Solution structure of calcium-free calmodulin

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The three-dimensional structure of calmodulin in the absence of Ca 2+ has been determined by three- and four-dimensional heteronuclear TROSY experiments, including ROE, isotope-filtering combined with reverse labelling, and measurement of more than 700 three-bond J-couplings. In analogy with the Ca 2+ -ligated state of this protein, it consists of two small globular domains separated by a flexible linker, with no stable, direct contacts between the two domains. In the absence of Ca 2+, the four helices in each of the two globular domains form a highly twisted bundle, capped by a short anti-parallel β-sheet. This arrangement is qualitatively similar to that observed in the crystal structure of the Ca 2+-free N-terminal domain of troponin C.

Calmodulin is a ubiquitous intracellular protein of 148 residues (Mr 16,700) that plays a critical role in coupling various Ca 2+-influx, caused by a stimulus at the cell surface, to events in the cytosol. It performs this role by binding to a host of intracellular enzymes in a calcium-dependent manner. The X-ray crystal structure of Ca 2+-ligated calmodulin resembles a dumbbell, in which the small globular amino- and carboxy-terminal domains are linked by a 26-residue α-helix, frequently referred to as the “central helix” (2). NMR, sedimentation experiments and small-angle X-ray scattering studies indicate that this helical linker is highly flexible in solution. However, NMR data also indicate that the structure of the globular domains of Ca 2+-ligated calmodulin in solution are very similar to those observed in the crystalline state (4). Each domain consists of a pair of α-helices, loop helix motifs, which are commonly called “EF-hands.” The “loops” of these two EF-hands are linked by a short antiparallel β-sheet. Attempts to grow apo-calmodulin crystals suitable for X-ray studies have failed, but a detailed model for its structure has been put forward based on the highly homologous (51% sequence identity) protein troponin C (ref. 8). In the X-ray structure of troponin C (ref. 9), the two EF-hands in the C-terminal domain are both ligated to Ca 2+, whereas the EF-hands in the N-terminal domain are not. Although the folds of the apo and Ca 2+-ligated domains are similar, a large difference in the relative orientation of the two α-helices is observed, which results in the formation of a pronounced hydrophobic pocket between the four helices in the Ca 2+-ligated state. Herzberg et al. proposed that this conformational difference constrains the basis for the activating Ca 2+-modu-

lating EF-hand proteins. The NMR and X-ray structures for complexes between calmodulin and two trinuclear light chain krone peptide fragments confirmed that the hydrophobic pockets play an important structural role in the binding of calmodulin to its target myosins (10,11). The EF-hand motif is found in a wide range of Ca 2+-binding proteins with very diverse functions. Structural information for most of these proteins is only available in the Ca 2+-ligated state or, as was the case for the N-terminal domain of troponin C, in the apo form. A recent solution NMR study of calmodulin D 9 , the first to directly compare the structures of the same protein in both the Ca 2+-free and Ca 2+-ligated states (12), although calmodulin D 9 has significant sequence homology with calmodulin, no large rearrangement of the α-helices was observed for this protein upon metal binding. This deviation from what the Herzberg model (12) predicted was ascribed to the function of calmodulin D 9 , which has a Ca 2+-buffering role rather than a regulatory one. A preliminary NMR study of the Ca 2+-ligated N-terminal domain of troponin C (ref. 14) supports the Herzberg conformational switch model. A preliminary report on the structure of the C-terminal domain of calmodulin α4 supports this qualitative support for the Herzberg model and for the apo calmodulin model of Strynadka and James. However, a recent study of the rotational dynamics of apo-calmodulin showed a difference in the “N NMR relaxation times of helices C and D attributed to motional anisotropy, that appeared incompatible with the nearly antiparallel orientation of helices C and D reported for the model structure.”
Here we present the three-dimensional structure of apo-calmodulin as determined by modern multidimensional NMR techniques, which permits a detailed study of the conformational changes that take place upon calcium binding. Many of the techniques and procedures used in the structure determination process are relatively new, and the experimental aspects of this study will therefore be emphasized, whereas a detailed description and an evaluation of the importance of the individual experiments for the quality of the final structure will be presented elsewhere.

Structural information from J-couplings

As with interproton distance constraints obtained from NOE's, dihedral constraints obtained from J-couplings contain important information for calculating the structure of a protein in solution. The sample of apo-calmodulin was enriched uniformly in 13C and 2H and has been used in our laboratory for the development of numerous new techniques for measuring J-couplings. As a consequence, a large number of three-bond J-couplings (over 700) was measured for apo-calmodulin, covering a wide range of different types. For example, three-bond J-couplings between aliphatic carbons were measured and indicated that all eight Mr residues have a χ1 angle of 180°. This permitted stereospecific assignments of all H-F methyl protons to be made, based on the large J-coupling between the Cα methyl carbon and H-F, and on the strong NOE between the Cα methyl proton and H-F. For two of these Mr residues (Mr 63 and Mr 130), J-couplings between the Cα methyl carbon and the backbone carbonyl and 2H indicate that the χ1 angle is subject to conformational averaging. These conclusions are confirmed by strong rotating-frame Overhauser effects (ROEs) between the backbone amides of these residues to HF and both H-F and 2H methyl protons. No physically reasonable conformation of the side chain can simultaneously satisfy these short distances and, as confirmed by measurement of H-F-HF, Cα-N, and Cα-N CO coupling, the χ1 angle of these two Mr residues is subject to conformational averaging. On the basis of 13C-13C and 13C-2H J-couplings, the same conclusion could be drawn about the χ1 angle of Val 55. Remarkably, the H-F-HF J-coupling for this residue is found to be large (−10 Hz), which would normally be taken as an indication of a sin 140° coifing, or a χ1 angle of 180°. A similarly large H-F-HF J-coupling is expected for the geometrically unfavorable χ1 = 0° rotamer, however. Based on the J-couplings and virtually identical parameters of both short- and long-range NOE's in the two Val 55 methyl groups, we conclude that the χ1 angle of this residue rapidly fluctuates between 0° and 180°, with both states approximately equally populated. In commonly used force-field parameterizations used for molecular dynamics calculations, population of the χ1 = 0° angle is energetically costly (∼5 kcal mol⁻¹). This suggests that Val 55 destabilizes the structure in this region of the protein, possibly for lowering the height of the energy barrier involved in the structural transition from the apo to the Ca²⁺-ligated state.
Interproton distance measurement from ROE

Measurement of the back-bone $^{13}$N amide relaxation times indicates that the overall motion of the protein is not quite isotropic but, on average, is described by a rotational correlation time, $\tau_R$, of ~8 ns (ref. 16). For such a relatively long $\tau_R$ value, NOE cross-peaks build up rapidly and indirect NOE effects (spin diffusion) can be a significant problem, making the NOE-derived distances appear shorter than their true value. In contrast to NOEs, ROEs are positive and increase monotonically with $\tau_R$, and indirect effects are opposite to spin relative to direct effects. For a ROE mixing time that is about 20% shorter than the average spin-labeled transverse relaxation time, $T_2$, which is close to optimal with respect to sensitivity, indirect NOE effects tend to be quite small. In practice, the indirect ROE contribution to a cross peak decreases the direct ROE and results in an overestimate of the true distance. When using upper-limit distance constraints, such an overestimate of the distance merely lessens the constraint imposed on the structure, and does not force hydrogen atoms to be more proximate than their true value.

Fig. 1 compares a cross-section through the four-dimensional (4D) NOE spectrum with the equivalent cross-section taken through the three-dimensional (3D) NOESY spectrum. Both panels show the protons that are in close proximity to the back-bone amide of Asp 42. The 4D spectrum is invaluable as it provides not only the H' shift of the second site, but also that of the directly attached $^{13}$C, allowing for a more unambiguous identification of those protons than could be made on the basis of their H’ shift alone. Comparison with the corresponding section of the 3D NOESY spectrum shows, however, that the interactions to Glu 41 H’ and Pro 43 H’ are dominated by indirect effects, and the corresponding distance constraints have not been used in the structure calculation. The Arg 42 amide also shows a strong positive cross peak with water, which is opposite in sign to the true ROE cross-peaks and is caused by rapid hydrogen exchange. Although a number of other back-bone amides exhibit negative ROE cross-peaks to water, all of these can be explained by the proximity of the back-bone amide protons to rapidly exchanging hydrols of Ser and Thr residues. Thus, we have found no evidence for tightly bound water molecules in the proximity of back-bone amides.

Reverse labelling of Phe residues

Besides determining the correct local geometry, which in our case is based primarily on the measurement of J-couplings and ROEs, identification of NOEs between residues that are far apart in the primary sequence is critical to determining as accurate a structure as possible. Such interactions are found primarily between hydrophobic residues in the core of the protein. We have used regular 4D $^{13}$C/$^{13}$C-separated NOE swap identifying interactions between aliphatic side chains, but as pointed out previously, this type of experiment provides less than optimal resolution when applying interaction involving Phe residues. NOEs to these residues are studied most conveniently using a reverse labelling procedure, where Phe residues are at natural abundance and the rest of the protein is enriched in $^{13}$C (ref. 21). This allows the interactions with these residues to be studied at high resolution and high sensitivity. The eight Phe residues in calmodulin provided nearly 200 long range NOE constraints, or about 50% of the total number of long-range constraints. Resonance assignment of the Phe aromatic ring protons were made using a combination of homonuclear $^{15}$N-based spin exchange experiments and a $^{13}$C-filtered NOE to correlate H' to the W and H".
Backbone dynamics

As reported previously, the secondary structure of apo-calmodulin is very similar to that observed in the Ca²⁺-ligated state. Hydrogen exchange measurements indicate, however, that the hydrogen bonding network in apo-calmodulin is considerably less stable, particularly in the C-terminal domain where all amides exchange with solvent in less than 1.5 minutes. In the first halves of the 'loop' regions of the four helix, 'loop'-helix calcium binding sites, rapid amide hydrogen exchange is observed and N relaxation parameters indicate higher-than-average amplitudes for the rapid internal motions of the backbone atoms. These more flexible loop regions each contain three residues that ligate Ca²⁺ in the X-ray structure, so their increased disorder in the absence of Ca²⁺ is not surprising. In this respect, it is interesting to note that in the homologous protein calbindin D₂₄, the canonical Ca²⁺-binding loop also shows an increase in backbone dynamics in the absence of Ca²⁺, whereas the other Ca²⁺-binding site, which is part of a so-called 'pseudo EF-hand', does not. In apo-calmodulin, the short B-strand and the first turn of the following helix (which constitute the second half of the Ca²⁺-binding loop and contain the three remaining protein-calciun coordination sites) do not show any pronounced increase in backbone dynamics.

As was found previously for Ca²⁺-ligated calbindin in solution, the C-terminal helix of the N-terminal globular domain (helix E) is a flexible linker, extending from Met76 to Ser81. This contrasts with the crystalline state of Ca²⁺-ligated calmodulin, where this linker is ordered and an integral part of the so-called 'central helix'. The Ca²⁺ chemical shifts for Met76 to Ser81 in apo-calmodulin are 1 ppm downfield from those of a random coil, and show sequential NOE connectivities between the N-terminal and the amide proton of residue 13, indicating that the linker adopts a helical conformation for a significant fraction of the time. However, it is also interesting to note that, in contrast to Ca²⁺-ligated calmodulin, weak NOE connectivities between the N-terminal and the amide of residue 13 are observed. The slow proton precession time of the helix backbone gives rise to NOE's that are close to random-coil values and to what is expected for as-rotor helix, and deviations from random coil (C²) shifts that are about threefold smaller than observed in an a-helix, we estimate that the Met76 to Ser81 linker adopts a helical conformation for about one third of the time. Considering the presence of observable a-helical NOE connectivities, this helical conformation must be relatively long-lived—at least several ms. The 31P spectrum of The 79 and 80 phosphocholine shows weak NOE's to (C=O) groups in the amide protons of Ser81 and Glu82, but no t (C=O) (t = 3) connectivities, suggesting that this region may adopt transiently a 3-point conformation. It is interesting to note that in the Ca²⁺-ligated state there is no direct NMR evidence for the transient presence of a helical conformation in this region of the polypeptide, even though in the a-helical conformation is observed in the X-ray structure of Ca²⁺-calmodulin. The significantly larger degree of rotational diffusion anisotropy observed for apo-calmodulin.

Table 1 Interhelical angles in calmodulin

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The C-terminal region (39-52) shows strong NOE to the (C=O) groups of the amide protons of Ser81 and Glu82, but no t (C=O) (t = 3) connectivities, suggesting that this region may adopt transiently a 3-point conformation. It is interesting to note that in the Ca²⁺-ligated state there is no direct NMR evidence for the transient presence of a helical conformation in this region of the polypeptide, even though in the a-helical conformation is observed in the X-ray structure of Ca²⁺-calmodulin. The significantly larger degree of rotational diffusion anisotropy observed for apo-calmodulin.
calmodulin \(^{9}\) relative to Ca\(^{2+}\)-calmodulin \(^{9}\) supports the conclusion that the linker has more of a helical character in the apo state compared to the Ca\(^{2+}\)-ligated state of the protein. Qualitatively, this also agrees with two digestion experiments, which indicated a higher sensitivity to proteolysis at Lys 77 in the Ca\(^{2+}\)-ligated state\(^{11}\).

No NOEs are observed between residues in the N-terminal domain and residues in the C-terminal domain, confirming that in solution, calmodulin also exists as two small globular domains, connected by a flexible tether which transiently adopts a helical conformation. The precision at which the structure of the C-terminal domain can be determined is severely limited by conformational averaging processes that take place on a microsecond time scale (vide infra). The N-terminal domain is not affected by such averaging and its structure has been determined at high resolution. We therefore first discuss this N-terminal domain, as it provides the clearest picture of a calmodulin EF-hand pair in the absence of calcium.

### Structure of the N-terminal domain

Fig. 2a shows a superposition of the back bones of the 25 lowest energy structures for the N-terminal domain. The r.m.s.d. relative to the mean of the non-hydrogen atom positions for residues 5 through 75 is 0.35 Å for the backbone and 0.71 Å for all non-hydrogen atoms. There are no consistent NOE violations larger than 0.15 Å, except for the flexible N-terminal residues and the side chains of Val 55 and Ile 63, for which \(J\) couplings indicate that \(\chi\) rotamer averaging is taking place. A ribbon diagram showing the structure of the N-terminal domain of apo calmodulin is shown in Fig. 3a. In the 3D structure, the N-terminal residues of helix A, and contact through Lys 18. The presence of an N-cap is identified by the characteristic \(J\) and \(\chi\) couplings shifts of the N-cap residues, one and two, 18. In addition, for all six N-caps identified in apo calmodulin NOEs are observed between the hydrophobic side chains of residues 1 and 11, which are predicted to have a hydrophobic interaction\(^{2}\).

The Phe 16 and Arg 26 from the L14-type hydrogen bonds to Phe 16 and Ser 17, respectively. The region from Lys 21 through Gly 25 shows an increase in backbone dynamical averaging and starts with a twist. The short \(\beta\)-strand (Thr 26, Thr 28) is well-defined and immediately precedes helix B, which starts with an N-cap at Thr 28. -He. -He.

### Table 2 Comparison of c-helical back bone atom positions

<table>
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<tr>
<th>apo CalM-N</th>
<th>apo CalM-C</th>
<th>model-1</th>
<th>model-2</th>
<th>Ca(^{2+})-CalM-N</th>
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<td>apo CalM-C</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>model-1</td>
<td>1.06</td>
<td>2.01</td>
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</tr>
<tr>
<td>model-2</td>
<td>1.09</td>
<td>1.99</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Ca(^{2+})-CalM-N</td>
<td>3.91</td>
<td>3.54</td>
<td>3.95</td>
<td>3.70</td>
</tr>
<tr>
<td>CalM-N</td>
<td>3.01</td>
<td>3.30</td>
<td>3.29</td>
<td>3.14</td>
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Comparison of N- and C-terminal domains

Compromise of the best-fit superposition of the α-helices of the N- and C-terminal domains shows rather similar structures for the two domains in the apo state (Fig. 4). However, this structural similarity is less than what was previously observed in the Ca²⁺-ligated state (Table 2). In part, the larger difference between the structure of the two domains in the apo state might be attributed to the relatively low precision of the C-domain solution structure. However, there are also a number of significant structural differences that fall well outside the range of uncertainty. One example is the absence of the pronounced kink in helix F, but its presence in helix B. Another example is the interaction between the aromatic ring of Phe 141 and backbone and side-chain atoms in helix G, whereas neither the homologous cytosine Phe 68, nor any other aromatic residue in the N-domains, shows such interactions with helix C (Fig. 2a).

Our study indicates that the C-terminal domain of apo calmodulin is considerably less stable than the N-terminal domain. Not only are the backbone amide hydrogen exchange rates much faster in the C-terminal domain, but there is also a measurable population of at least one alternate conformation. This alternate conformation has a lifetime of several hundred microseconds and, as a result of this short lifetime, it does not give rise to observable resonances. It is interesting to note that trypsin digestion experiments show rapid cleavage at residues Arg 106 in

![Fig. 5] Comparison of the backbone conformation of the N-terminal domain of the apo calmodulin NMR structure (yellow) with the model of Strynaskag and Jansen (plum).

![Fig. 6] Superposition of the N-terminal domain of apo calmodulin (yellow) with the X-ray structure of the N-terminal domain of Ca²⁺-ligated calmodulin (magenta).
apo calmodulin\textsuperscript{a}, near the middle of helix E. The apo calmodulin back bone must have a non-helical structure in this region at least part of the time in order to be accessible to the active site of the protease. Based on these considerations, we propose that helix E is not formed in the minor conformation. Qualitatively, this also agrees with a microcalorimetry study\textsuperscript{5}, which indicated that the melting behavior in the absence of Ca\textsuperscript{2+} for the C-terminal domain is not compatible with that of a single well-ordered structure.

**NMR structure versus model**

The N-terminal domain of apo calmodulin closely resembles that of the Ca\textsuperscript{2+}-free N-terminal domain of troponin C (ref. 9) and that of the apo-C structure (Table 2). The apparent disagreement between the orientation of helix D in the model and "N" relaxation data recorded for apo calmodulin\textsuperscript{3} is caused, in part, by the algorithm used for measuring the interhelical angles. Whereas Streitwieser and Jagga\textsuperscript{7} reported a C/D interhelical angle of 175\degree, their model coordinate correspond to a C/D angle of only 114\degree when using the algorithm outlined in the footnote to Table 1. The C/D angle measured for the NMR apo-calmodulin structure is somewhat smaller (130\degree) and the difference in the orientation of helix C and D is sufficiently large to explain their different "N" relaxation behavior.

The back bone of the N-terminal domain NMR structure and of the apo calmodulin model\textsuperscript{3} are superimposed in Fig. 5 and differ by only 1.13\AA for the backbone and 1.87\AA for 20 non-hydrogen atoms, including surface-exposed flexible side chains. For example, several of the details regarding the 3h extension of helix A and the pronounced kink at Glu 31 are virtually identical to features seen in Ca\textsuperscript{2+}-free troponin C, and predicted by the apo calmodulin model. The main differences between the model and our structure are found in the slightly smaller angle between helices C and D (130\degree versus 142\degree), and the side-chain orientations of Leu 18, Glu 31, Met 51, and Met 71. The packing of the five Phe side chains is virtually identical to what is observed in the NMR structures.

Considering that the spread of the calculated C-terminal domain NMR structures relative to their mean is rather large (0.64\AA for residues 82–146), the 2.1 A pairwise r.m.d., for the back-bone atoms between the model and the NMR structure indicates that the two structures and the calculated structures are essentially the same with a relatively large number of significant differences in the packing of the hydrophobic residues is found, however, including the position of four of the arginines (Phe 89, Tyr 99, Tyr 138, and Phe 141). The start of the second Ca\textsuperscript{2+}-binding loop, including residues Asp 129–Asp 131, and in particular the position of the Leu 130 side chain, also differs significantly from the model of Strynadka and James.

The conformational switch

In apo calmodulin, sequential helix pairs in the N-terminal domain (A/B, N/C, D/E, and F/G) show the corresponding pairs in the C-diis aline, on average average interhelic angle of 130\degree. Thus, in the absence of calcium, each calmodulin domain consists of a strongly twisted but tightly packed bundle of four intertwined helices. Upon binding of Ca\textsuperscript{2+}, most of the change occurs within each of the EF-hand. As can be seen from Fig. 6, helices A and D remain in similar positions relative to one another as do B and C, but the A/B and C/D helix angles decrease nearly 30\degree (Table 1), and a similar change is seen for the two EF-hands in the C-terminal domain. The "knee" in this EF-hand rearrangement occurs at the end of the first B-strand in the first calcium-binding site, whereas in the second Ca\textsuperscript{2+}-binding site it occurs just prior to the B-strand. In the first Ca\textsuperscript{2+}-binding site of the N-terminal domain, the knee includes a sharp kink in helix B at Glu 31, but such a kink is not observed in any of the other helices.

The structural rearrangement upon binding of Ca\textsuperscript{2+} results in a pronounced hydrophobic pocket on the surface of each domain, which is absent in apo calmodulin. The hydrophobic pockets are formed by the NMR and X-ray structures of calmodulin complexed with their different synthetic target peptides, where each of these two pockets is fill by a long hydrophobic amino acid side chain of the peptide(C12).

The increased back-bone dynamics in the Ca\textsuperscript{2+} binding "loop" regions in apo calmodulin lowers the precision at which these structures can be determined. In addition, precision of the C-terminal domain is decreased by the conformational averaging process mentioned above. For the second Ca\textsuperscript{2+}-binding site, multiple side-chain conformational observables for Val 55 and Glu 71 also decrease the precision of the structure in this region. Nevertheless, the back-bone mean of residues 36–61 superimpose on those in the Ca\textsuperscript{2+}-ligated X-ray structure with an r.m.d. of 0.50\AA and residues 63–67 fit within 0.22\AA. Despite the increased amplitude of the back-bone dynamics, the average structure of the first Ca\textsuperscript{2+}-binding site

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### Table 3 Structural statistics and atomic r.m.s. differences

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<th>C-domain (76-148)</th>
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<td>E_r (kcal mol⁻¹)</td>
<td>0.125</td>
<td>0.295</td>
</tr>
<tr>
<td>E_r (kcal mol⁻¹)</td>
<td>0.5</td>
<td>2.2</td>
</tr>
<tr>
<td>E_r (kcal mol⁻¹)</td>
<td>0.74</td>
<td>2.9</td>
</tr>
<tr>
<td>E_r (kcal mol⁻¹)</td>
<td>109</td>
<td></td>
</tr>
</tbody>
</table>

| ϕ-α vs ϕ-α                                    | 0.23           | 0.71             |
| 1.24                                          | 0.64           |

### Methods

Recombinant Aspergillus calmodulin was overexpressed in E. coli (strain [AR]B) containing the expression vector pTACΔ3. A total of 5×10⁷ RMBI samples was used in the gelshift assay, each at a concentration of 1×10⁻⁶ M, 100 mM KCl, 1.5 mM NaCl, 10 mM Tris-acetate, pH 7.4. One sample contained 7.6 μg of uniformly 14C-labeled calmodulin in 25 μL 95% H₂O, 5% D₂O, using [14C]MnCl₂ (New England Nuclear, M 148), 0.1% sodium azide, and 0.05% Tween 80. Two samples of uniformly 14C-labeled calmodulin were introduced, one at 90% H₂O, one at 98% H₂O, each in a 9-μL-MBR sample tube. Two of these 'reverse-labeled' samples, dissolved in 98% H₂O, 90% H₂O, were introduced at 1% H₂O, at 1% H₂O, for the which was recombinantand at 1% H₂O, each in 9-μL-MBR sample tube. Two 'reverse-labeled, sample dissolved in 98% H₂O, 90% H₂O, were introduced at 1% H₂O, at 1% H₂O, for the which was recombinant and at 1% H₂O, each in 9-μL-MBR sample tube. Two 'reverse-labeled, sample dissolved in 98% H₂O, 90% H₂O, were introduced at 1% H₂O, at 1% H₂O, for the which was recombinant and at 1% H₂O, each in 9-μL-MBR sample tube. Two 'reverse-labeled, sample dissolved in 98% H₂O, 90% H₂O, were introduced at 1% H₂O, at 1% H₂O, for the which was recombinant and at 1% H₂O, each in 9-μL-MBR sample tube. Two 'reverse-labeled, sample dissolved in 98% H₂O, 90% H₂O, were introduced at 1% H₂O, at 1% H₂O, for the which was recombinant and at 1% H₂O, each in 9-μL-MBR sample tube. Two 'reverse-labeled, sample dissolved in 98% H₂O, 90% H₂O, were introduced at 1% H₂O, at 1% H₂O, for the which was recombinant and at 1% H₂O, each in 9-μL-MBR sample tube.


