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Multicenter evaluation of dystrophin exon 51 skipping

Introduction

Duchenne muscular dystrophy (DMD) is generally caused by mutations in the *DMD* gene that abolish dystrophin protein production or its correct assembly[1]. Some out-of-frame deletions can be corrected by targeted antisense oligonucleotide (AON) mediated exon skipping[2]. Exon skipping enables the production of a shorter, reframed mRNA that can be translated into smaller, but potentially still functional dystrophins. The functionality of these dystrophins is underlined by the fact that they are found in the milder Becker muscular dystrophy[3]. Skipping of exon 51 would be applicable to the largest group of patient genotypes (14% of all DMD patients) and is evaluated in clinical trials[4,5]. Exon skipping (exon exclusion) determined at the mRNA level has been used in the past to provide proof of principle and to evaluate AON efficiency[6]. However no standard operating procedure is in place to quantify exon skipped mRNAs, thus hampering the comparison between different laboratories and different clinical trial samples.

Experimental Setup

Five independent laboratories working on DMD exon skipping research were involved to comparatively evaluate exon 51 skipping with PCR technologies in a set of blinded RNA samples. Samples were provided by a single central lab (BIOCRUCES) after transfection of 2 DMD patient derived immortalized myotubes with an exon 51 targeting AON[7]. Cells were transfected with 50, 200 or 400 nM AON in order to achieve low, medium and high levels of exon skipping. Untransfected cells were taken as controls. Each lab shared its protocol with other participating labs and the different protocols were replicated independently in multiple labs. Samples were unblinded once all data had been gathered.

Protocol Developed By	Method	RNA (ng) needed	Tested by				
			LUMC	UNIFE	UCL	BIOMARIN	RHUL
LUMC	Nested and one round PCRs, quantification by agarose gel densitometry	400	X	X	X		X
UNIFE	One round PCR, quantification by Agilent Bioanalyzer	300	X	X			X
UCL	RT-qPCR (TaqMan assay)	1500	X	X	X		
BIOMARIN	Digital droplet qPCR	375				X	
RHUL	Nested PCR, quantification by agarose gel densitometry	500					X

Table 1. An overview of the different technologies used to quantify exon 51 skipping.

Statistical analysis of the results was performed using a univariate linear model for each cell line, where the exon skipping percentage was the dependent variable, while the technique, performing lab and sample name (E, I, O, U) were considered as factors. Bonferroni correction was used for post-hoc comparisons.

Results

Results for a cell line carrying a deletion involving exons 48 to 50 are presented:

- Exon skipping was observed and quantified with all the methods (Table1).

- Digital droplet PCR enabled absolute quantification of exon skipping independent of amplification efficiency, and therefore was considered a reference for the other technologies.

- All technologies and labs identified O as the untransfected control with physiological exon skipping levels beween 0 and 0.6%. These values were lower than all the other samples ($p<0.001$).

- All technologies and labs identified U as the sample transfected with the lowest AON concentration.

- E and I were the samples with higher exon skip %. Although no difference was found between E and I ($p=0.06$), only I was statistically higher compared to U ($p<0.01$), suggesting I to be the sample transfected with 400 nM AON.

- Although RHUL and UCL methods were not tested by other labs, they delivered results comparable to the digital PCR.

- UNIFE and LUMC nested protocol overestimated exon skip % compared to the digital PCR ($p<0.05$ and $p<0.001$, respectively)

- Considerable variation was visible among performing labs with UNIFE overstimating exon skip % when performing the LUMC protocol, compared to the other labs ($p<0.01$).

- The skipped fragment was not visible on the gel after the LUMC single round protocol.

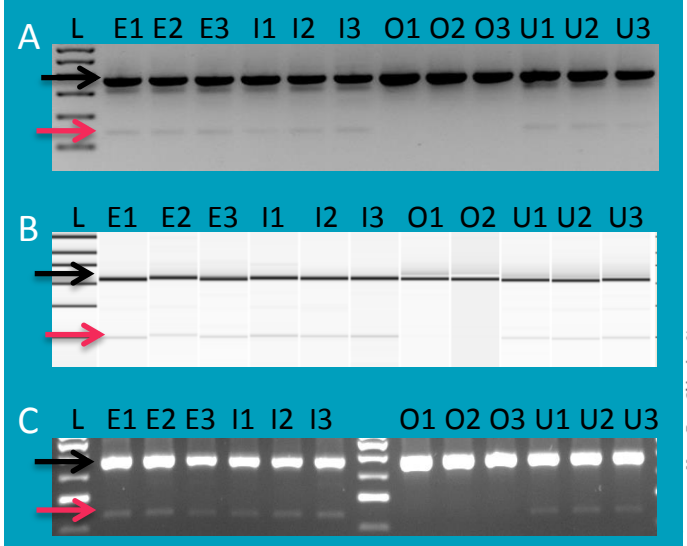

Figure 1. Examples of the obtained fragments with LUMC nested protocol (A), UNIFE protocol (B) and RHUL protocol (C). L stands for molecular ladder. Black arrows indicate the unskipped fragment containing exon 51, while red arrows indicate skipped fragments.

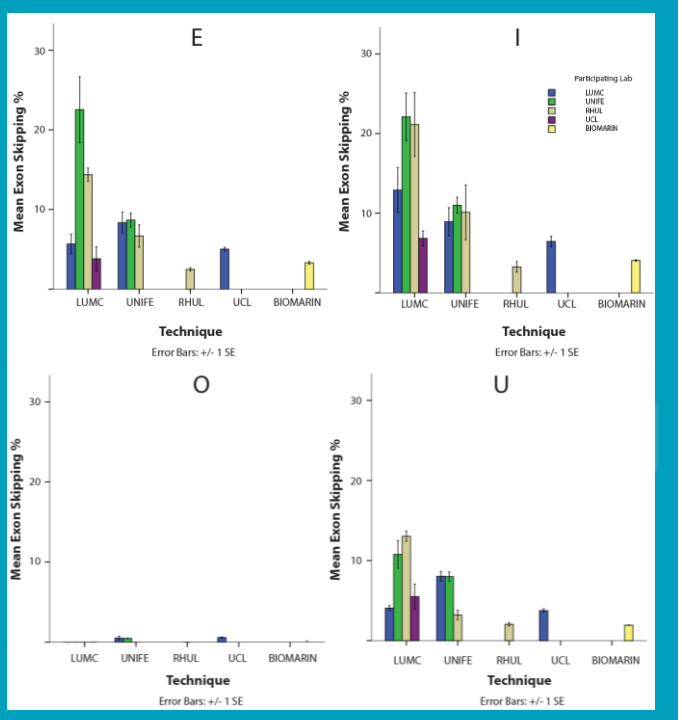

Figure 2. Comparison of exon skipping % obtained with the different techniques.

Conclusions

- **The experimental set-up enabled us to identify strengths and weaknesses of the different methods and to assess overall reproducibility**
- **Exon skipping levels were lower than expected; a second round of transfection has been performed to obtain samples with higher exon skip % (data not shown)**
- **Results indicated considerable variation across labs and techniques**
- **Quantification with the Agilent Bioanalyzer is more reproducible compared to densitometry as it reduces the variation introduced by the operator when the image of the gel is obtained**
- **Digital PCR enables reliable and sensitive quantification of exon skipping levels**
- **Nested amplification leads to an overestimation of the exon skip levels compared to a single round amplification**
- **The definition of a SOP, shared quantification standards and a central lab performing the RNA analysis will enable better comparison of the exon skip levels across clinical trials**

References and footnotes

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7. This AON was not Drisapersen

Sponsors

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