Instruction Manual

Roti®-Mark WESTERN Set (Art. No. 0947.1) and MINI-Set (Art. No. 0947.2)

Roti®-Mark WESTERN Marker (Art. No. 0948.1)

Roti®-Mark WESTERN HRP-Conjugate (Art. No. 0949.1)

Protein molecular weight marker and anti-marker antibody for chemiluminescent detection on Western blots.

I. Content

The Roti®-Mark WESTERN Set (Art. No. 0947.1) contains:

- 0.5 ml ready-to-use Roti®-Mark WESTERN marker (Art. No. 0948.1)
- 0.1 mg Roti®-Mark WESTERN HRP-conjugate, anti-WESTERN marker antibody (rabbit) (Art. No. 0949.1) reconstituted in 500 µl buffer (see page 5, IX. Composition)

The Roti®-Mark WESTERN MINI-Set (Art. No. 0947.2) contains:

- 50 µl ready-to-use Roti®-Mark WESTERN marker (cannot be ordered separately)
- 0,01 mg Roti®-Mark WESTERN HRP-conjugate, anti-WESTERN marker antibody (rabbit) (cannot be ordered separately), reconstituted in 50 µl buffer (see page 5, IX. Composition)

II. Storage

II.1 Roti®-Mark WESTERN Set

Please store the Roti®-Mark WESTERN set at -20 °C before use.

II.2 Roti®-Mark WESTERN Marker

The marker can be stored at 4 °C for a short period (a few days). Longer storage should be at –20 °C. We recommend freezing aliquots to avoid repeated freezing and thawing.

II.3 Roti®-Mark WESTERN HRP-Conjugate

0947 and 0949: The conjugate solution is stable at 4 °C for several weeks. The conjugate must be kept at -20 °C or -80 °C for long-term storage and aliquoted in order to avoid repeated freezing and thawing.
Please note: In some cases, the conjugate may be delivered in lyophilized form. Then, the lyophilized antibody conjugate can be stored at 4 °C prior to reconstituting. Before it is used for the first time, the lyophilized antibody Roti®-Mark WESTERN HRP-conjugate should be reconstituted in 250 µl sterile H_2O by incubating it for 5 minutes at room temperature. The lyophilisate already contains stabilising compounds according to the buffer given in section IX. Mix the antibody solution carefully and aliquot it. For storage see above.

III. Preparation

III.1 Roti®-Mark WESTERN Marker

Slightly heat Roti®-Mark WESTERN marker before using to solubilise precipitated SDS. Aggregates which may be deposited at the stacking gel / resolving gel border during the gel run can be resolved before loading the gel by heating samples at max 60 °C for 2-5 minutes.
Please note: The marker should not be heated above 80 °C.

IV. Gel loading

Recommended gel loading amount for mini gels (10 x 10 cm and 0.75 – 1 mm thick): **5 µl**
The loading amount varies with the gel thickness, C/T-ratio, staining and width of the comb teeth, with the blot conditions and the detection substrate used. It should be specifically established based upon the mentioned 5 µl. The presence of other antibodies during the detection reaction can also change the intensity of the detection and, subsequently, the amount of marker required. For example, simultaneous application of anti-rabbit secondary antibodies may well lead to an intensification of the marker signal.
1 x concentrated loading buffer according to Lämmli (with SDS and DTT) can be used to dilute the marker (e.g. diluted Roti®-Load 1, Art. No. K929.1).

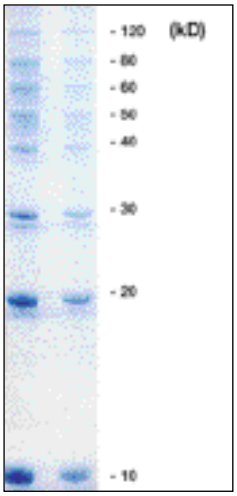


Fig. 1: Roti®-Mark WESTERN Marker. Amount 10 µl / 5µl, 12% SDS-PAGE, Staining with Roti®-Blue (Art. No. A152.1).

Please note:
The band intensity of the Roti®-Mark WESTERN marker was optimised in order to gain equal band signals after Western detection. As the larger proteins yield unusually strong colour and light intensity, the smaller proteins were adapted in amount and are represented in the mixture with over proportionally large protein amounts. As a result, the staining of the lower bands in the gel is stronger and weaker in the upper bands.

V. Antibody-dilution

Recommended dilution for WESTERN marker detection on Western blots: **1:1000**
The applied dilution varies with the blot conditions, e.g. the type of membrane, the blocking solution, the secondary antibody used, and the detection substrate used in the assay. The Roti®-Mark WESTERN HRP-conjugate can be applied together with other specific antibody conjugates or secondary antibodies. Please note that the optimal conditions must be determined for each antibody and each combination of antibodies used. Simultaneous use of another anti-rabbit secondary antibody is basically unproblematic; however, we recommend using a higher dilution of the secondary antibody (1:4000) as well as of the marker-anti body, owing to intensification (see Trouble Shooting).

VI. Compatibility

The Roti®-Mark WESTERN Market Set is compatible with

- All gel solutions and ready-to-use gels available on the market
- PVDF and nitrocellulose membranes
- Semi-dry and tank blotting systems
- Powdered milk and Roti®-Block as a blocking reagent.
  Please note: Using BSA can lead to an enhanced background
- PBS/PBST and TBS/TBST as buffer systems
- Colour detection and chemoluminescence detection with HRP

VII. Application

Upon completion of the gel run, the gel should be blotted onto a filter membrane using the standard methods. Unspecific binding sites should be subsequently blocked with a suitable blocking reagent. After the blot has been incubated in Roti®-Mark WESTERN HRP-conjugate antibody solution and washed, detection can take place with a chemoluminescent substrate or via colour reaction. Incubation with Roti®-Mark WESTERN HRP-conjugate may, if necessary, be performed together with a secondary antibody. We recommend using the following protocol for standard application of the Roti®-Mark WESTERN set (accessories can also be found under section X. Recommended Reagents):

VII.1 Separation of 5 µl Roti®-Mark WESTERN marker in a SDS-PAGE alongside your samples, followed by blotting of the gel onto a PVDF-membrane (e.g. Roti®-PVDF). We recommend an electrical field of 1 mA/cm^2 membrane for 2 hours for a semi-dry blot so that even the largest marker protein of 120 kD is transferred quantitatively onto the membrane. A blotting time of 60 to 90 minutes is sufficient for the other marker proteins.

VII.2 After transfer has been completed, the membrane should be rinsed shortly in TBST or PBST (see X) and then blocked for one hour in TBST or PBST with 1% low-fat powdered milk, or in 1 Roti®-Block, under gentle agitation.

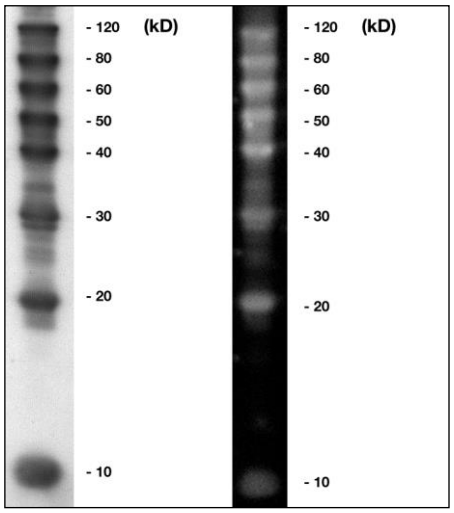
VII.3 Primary antibody incubation in blocking solution, at room temperature, in general for 1 hour under gentle agitation. Apply your specific sample antibody here. If you are only carrying out a single antibody incubation, steps 3 and 4 are not applicable.

VII.4 Wash in TBST or PBST 4 times at 5 minutes each, at room temperature under gentle agitation.

VII.5 Secondary antibody incubation. Use a mixture consisting of your own secondary antibody and the reconstituted Roti®-Mark WESTERN HRP-conjugate at a dilution of 1:1000 in blocking solution for 1 hour at room temperature under gentle agitation.

VII.6 Wash in TBST or PBST 4 times for 5 minutes each, at room temperature under gentle agitation.

VII.7 Colour or chemoluminescence detection for visualisation of bands. Incubate the colour reaction until the desired band intensity has been reached. Following applies to chemoluminescence detection: absorb supernatant substrate solution with a filter paper after incubation (e.g. in Roti®-Lumin) and immediately detect chemoluminescence with a camera or X-ray film (see Figure 2). Typical exposure time when using a camera is between 20 seconds and 3 minutes. Exposure time may be even shorter when using an X-ray film under the given conditions.



a) b)

Fig 2: Roti®-Mark WESTERN marker on Roti-PVDF membrane after chemoluminescence detection with Roti-Lumin, Roti®-Mark WESTERN HRP-conjugate 1:1000.

a): 5 µl Roti®-Mark WESTERN marker, blocking with 1 x Roti®-Block, detection via X-ray film, exposure time 15 seconds

b): 2.5 Roti®-Mark WESTERN marker, blocking solution 1 % low-fat milk in TBST, detection via CCD-camera, exposure time 45 seconds

VIII. Trouble-Shooting

VIII.1 Too strong or too weak signal of marker bands.

- The loading amounts specified for marker and antibody dilution are standard values and are to be adapted to the system used. They can vary depending on the blot membrane applied, the transfer buffer, blocking solution, peroxidase-substrate and detection system.
- Avoid overloading the gel. The application of 5 µl marker for standard mini-gels is in most cases adequate. A too large amount of marker may lead to a weakening of the signal by the following mechanism: a large amount of antibody conjugate binds to the high amount/number of marker protein molecules; this leads to a

very high peroxidase activity which can increase so much that the substrate is converted faster than it can be supplied from the remnants in the surrounding area.

- A similar effect can result from using anti-rabbit secondary antibodies, since these bind to the rabbit blood-borne Roti®-Mark WESTERN marker antibody, thus intensifying the enzyme activity in the marker bands. For this special combination of detection systems we therefore recommend a stronger dilution of Roti®-Mark WESTERN HRP-conjugate of 1:4000.

- The amount of marker specified for loading and the antibody dilution used also depends on the specific protein to be detected. If the specific signal is expected to be weak and must therefore be exposed for a particularly long time during detection, less marker (2.5 µl or less) should be used, possibly combining it with a higher antibody dilution.

- Marker band signals which are too weak can be caused by bad transfer efficiency during blotting. Please observe the blot times and the manufacturers' instructions. Test the protein transfer by staining the gel or membrane after blotting (e.g. with Roti®-Blue, Roti®-Red or Roti®Green) and, if necessary, use other transfer buffers or increase the time. The 120 kDA protein band signal may be missing when using less than 1.25 µl marker in combination with a short blotting time.

VIII.2 The specific signal is too weak

- When using Roti®-Mark WESTERN HRP-conjugate together with anti-rabbit antibodies the signal strength of the specific protein may be influenced. As described in Vlll.1, it is possible that anti-rabbit antibodies may bind to the Roti®-Mark WESTERN HRP-conjugate, and thus the antibody amount available for specific reaction is lowered. In such cases we recommend, first of all, reduction of the concentration of Roti®-Mark WESTERN HRP-conjugate used (e.g. dilution of up to 1:4000) and doubling of the concentration of your own secondary antibody.

VIII.3 High background

- Please ensure thorough washing after antibody incubation.

- Test other blocking solutions. Blocking with 3 % BSA, which is occasionally applied, often leads to a high background. Rather use, for example, 1 % low-fat powdered milk in TBST or in PBST or 1 x Roti®-Block.

VIII.4 The strength of the marker band signal varies greatly

- Uniform band intensity in chemoluminescence is not equally present for all detection systems. The signal development of 10 kD and 20 kD bands may need longer than that of the remaining ones with some substrates. The 120 kD signal may be missing when using less than 1.25 µl marker in combination with a short blotting time.

VIII.5 Additional bands

- Additional bands, which are generally hardly visible in the gel, may appear after detection above 120 kDa. This is an agglomerate of the marker protein whose appearance is dependent on the batch. Other additional weak bands may appear above and below the 30 kDa protein band. This is a side reaction of the HRP-conjugate marker-specific antibody with *E. coli* specific proteins, which enter the marker during the manufacturing process (recombinant expression in *E. coli*). Detection is in no way impeded nor is analysis made more difficult by these light additional bands.

IX. Composition

The marker contains eight recombinant proteins produced in *E. coli* with defined, regular molecular weight, pre-reduced and acylated. The proteins guarantee a maximised constancy of band strength in chemoluminescence. The actual percentage by mass of the individual protein is 0.1 to 0.2 mg/ml and is batch-dependent. Determining protein amounts with this marker is therefore not recommended. The proteins are available dissolved in 50 mM Tris-HCl (pH 6.8), 2 % SDS, 0.01 % bromophenol blue, 10 % saccharose, 8.7 % glycerol.

The antibody conjugate consists of 0.1 mg or 0.01 mg horseradish peroxidase-conjugated lgG-fraction of rabbit anti-Roti®-Mark WESTERN marker (concentration 0,2 mg/ml) in buffered solution (phosphate buffered sodium-/potassium salt solution, pH 6.5, with ca. 5 % BSA, 30 % glycerol, gentamycin, cytochrom C and thimerosal, overall 500 µl and 50 µl, respectively).

X. Recommended reagents

Roti®-Blot 1	Transfer buffer for semi-dry blotting	L509.1
Roti®-PVDF	PVDF-blot membrane	T830.1
Roti®-Blue	Colloidal coomassie staining	A152.1
Roti®-Red	Highly sensitive fluorescence staining for gels	1045.1
Roti®-Green	Highly sensitive fluorescence staining for gels and blots	1000.1
Roti®-Block	Protein-free blocking solution	A151.1
Roti®-Lumin	HRP-detection reagent for chemo-luminescence	P078.1
Roti®-Stock 10 x PBS	10 x PBS stock solution, autoclaved and sterile-filtered	1058.1
Roti®-Stock 10 x TBS	10 x TBS stock solution, autoclaved and sterile-filtered	1060.1
Roti®-Stock 10 x PBST	10 x PBST stock solution, autoclaved and sterile-filtered	1059.1
Roti®-Stock 10 x TBST	10 x TBST stock solution, autoclaved and sterile-filtered	1061.1

Roti®-Mark WESTERN Set	1 Set 1 MINI-Set	Warning H319 P305+P351+P338-P337+P313	0947.1 0947.2
Roti®-Mark WESTERN Marker	0.5 ml	Warning H319	0948.1
Roti®-Mark WESTERN HRP-Conjugate	0.1 mg / 500 µl		0949.1

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