1.3. Monoclonal dystrophin antibodies for heart tissue immunostaining and western blot

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A. OBJECTIVE

A large collection of epitope-specific dystrophin monoclonal antibodies has been generated to react with human dystrophin (Lam et al, 2014; Morris et al, 2011). However, the reactivity of these antibodies varies in the murine and canine heart/skeletal muscle samples (Tables 1 to 4) (Kodippili et al, 2014). The protocol here describes their use for studying cardiomyopathy in murine and canine models of DMD.

B. CAUTIONS

B1. Immunofluorescence staining

- Proper initial freezing, subsequent handling and sectioning of tissue samples will eliminate bad freezing artifacts.
- Avoid repeated thawing and freezing of stored tissues/slides.
- Always include primary antibody only and secondary antibody only controls.
- All sections/samples must be imaged using identical settings on the same fluorescence microscope for accurate comparison/analysis.
- Do not let your sections dry out at any stage in the procedure since it will result in non-specific binding of antibodies and lead to high background staining. During the overnight incubation step with the primary antibody, place the slides in a slide box and add some water to the box to create a moist chamber. This will prevent your slides from drying out during overnight incubation.

B2. Western blotting

- For proper analysis of results, always include positive and negative controls and protein loading controls.
- Always wear gloves when handling the PVDF membrane in order to reduce non-specific/background signals.
- When placing the membrane in the transfer sandwich, take care to prevent air bubbles from forming. Air bubbles will block the transfer of proteins from the gel to the membrane.
- During the detection step, closely follow package instructions of chemiluminescent substrate solutions.
- If signal is too weak, consider using a stronger chemiluminescent substrate solution.

C. MATERIALS

C1. Immunofluorescence staining

Hydrophobic PAP pen
Fab-c solution (for blocking)

1x PBS
20% Goat serum
Primary antibodies (Dystrophin mAbs)
Fluorophore conjugated goat anti-mouse secondary antibodies
Pipettes and pipette tips
Slide box
Cover slips
CITIFLUOR antifadent mountant solution
DPX mountant (Sigma, cat log # 06522) or nail polish (optional)
Kimwipes

C2. Western blotting

3% Stacking/6% separating SDS gels
Gel loading buffer (recipe below)
Protein dissociation buffer lacking SDS (recipe below)
2-Mercaptoethanol
5% Bromophenol blue solution
Microfuge
Wet electroblotting system for mini or midi sized gels
PVDF membrane (or Nitrocellulose)
10x running buffer (recipe below)
10x transfer buffer (recipe below)
Power supply
TBST (Tris buffered saline, 0.1% Tween-20; recipe below)
Nonfat dry milk powder
Shaker/rocker
95°C heating block
Paper towels/kimwipe
Saran wrap
Pyrex dishes
Pipettes and pipette tips

C3. Recipes <u>Homogenization buffer</u>

SDS	10 g (final 10%)
0.5M EDTA	1 ml (final 5mM)
0.5M Tris-HCl (pH 6.8)	12.5 ml (final 62.5 mM)

Bring the final volume to 100 ml using dH_2O
Add 1:100 protease inhibitor to the mix before use.

<u>10x running buffer</u>

Trizma base	300 g
Glycine	1441.6 g
SDS	100 g

Bring the final volume to 10L using dH_2O

<u>1x running buffer</u>

10x running buffer	100 ml
dH_2O	900 ml

<u>10x transfer buffer</u>

Trizma base	606 g

Glycine	2883.2 g
SDS	100 g

Bring the final volume to 10L using dH_2O

1x transfer buffer

10x transfer buffer	100 ml
dH_2O	800 ml
Methanol	100 ml

Protein dissociation buffer lacking SDS

0.5M Tris pH 6.8	12.5 ml
Glycerol	10 ml
dH_2O	66.5 ml

Gel loading buffer

Protein dissociation buffer	180 µl
2-Mercaptoethanol	20 µl
5% bromophenol blue	3-4 µl

10x TBS

Tris base	48 g
NaCl	176 g
dH_2O	1800 ml

pH to 7.6 using 12 N HCl and bring the final volume to 2 L using dH2O

0.1% TBST

10x TBS	2 L
dH2O	18 L
Tween-20	20 ml

D. METHODS

D1. Immunofluorescence staining

1. Remove slides from -20°C and allow them to warm up to room temperature.
2. Label the slides with the experimental information and circle each section with the hydrophobic PAP pen.
3. Prepare Fab-c in PBS by mixing each at a 1:1 ratio. Add sufficient Fab-c in PBS solution to the slides to cover each section. Cover the slides and incubate at room temperature for 1 hour.
4. At the end of this 1-hour incubation, aspirate out the Fab-c in PBS solution and immediately cover each section with PBS and let it sit for 5 minutes at room temp.
5. Remove and replace with PBS two more times for a total of 3 washes.
6. Prepare 20% goat serum blocking solution in PBS. Cover the sections with the blocking solution, cover the slides and incubate at room temp for 30 minutes.
7. Remove 20% goat serum blocking solution and wash with PBS three times as in step 4 (each time for 5 minutes).

8. While washing the slides in PBS, prepare the primary antibody solution. Dilute your primary antibody of choice in PBS + 1% goat serum.
9. Cover sections with your primary antibody, cover the slides and incubate at 4°C, overnight.
10. Next day, let the slides warm up to room temperature, wipe the bottom of the slides with Kimwipes and aspirate out the primary antibody solution.
11. Wash the slides with 1% goat serum in PBS, 3 times, for 5 minutes each.
12. While washing the slides in 1% goat serum + PBS, prepare your secondary antibody solution in 1% goat serum + PBS solution.
13. Add the secondary antibody solution and incubate at room temperature for 30 minutes. Keep slides covered from this point forward, as the secondary antibody will react to light.
14. Remove secondary antibody. Add 1 drop of Citifluor to each section and cover the slide with the appropriate sized cover slip.
15. Seal the edges of the cover slip with DPX mountant and let the slides air dry for one hour while covered.
16. Transfer the slides to the -20°C freezer, until you are ready to view the slides on a fluorescence microscope.

D2. Western blotting

1. Use tissue that was snap frozen immediately following dissection to prepare protein samples.
2. Cut 50 mg of tissue from the original tissue sample, transfer to a mortar, fill it halfway with liquid nitrogen and start grinding the tissue using a liquid nitrogen-cooled pestle. Repeat 3-4 times until the tissue has been ground to a fine powder.
3. Add 100 µl of homogenization buffer and start homogenizing. Repeat until you have added 750-1000 µl of homogenization buffer. Transfer homogenate to an Eppendorf tube.
4. Spin homogenate at 14,000 rpm for 2 minutes.
5. Transfer supernatant to a labeled Eppendorf tube and determine protein concentration using the Bio-Rad DC protein assay kit.
6. Take 30-50 µg of protein and add an equal volume of gel loading buffer. Vortex to mix and centrifuge briefly.
7. Place protein sample in 95°C heating block for 3 minutes. Remove from the heating block and let the sample cool down for a few minutes.
8. Load protein sample on a 3% stacking/6% separating SDS-polyacrylamide gel and run for 4 hours at 100 V (use running buffer for gel electrophoresis).
9. Transfer the protein to a polyvinylidene fluoride (PVDF) membrane for 10 hours at 60 V using transfer buffer (wet transfer method). Activate the PVDF membrane with methanol briefly before you prepare the transfer sandwich.
10. Following transfer, remove membrane from transfer cassette and block with 5% milk in 0.1% Tris-buffered saline-Tween (TBST) solution for one hour at room temperature.

11. Incubate the PVDF membrane with your dystrophin mouse monoclonal primary antibody of choice (1:100 dilution in 5% milk/0.1% TBST overnight at 4°C).
12. Next day, wash the membrane in TBST three times, for 5 minutes each, and then incubate with the peroxidase conjugated goat anti-mouse secondary antibody (1:2,000 dilution in 0.1% TBST) for one hour at room temperature.
13. Wash the membrane again in TBST three times, 5 minutes each, and detect the signal on the membrane by a chemiluminescent detection method of your choice.

E. EVALUATION AND INTERPRETATION OF RESULTS

- For immunofluorescence staining: view slides on a fluorescence microscope using the appropriate filter to confirm staining. Image all sections under identical conditions.
- For western blots: visualize protein on membrane, using X-ray film or a digital imaging system.

F. REFERENCES

- Kodippili K, Vince L, Shin JH, Yue Y, Morris GE, McIntosh MA, Duan D (2014) Characterization of 65 epitope-specific dystrophin monoclonal antibodies in canine and murine models of Duchenne muscular dystrophy by immunostaining and western blot. *PLoS One* **9:** e88280

- Lam LT, Man NT, Morris GE (2014) Monoclonal antibodies for clinical trials of Duchenne muscular dystrophy therapy. *Neuromuscul Disord* **24:** 195-200

- Morris GE, Man NT, Sewry CA (2011) Monitoring Duchenne muscular dystrophy gene therapy with epitope-specific monoclonal antibodies. *Methods Mol Biol* **709:** 39-61

G. Tables

Table 1. Dystrophin monoclonal antibodies suggested for mouse heart immunofluorescence staining.

Table 2. Dystrophin monoclonal antibodies suggested for mouse heart western blot.

Table 3. Dystrophin monoclonal antibodies suggested for dog heart immunofluorescence staining.

Table 4. Dystrophin monoclonal antibodies suggested for mouse heart western blot.

Table 1. Dystrophin monoclonal antibodies suggested for mouse heart immunofluorescence staining.

Antibody name	Normal mouse heart	Mdxt mouse heart
MANEX1A	+++	_
DysB	++	_
MANDYS16	+++	_
MANDYS1	++	_
MANDYS8	+++	_
MANDYS105	+++	_
MANDYS110	++	_
MANDYS111	++	_
MANEX44A	+++	_
MANEX45A	++	_
MANEX46B	+++	_
MANEX4850B	+++	_
MANEX50	+++	_
MANHINGE4A	++	_
MANCHO19	++	_
MANDRA9	++	_
MANCHO11	++	_
MANEX7374A	++	_
MANDRA17	++	_
MANDRA16	+++	_
MANDRA4	++	_
Dys-2	++	_

Table 2. Dystrophin monoclonal antibodies suggested for mouse heart western blot.

Ab Name	Normal mouse heart	Mdxt mouse heart	Comments
MANEX1A	Pos	_	Weak cross-reactive band at 250 kDa
MANEX7B	Pos	_	
MANHINGE1A	Pos	_	
MANEX1011A	Pos	_	
MANEX1216B	Pos	_	Weak cross-reactive band at 380 kDa
MANEX1216A	Pos	_	
MANDYS19	Pos	_	
MANDYS18	Pos	Weak	
Dys-1	Pos	_	
MANDYS17	Pos	_	
MANDYS1	Pos	_	Weak cross-reactive band at 100 kDa
MANDYS8	Pos	_	
MANDYS141	Pos	_	Weak cross-reactive band at 170 and 100 kDa
MANDYS110	Pos	_	
MANDYS124	Pos	_	
MANDYS122	Pos	_	
MANEX44A	Pos	_	Strong cross-reactive band at 200 kDa
MANEX44B	Pos	_	
MANEX45B	Pos	_	
MANEX50	Pos	_	
MANHINGE4A	Pos	_	Weak cross-reactive band at 400 kDa
MANDRA9	Pos	_	Weak cross-reactive band at 230 kDa
MANDRA2	Pos	_	
MANCHO11	Pos	Pos	
MANEX7374A	Pos	_	
MANDRA17	Pos	_	
MANDRA14	Pos	_	
MANDRA6	Pos	_	
MANDRA12	Pos	_	
MANDRA13	Pos	_	
MANDRA16	Pos	Pos	
MANDRA3	Pos	Weak	
MANCHO18	Pos	_	
MANDRA5	Pos	_	
Dys-2	Pos	_	

Table 3. Dystrophin monoclonal antibodies suggested for dog heart immunofluorescence staining.

Ab Name	Normal dog heart	DMD dog heart
MANEX1A	+++	_
DysB	+++	_
MANDYS19	+++	_
MANDYS18	++	_
Dys-1	++	_
MANDYS16	++	_
MANDYS1	++	_
MANDYS8	+++	_
MANDYS105	+++	_
MANDYS110	+++	_
MANDYS104	++	_
MANDYS101	++	_
MANDYS107	+++	_
MANDYS111	+++	_
MANDYS102	++	_
MANDYS106	++	_
MANDYS103	++	_
MANEX44A	+++	_
MANEX45A	+++	_
MANEX46A	++	_
MANEX46B	+++	_
MANEX47	+++	_
MANEX4850A	+++	_
MANEX4850B	+++	_
MANEX4850D	++	_
MANEX50	+++	_
MANHINGE4A	++	_
MANCHO19	++	_
MANDRA9	++	_
MANCHO11	++	_
MANEX7374A	++	_
MANDRA17	++	_
MANDRA6	++	_
MANDRA16	+++	_
MANDRA4	++	_
Dys-2	++	_

Table 4. Dystrophin monoclonal antibodies suggested for mouse heart western blot.

Ab Name	Normal dog heart	DMD dog heart	Comments
MANEX1A	Pos	_	
MANHINGE3C	Pos	_	Strong cross-reactive band at 300 kDa
MANHINGE1A	Pos	_	
MANEX1011A	Pos	_	
DysB	Pos	_	Strong cross-reactive band at 300 kDa
MANEX1216A	Pos	_	Weak cross-reactive band at 400 kDa
MANDYS19	Pos	_	
MANDYS18	Pos	Weak	
Dys-1	Pos	_	
MANDYS17	Pos	_	
MANDYS1	Pos	_	Weak cross-reactive band at 100 kDa
MANDYS8	Pos	_	
MANDYS141	Pos	_	Strong cross-reactive band at 100 kDa
MANDYS110	Pos	_	
MANDYS101	Pos	_	
MANDYS107	Pos	_	
MANDYS124	Pos	_	
MANDYS122	Pos	_	
MANEX44A	Pos	_	Strong cross-reactive band at 200 kDa
MANEX44B	Pos	_	
MANEX45A	Pos	_	
MANEX46A	Pos	_	
MANEX46B	Pos	_	
MANEX47	Pos	_	
MANEX4850A	Pos	_	
MANEX4850B	Pos	_	
MANEX4850D	Pos	_	
MANEX50	Pos	_	Several cross-reactive bands at 350, 200, 175 kDa
MANHINGE4A	Pos	_	Weak cross-reactive band at 400 kDa
MANDRA9	Pos	_	Strong cross-reactive band at 230 kDa
MANDRA2	Pos	_	
MANCHO11	Pos	Pos	
MANEX7374A	Pos	_	
MANDRA17	Pos	_	
MANDRA14	Pos	_	
MANDRA6	Pos	_	
MANDRA13	Pos	_	
MANDRA16	Pos	Pos	
MANDRA3	Pos	Weak	
MANCHO18	Pos	_	
MANDRA5	Pos	_	
MANDRA4	Pos	_	
Dys-2	Pos	_	