only) for 30 min at RT to allow for complex formation.
12. Add 500 μ l of Opti-MEM to each tube for a final transfection volume of 1 ml per well of the 6-well plate.
13. Add 1 ml of transfection mix to each well of the 6-well plate and 50 μ l of mix to each well of the 96-well plate. Incubate for 5 h at 37 °C and 5% CO₂.
14. Five hours after AON transfection, medium is changed to differentiation medium, and plates are placed again in the incubator.

Fig. 3 Examples of plate distribution for exon skipping evaluation at protein level. **(a)** The four experimental conditions are distributed as follows in the 96-well plate: 2 columns for control (Lipofectamine only), 2 columns for DM (differentiation medium only), 4 columns for 100 nM AON, and 4 columns for 300 nM AON, both transfected with Lipofectamine. **(b)** The four experimental conditions are distributed as follows in the 96-well plate: 1 row for control (Lipofectamine only), 1 row for DM (differentiation medium only), 3 rows for 100 nM AON, and 3 rows for 300 nM AON, both transfected with Lipofectamine. **(c)** Reduction of the edge effect by filling edge wells with 100 μ l of preferred buffer, but not cells. Then, the four experimental conditions are distributed as follows in the 96-well plate: 1 row for control (Lipofectamine only), 1 row for DM (differentiation medium only), 2 rows for 100 nM AON, and 2 rows for 300 nM AON, both transfected with Lipofectamine. Each experimental condition will be probed with different antibodies in the 800-nm channel (green): the majority of wells will be probed with a dystrophin antibody mix, some with an MF20 antibody (experimental quality control to assess differentiation), and the remaining wells will have no primary antibody to eliminate background. In the 700-nm channel (red), every well will be probed with a cell number stain (CellTag™ 700 Stain) to allow for cell number normalization. Created with [Biorender.com](https://biorender.com)

15. After 48 h, cell pellets are collected from 6-well plates for RNA extraction using RNeasy Mini Kit to be used in the nested PCR (*see* Subheading 3.2).
16. Incubate the 96-well plates for 7 days and fix with ice-cold methanol. Myoblots can be performed immediately, or plates

may be stored in PBS at 4 °C until analysis (*see* Subheading 3.3).

3.2 Nested PCR and Gel Image Analysis Method

3.2.1 Reverse Transcription

1. After RNA extraction, RNA concentrations are measured with Nanodrop and 1 µg of template RNA is used for reverse transcription (RT).
2. RT is performed following the SuperScript™ IV First-Strand Synthesis System manufacturer's instructions with a specific primer (*see* **Note 5**), as described in Table 1.

3.2.2 Nested PCR

1. For the first PCR, prepare the PCR mastermix and add 2 µl of each primer of the first set of primers at 10 µM. In the example they target exon 48 and 54 (*see* Fig. 4a for primer location strategy).
2. Add 3 µl of cDNA (direct RT product) to the PCR mastermix.
3. Run the first PCR as follows: 94 °C for 5 min, 30 cycles of 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 1 min and 20 s, and finally, 72 °C for 7 min.
4. Three microliters of the first PCR product are used as template in the second PCR, which has the same PCR mastermix plus 2 µl of each of the second pair of primers at 10 µM (49F and 53R in the example) (*see* **Note 6**).
5. The second PCR (or nested) should be run as follows: 94 °C for 5 min, 35 cycles of 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 1 min and 20 s, and finally, 72 °C for 7 min.
6. Run a 2% agarose gel with the whole PCR product (50 µl) for 1 h at 100 mV.
7. Place gel on the transilluminator of the gel documentation system and acquire the image. A representative image is shown in Fig. 4b. This figure shows the skipped and non-skipped product highlighting the bands selected for quantification.
8. Cut the relevant PCR bands for DNA extraction and verification by Sanger sequencing.

3.2.3 Band Semi-Quantification

For the image analysis, if the available gel documentation system has quantification software you may use this, if not you may quantify the intensity of the bands with image J (<https://imagej.nih.gov/ij/download.html>):

1. Select the lanes that include the relevant bands using the region of interest (ROI) selection tool.
2. Use the gel analysis tool to create Analyze>Gels>Plot lanes.
3. Draw lines to separate the peaks corresponding to the required bands and use the wand tool to select them.

Fig. 4 Nested PCR results. **(a)** Skipped and non-skipped product schematic representations. Primers 48F and 54R were used in the first PCR and primers 49F and 53R in the second. Skipped product size is reduced due to the lack of exon 51 and 52 while the non-skipped product is just lacking exon 52. Created with [Biorender.com](https://www.biorender.com). **(b)** Agarose gel image from AON transfection. Fifty microliter of PCR product were examined in a 2% agarose gel stained with SYBRSafe. Image was captured in a Gel Doc™ EZ Imager (BIORAD). Red boxes indicate the quantified bands. The described relative percentage of exon skipping for 100 nM AON was 95.41% and for 300 nM AON was 53.62%

4. Analyze by clicking Analyze>Gels>LabelPeak to obtain each peak's area.
5. Normalize peak areas by amplicon sizes and calculate exon skipping percentages according to the formula [17]:

$$\text{Exon skipping\%} = \frac{(\text{normalized peak area skipped fragment})}{(\text{normalized peak area skipped fragment}) + (\text{normalized area non - skipped fragment})} \times 100$$

3.3 Myoblot Method

In our example, we are interested in dystrophin restoration, and the plate will be probed with the different antibodies as described in Fig. 3a.

3.3.1 Myoblot Procedure

1. If plates were stored at 4 °C, remove PBS. If not, remove methanol and wash with PBS.
2. Add 100 µl of Permeabilization Buffer (PB) per well.
3. Place the plates in an orbital shaker at RT for 5 min.
4. Remove PB and repeat four times the previous process.
5. Remove PB and add 50 µl of Intercept® (PBS) blocking buffer per well.
6. Incubate for 2 h in the orbital shaker at RT.
7. Remove blocking buffer.
8. Add 50 µl of the corresponding primary antibodies diluted in the blocking buffer. In the example (Fig. 3a), plate distribution was the following:
  - (a) Sixty wells for dystrophin detection. Add a dystrophin-Ab mix: Mandys1, Mandys106, and Dys1 at 1/100 dilution.
  - (b) Twenty-four wells for the differentiation marker. Add MF20 antibody at 1/50 dilution.
  - (c) Twelve wells as negative controls without any primary antibody. Add 50 µl of blocking buffer.
9. Incubate overnight at 4 °C in the orbital shaker.
10. Remove antibodies and add 100 µl per well of Washing Buffer (WB).
11. Wash for 5 min in the orbital shaker at RT.
12. Remove WB and repeat the wash four times.
13. For dystrophin detection, an amplification of the signal is needed. Biotin antibody (1/2000 dilution in blocking buffer) is used for signal amplification in those wells where dystrophin mix is added and also to 6 wells of the negative controls: after 1 h incubation at RT with this antibody, WB washes are repeated four times more as indicated in steps 10–12.
14. Add 50 µl of the corresponding secondary antibody diluted in the blocking buffer.
  - (a) Add CellTag™ 700 Stain to all secondary antibody mixes at 1/1000 dilution.

- (b) For dystrophin mix amplified with biotin: add IRDye 800CW Streptavidin at 1/2000 dilution. Also added to 6 wells of the negative controls where biotin was added before (background control for the streptavidin secondary).
  - (c) For MF20: add IRDye 800CW Goat anti-Mouse IgG at 1/500 dilution. Also added to 6 wells of the negative controls (background control for the goat antibody).
15. Incubate for 1 h at RT in the orbital shaker.
  16. Remove the secondary antibodies and add 100 µl of WB per well.
  17. Wash for 5 min at the orbital shaker at RT.
  18. Remove the WB and repeat the wash process four times.
  19. Add 100 µl of PBS per well and prepare the Odyssey CLx Scan (*see* **Note 7**).

3.3.2 Myoblot Analysis

1. In the Image Studio™ Software from your computer, select “In Cell Western analysis” and start the acquisition.
2. Once acquired, align channels using the Image Studio™ Software.
3. Select all the wells in the “ICW wells” tab, and in the lower menu, go to “Grid Sheet” Tab and click in Copy (*see* **Note 8**).
4. Paste intensity measurements in an Excel sheet. Figure 5a shows an example of the raw data directly copied, without any modification, to an Excel sheet.
5. Revise the plate image and the raw intensity measurements to discard any outliers such as scraping the cells with the pipette tip or antibody specks, as in the case of the highlighted value represented in Fig. 5b, in which the antibody specks increase the 800-nm signal of the well. These wells are not included in the calculations, and they are highlighted for auditing purposes.
6. As shown in Fig. 5a, select in the 800-nm channel the rows without primary antibody to calculate the average background intensity.
7. Subtract from each sample’s intensity measurement acquired at the 800-nm channel the corresponding average background intensity value.
8. Normalize the previous values, dividing them by the corresponding intensity value measured in the 700-nm channel (CellTag™ 700 Stain), as shown in Fig. 5a (*see* **Note 9**).
9. Calculate average and SEM for each condition tested (i.e., non-treated, 100 nM AON and 300 nM AON).

Fig. 5 In cell Western analysis. **(a)** Readings performed by the Odyssey Scan at 700 nm (Red, CellTag™ 700 Stain) and 800 nm (Green, Dystrophin, MF20 and background) provide a single intensity value for each well at each channel. To normalize each value, the corresponding background average value (no primary antibody) is subtracted from each value and later divided by its corresponding one for the CellTag™ 700 Stain signal (700 nm value). Created with [Biorender.com](#). **(b)** Example of omitted wells by antibody specks (marked with arrows) and its corresponding eliminated value. **(c)** Bar graph of data: average ± SEM of the two AON concentrations against control (lipofectamine only) and analysis by two-way ANOVA with multiple comparisons (**** $p < 0.0001$)

10. Evaluate MF20 values (as a measurement of culture differentiation) to critically assess the experiment: poor differentiation of the cultures may render unacceptable results (*see* **Note 10**).
11. Plot mean ± SEM in a bar graph as represented in Fig. 5c.

3.4 Concluding Remarks

The two methods described are complementary in their assessment of the exon skipping capacity of an AON: semi-quantification of the skipped product at RNA level plus dystrophin quantification at

protein level. As seen in the example we used, they show concordant results supports AON suitability for dystrophin restoration in preclinical studies.

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4 Notes

1. Different RNA extraction and RT-PCR kits or techniques may be used according to the usual protocols of the laboratory.
2. The AON used in this example skips exon 51, and the cell culture is missing exon 52. We are using primers in the vicinity; these will need to be adapted to the specific regions studied. Many different primer combinations may be used, but they should always produce a larger product in the first round and a smaller one in the second one. In our example, the first PCR primers (Table 1) target exons 48 (forward) and 54 (reverse), and the product of this reaction will be the template of the second PCR, using primers 49 (forward) and 53 (reverse). The final PCR product that will be visualized by gel electrophoresis will include exons 49, 50, 51, and 53 if not skipped, and the skipped product contains exons 49, 50, and 53 (Fig. 4a).
3. Cell number in 96-well plates for myoblot analysis should be adjusted as required depending on the proliferation rate and differentiation facility of the cell culture used. To improve cell attachment, it is advisable to incubate the plates for an hour at RT.
4. An edge effect might be observed when analyzing some plates. In that case, the plate layout in Fig. 3c is recommended in order to avoid non-reliable well signals. Blank wells should be filled with 100 µl of the preferred buffer to prevent evaporation.
5. Random or hexamer primers may be used for the RT reaction, but in our hands specific primers work best for *DMD* amplification.
6. In case some regions are difficult to amplify, one of the primers (forward or reverse) can be used again in the second PCR (e.g., first PCR primers 48F+54R and second PCR primers 48F + 53R).
7. Plates can be read with or without liquid (e.g., PBS or WB) indistinctly, although it is important to evaluate the Odyssey CLx Scan focus at least once with the desired condition in the plates selected for ICW.
8. This process needs to be done individually for each channel (700 nm and 800 nm measurements), as each time one set of values (700 nm or 800 nm) is added to the clipboard. Pay special attention to which channel is active at the time of copying the data.

9. CellTag™ 700 Stain accumulates in both nucleus and cytoplasm of permeabilized cells and provides a linear fluorescent signal across a wide range of cell types and cell numbers (<https://www.licor.com/bio/reagents/celltag-700-stain-for-in-cell-western-assays>). It is highly recommended to test linearity of fluorescent signal for each cell line of interest by staining different cell number wells with CellTag™ 700 Stain. Also, it is advisable to revise raw and normalized data values to assess normalization and variability for each experimental condition.
10. Assessing the cultures by visual examination is a good first quality control. MF20 values are expected to be similar and consistent for the experiment shown, and values may vary depending on the cell type, as patient's cells usually differentiate worse than controls.

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