APPENDIX e-1. PCR PATIENT-SPECIFIC PRIMERS

RT-PCR Primer Tables

Each DMD patient enrolled in the clinical trial had a known genetic deletion mutation in the *DMD* gene. This known genetic deletion identified the individual patient as having a mutation amenable to skipping of exon 51 by treatment with eteplirsen. This patient-specific information allowed for the selection of patient-specific PCR primers (Tables e-1–3).

APPENDIX e-2. WESTERN IMMUNOBLOT METHOD DETAILS

Nine to 12 muscle sections, 20-µm thick and approximately 5 mm in diameter, were collected in a 1.5-mL microfuge tube. Samples were immersed in 133-µL ice-cold homogenization buffer (4M urea; Teknova, Hollister, CA), 125-mM tris pH 6.8 (Amresco; Solon, OH), 4% sodium dodecyl sulfate solution (Fisher; Loughborough, UK), and 1 complete mini protease inhibitor tablet/7-mL buffer (Roche; Basel, Switzerland). Samples were homogenized at room temperature by grinding using an Eppendorf micropestle (Eppendorf; Hamburg, Germany). Samples were twice sedimented in a centrifuge for 1 minute at 15,000 g at room temperature and were stored on ice or at −80°C. A total protein assay of the tissue extract was performed with the RC DC Protein Assay Kit II per manufacturer's instructions (BioRad; Hercules, CA). Samples were prepared such that 35 µL of sample would contain the desired amount of protein using 25 µL of protein lysate, 7 µL NuPAGE LDS Sample Buffer (Life Technologies; Carlsbad, CA), and 3 µL NuPAGE Reducing Agent (Life Technologies). After samples had been prepared, each was placed in a 95°C heating block for 5 minutes. The sample was loaded onto a NuPAGE Novex 10 well, 1-mm mini 3% to 8% polyacrylamide tris-acetate gel (Life Technologies). Protein transfer onto Invitrolon polyvinylidene difluoride (PVDF) membranes (Life Technologies) was accomplished after 75 minutes with current generating 30 V at room temperature. After protein transfer, the PVDF membrane was immersed in TTBS buffer (1X tris-buffered saline [TBS; Amresco]), 0.1% (V/V) TWEEN-20 (Thermo Scientific; Waltham, MA). The membrane was then transferred to blocking buffer (5% [W/V] nonfat dry milk [Lab Scientific; Highlands, NJ] in TTBS) and rocked overnight at 4°C. After blocking, PVDF was incubated for 60 minutes at room

temperature in DYS1 (Leica; Wetzlar, Germany) diluted 1:20 using blocking buffer. Primary incubation was followed by 6 washes, 5 minutes each, with TTBS. Anti-mouse IgG-conjugated horseradish peroxidase (GE Healthcare; Little Chalfont, UK) was diluted 1:40,000 using blocking buffer for 45 minutes. Secondary incubation was followed by 6 washes, 5 minutes each, in TTBS. The PVDF membrane was incubated for 5 minutes in ECL Prime Buffer (GE Healthcare), wrapped in plastic wrap, and placed in an autoradiography cassette for film exposure. Developed film was scanned and analyzed using Image Quant TL Plus software version 8.1 (GE Healthcare) and linear regression analysis was performed using GraphPad software. To determine the percent dystrophin in a sample, dystrophin band intensities as well as the input percent of normal control (NC) were log-transformed, and linear regression analysis was performed on the log-transformed data. The percent NC was then calculated using the equation:
%NC =10^((LOG(band intensity)-y intercept)/slope).

APPENDIX e-3. FLAGSHIP PATHOLOGIST RESULTS

Table e-4 depicts the individual analysts and mean analyst values for the eteplirsen and untreated control sample results. There was also strong agreement across the 3 analysts on an individual treated or control tissue basis. The fold increase of treated patients over untreated control tissue was 15.8.

APPENDIX e-4. METHODS: INVERTED IMAGES

Original fluorescent images were inverted from fluorescent RGB images to brightfield RGB image base100 images using the MuscleMap image invert algorithm (Flagship Biosciences, Westminster, CO). To generate the enhanced inverted base100 image (Invert Base100), the algorithm produces a nonlinear mapping of RGB fluorescent values that will specifically enhance low-contrast objects in the image (figure e-4). It does this by scaling the RGB fluorescent values using the following formula: I’ = 1 – 100^(-I) normalized by the maximum value of 1 – 100^(-1) for each of the channels independently. This results in low-intensity values being stretched and therefore perceived as having higher intensity and contrast.

APPENDIX e-5. BECKER MUSCULAR DYSTROPHY (BMD) AND NON-BMD/DMD CONTROLS

In addition to the patient and control samples, 3 Becker muscular dystrophy (BMD) and 3 non-BMD/DMD control samples were evaluated using both Western blot and Bioquant assays. Western blot evaluation of dystrophin quantities showed that the BMD samples ranged from 2.31% to 32.19%, and non-dystrophic muscle samples ranged from 51.97% to 95.84% of normal tissue levels (Table e-6). The resulting correlation analysis of the clinical, BMD, and non-DMD samples indicated good agreement using these 2 assays across a broad range of dystrophin levels ($R^2 = 0.9135$; Pearson correlation coefficient = 0.709, $p = 0.015$; figure 4). It is noteworthy that one of the BMD samples showed dystrophin protein expression values in the range of many of the eteplirsen-treated, study week 180 DMD patient samples when evaluated by Western blot and Bioquant analyses.