Versatile Modular Antibodies for Sensitive and Specific Detection of Poly-ADP-Ribose

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Abstract

ADP-ribosylation is a chemically versatile modification of proteins, RNA and DNA that regulates various important signaling pathways, many of which are implicated in human diseases. Despite being discovered 60 years ago in the form of poly-ADP-ribosylation - the most studied form of this modification - investigating ADP-ribosylation at the molecular level has historically been challenging. By applying serine ADP-ribosylation-based antibody engineering technology, we have developed the first site-specific, as well as sensitive, mono-ADP-ribosylation modular antibodies. Here, we extend the scope of this technology to poly-ADP-ribosylation. By combining serine poly-ADP-ribosylated peptides as antigens, PARP1 serine mono- and poly-ADP-ribosylation for validation, phage display and the SpyTag protein ligation systems, we developed modular antibodies that are highly specific for poly-ADP-ribosylation. SpyTag-based coupling of horseradish peroxidase at specific positions, distant from the antigen-binding region of the antibody, yields a format that simplifies immunoblotting while dramatically enhancing poly- ADP-ribosylation detection sensitivity. Additionally, the creation of synthetic immunoglobulin formats – mouse, rabbit and human – enables straightforward co-detection of mono- and poly-ADP-ribosylation in cells by immunofluorescence. Our new tools improve and simplify the detection of poly-ADP-ribosylation, particularly in immunoblotting, enabling specific investigations of this key cellular signal in the context of other distinct forms of ADP-ribosylation.

Introduction

ADP-ribosylation is a versatile post-translational modification (PTM) that regulates numerous cellular processes, including DNA repair, transcription, signal transduction, and cell death[1-4]. It involves the enzymatic transfer of ADP-ribose units from nicotinamide adenine dinucleotide (NAD^+) to target molecules, mediated primarily by members of the ADP-ribosyltransferase (ART) enzyme family[5]. This dynamic modification can occur as mono-ADP-ribosylation (mono-ADPr), where a single ADP-ribose unit is present, or poly-ADP-ribosylation (poly-ADPr), involving the formation of linear or branched chains of ADP-ribose. The reversibility of this process, mediated by ADP-ribosyl hydrolases, underscores its regulatory significance in maintaining cellular homeostasis[6,7]. First identified in the 1960s, ADP-ribosylation has since emerged as a critical player in cellular stress responses, particularly in the context of DNA damage repair[8,9]. Poly(ADP-ribose) polymerase 1 (PARP1), the most studied member of the PARP family, is activated in response to DNA strand breaks, orchestrating the recruitment of DNA repair machinery and chromatin relaxation. Beyond DNA repair, ADP-ribosylation influences chromatin remodeling, RNA processing, and immune signaling. Notably, dysregulation of ADP-ribosylation is implicated in various diseases, including cancer, neurodegenerative disorders, and inflammatory conditions, making it an attractive target for therapeutic intervention[10-14].

The biological complexity of ADP-ribosylation stems from the diversity of its enzymes, substrates, and cellular contexts. The PARP family alone consists of 17 members in humans, of which four are known to synthesize poly-ADPr (PARP1, PARP2, PARP5a/TNKS1, and PARP5b/TNKS2), each exhibiting distinct substrate specificities and cellular functions[15]. Moreover, emerging evidence highlights non-canonical roles of ADP-ribosylation, such as its involvement in liquid-liquid phase separation and the regulation of stress granules[16-18].

ADPr is one of the most chemically complex PTMs, with conjugation chemistries ranging from O-glycosidic (serine and tyrosine), S-glycosidic (cysteine), N-glycosidic (arginine) to ester (aspartate and glutamate) linkages. Strikingly, some of the main conjugation chemistries have remained elusive for decades. A prime example of this is serine ADP-ribosylation, which, despite being the most abundant - though not the exclusive target residue in PARP1/2 signaling - was only discovered less than ten years ago[19-22].

The structural complexity of ADP-ribosylation arises from the unique structure of ADP-ribose and its ability to form both mono-ADPr and intricate polymer chains. The polymeric forms, particularly poly-ADPr, can adopt linear or branched conformations, creating a diverse array of structural motifs[23,24]. These variations influence the binding affinity and functionality of

effector proteins, known as "readers," which recognize specific poly-ADPr structures through macrodomains, PAR-binding zinc fingers, and other motifs[25-28]. Additionally, the rapid synthesis and degradation of these chains add a temporal layer of complexity, making ADP-ribosylation a highly dynamic and versatile regulatory mechanism.

The inherent biological and chemical/structural complexity of ADPr has historically hindered its study and the development of appropriate analytical tools. This lack of tools has historically remained a significant limitation in many studies. Nevertheless, the critical role of poly-ADPr in modulating essential biological processes, particularly PARP1 signaling in the DNA damage response, has driven the development of various approaches and tools to study this modification since its discovery in the 1960s[29].

ADPr research methods and tools can be broadly categorized into indirect and direct approaches. Indirect methods include the use of small molecule inhibitors to block poly-ADPr writers[30], or genetic and RNA interference-based techniques to modulate the occurrence and persistence of this modification by targeting readers or erasers. Direct approaches aim to study poly-ADPr either *in-vitro* or *in-vivo*, including artificial construction or modification of poly-ADPr[31,32] and its targets to unravel its structural complexity and biological functions. Early techniques utilized NAD^+ analog probes[33], which remain in use today with further optimizations, such as Et-DTB-NAD^+[34-36]. In addition to chemical probes, enrichment strategies originally designed for other post-translational modifications (e.g., phosphoenrichment) have been adapted for poly-ADPr[37]. Elegant methods such as Enzymatic Labeling of Terminal ADP-Ribose (ELTA) offer another avenue for studying this modification by leveraging enzymes like OAS1 and modified dATP analogs to chemically tag and track poly-ADPr chains[38].

Among direct methods to study endogenous poly-ADPr levels, antibodies and reagents specific to poly-ADPr have emerged as promising tools to advance the field. Notably, many of the most frequently cited poly-ADPr antibodies have been polyclonal, which are challenging to produce consistently, leading to their discontinuation. This left the field reliant on monoclonal antibodies, such as 10H and the much less commonly used 16B. The 10H antibody, which was the most widely used tool in the early decades of ADPr research, primarily recognizes long poly-ADPr chains starting at 10–20 units and has low sensitivity compared to reagents developed in the last decade.

Recently, the Kraus laboratory pioneered the conversion of established ADP-ribose reader domains into Fc fusion, antibody-like recombinant reagents. One widely used tool is the AF1521 domain, which has been applied in various studies. Its mutated from, with increased

100 affinity is used particularly for enrichment followed by mass spectrometry analysis[39,40]. While
101 this approach has identified numerous ADPr sites, it cannot distinguish between mono- and
102 poly-ADPr because the domain binds to the terminal unit, enriching both forms
103 indiscriminately. Under certain conditions, both the original and affinity-matured AF1521
104 domain exhibit hydrolytic activity towards aspartate and glutamate ADPr[39]. Another approach
105 employs the WWE domain of RNF146, which specifically binds poly-ADPr. More precisely, it
106 targets iso-ADPr, a structure unique to poly-ADPr chains, thereby avoiding misinterpretation
107 by not interacting with mono-ADPr[41]. This innovation added a more selective tool for studying
108 poly-ADPr[42,43]. However, the diversity of poly-ADPr-specific domains, each potentially evolved
109 to recognize distinct poly-ADPr structures, suggests that individual domains might exhibit
110 specificity for certain chain types or protein targets. Even among WWE domains, differences
111 in binding specificity and affinity have been observed, as exemplified by the two WWE
112 domains in PARP13[44]. As a result, the application of these protein domain-based tools may
113 be beneficial to study specific research questions rather than offering the broad applicability
114 of antibodies. In fact, a so-called 'Poly/Mono-ADP Ribose' rabbit monoclonal antibody is
115 frequently used to detect poly-ADPr. While it is sensitive and specific for ADP-ribose, this
116 antibody is not specific for poly-ADP-ribose, as it also recognizes mono-ADP-ribose[45].

117 By converting our biological discovery of serine ADPr by HPF1/PARP1[19,20] into a technology
118 for generating ADPr peptides we have developed several anti-ADPr modular antibodies[21,46-
119 49]. This includes the first - and so far only - site-specific ADPr antibodies[46], as well as a
120 sensitive and specific mono-ADPr antibody that has proven effective in detecting not only
121 serine mono-ADPr[47] but also beyond PARP1 signaling, including interferon signaling[50-52].
122 Importantly, serine ADPr reactions not only yield antigens for the initial selection of antibody
123 candidates via phage display but also play a crucial role in subsequent steps, enabling in-
124 depth validation and characterization of the best antibody candidates. While phage display
125 used in our approach offers several advantages compared to traditional animal-based
126 approaches for generating antibodies, including the rapid initial identification of multiple
127 binders to the antigen of choice, it has the drawback of yielding recombinant antibodies in non-
128 standard formats. While conversion to standard immunoglobulins and other formats is
129 feasible, cloning-based conversion is impractical and time-consuming, counterbalancing some
130 of the advantages of recombinant antibodies. This has changed dramatically with the
131 application of SpyTag/SpyCatcher protein ligation technology to recombinant antibodies. This
132 biochemical format conversion enables the almost instantaneous generation of a versatile
133 family of formats, including rabbit, mouse and human synthetic immunoglobulins, as well as
134 site-specific labeling with horseradish peroxidase (HRP), biotin, and other tags[53]. In particular,
135 a bivalent modular antibody carrying three HRP molecules has proven to be a much more

136 sensitive, simpler and faster immunoblotting approach compared to standard protocols based
137 on secondary antibodies[53], significantly improving detection of ADPr[21,47].

138 Here, we extend the scope of our serine ADPr technology to poly-ADPr, combining it with a
139 proven phage display recombinant antibody platform and the SpyTag protein ligation system
140 to develop sensitive and specific poly-ADPr antibodies. This advance enables versatile,
141 precise and consistent detection across a variety of experimental applications, such as
142 immunofluorescence, immunoblotting and immunoprecipitation. In this study, we present the
143 characterization and validation of new poly-ADPr recombinant antibodies, reasoning that the
144 modular nature of this antibody, as well as our previously introduced complementary
145 antibodies, will enable more versatile and sensitive ADPr detection. Despite recent advances,
146 many aspects of ADP-ribosylation remain poorly understood, with outstanding questions
147 regarding its substrate specificity, spatial and temporal regulation. Furthermore, recent
148 discoveries of novel ADP-ribosylation readers, writers, and erasers promise to reshape our
149 understanding of this modification and its broader impact on cellular physiology.

150 **Material and Methods**

151 Resource availability

152 *Lead contact*

153 Further information and requests for resources and reagents should be directed to and will
154 be fulfilled by the lead contact, Dr. Ivan Matić (imatic@age.mpg.de)

155 *Material availability*

156 Antibodies, Reagents and BiSpyCatcher

157 The α-Poly-ADPr antibodies generated in this study (AbD64138 and AbD64235) are not yet
158 commercially available but requests can be forwarded to the lead contact. Previously
159 published antibodies converted to the SpyTag formats (AbD43647) and SpyCatchers are
160 available through BioRad Laboratories (unconjugated, requires conjugation to a SpyCatcher:
161 TZA020; HRP-conjugated, ready-to-use: TZA020P)

162 Uncoupled SpyTagged antibodies were conjugated as described below to following
163 SpyCatchers: bivalent-HRP Format (BiSpyCatcher2 #TZC002P, Bio-Rad); bivalent synthetic
164 IgG Format mouse (Mouse IgG2a-FcSpyCatcher3 # TZC012, Bio-Rad); bivalent synthetic IgG
165 Format rabbit (Rabbit IgG-FcSpyCatcher3 # TZC013, Bio-Rad)

166 Commercial antibodies and reagents used in this study: α-Poly/Mono-ADP Ribose (D9P7Z)
167 (Cell Signaling Technology, #89190), α-Poly (ADPr-ribose) monoclonal antibody (10H) (Enzo
168 Life Sciences, ALX-804-220-R100), α-Poly-ADP-ribose binding reagent (Merck, MABE1031)

169 Experimental Model

170 *Cell Culture and drug treatments*

171 U2OS cell lines were obtained, authenticated by STR profiling and confirmed mycoplasma
172 free by ATCC cell line authentication service. Cells were cultured in Glutamax-DMEM
173 supplemented with 10% bovine serum and 100U/ml penicillin/Streptomycin at 37°C and 5%
174 CO_2.

175 To induce DNA damage, the cell medium was aspirated and replaced with 37°C complete
176 DMEM containing 2mM H_2O_2 for 10 min. For PARP inhibition (Olaparib, Cayman Chemical)
177 cells were treated with 1 μM Olaparib in complete DMEM for 30 min prior to DNA damage
178 treatment, followed by 10 min 2mM H_2O_2 & 1μM Olaparib treatment.

179 Method details

180 *Antibody SpyCatcher Coupling*

181 All Bio-Rad antibodies have been obtained unconjugated in a monovalent format and were
182 conjugated to BiSpyCatchers inhouse leading to bivalent HRP conjugated or bivalent

183 synthetic IgG antibody formats. In brief antibody stocks were diluted to 1 mg/ml in PBS and
184 incubated with 1/10th the volume of unconjugated antibody with BiSpyCatcher for 1 hr at RT,
185 i.e. 10 µl of BiSpyCatcher was added to 100 µl of 1mg/ml unconjugated antibody. Afterwards
186 they were aliquoted and stored at -20°C.

187 *Expression and purification of recombinant proteins*
188 Proteins were expressed in *Escherichia coli* (*E.coli*) and purified essentially as previously
189 described[54], but with addition of a size-exclusion chromatography purification using HiLoad
190 16/60 Superdex 75 column. PARP1 wild-type was expressed and purified as previously
191 reported[54,55]. PARG was purified as described[56]. HPF1 was expressed and purified as
192 reported[57].

193 *In vitro Modification of Proteins*
194 Recombinant PARP1 protein (0.5 µM) was incubated with 2 mM NAD^+ in the presence of
195 HPF1 (5 µM). The reaction buffer contained 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM
196 $MgCl_2$ and 1 ng/µL sonicated DNA. Reactions were allowed to proceed for at 30 min at RT,
197 before being stopped by the addition of 1 µM Olaparib. To create mono- and poly-ADP-
198 ribosylated protein samples were split and one half was further incubated with 3 µM PARG for
199 1 hr at RT and stopped by the addition of Laemmli buffer. Samples were boiled and run on an
200 8% Bis-Tris gel (Invitrogen).
201 For Dot-blotting the same reaction as above was performed without splitting the reaction after
202 stopping with Olaparib. No loading buffer was added. A dilution series of the sample was
203 performed using reaction buffer and the same volumes (1 µl) of the probe was pipetted onto
204 a dry nitrocellulose membrane. The membrane was left to dry and was proceeded with 5%
205 non-fat dried milk blocking and regular immunoblotting steps as described below.

206 *SDS Sample Preparation and Immunoblotting*
207 U2OS cells were treated as indicated, lysed in SDS buffer (4% SDS; 50 mM HEPES pH 7.2,
208 150 mM NaCl, 5 mM $MgCl_2$) and incubated for 5 min at RT with recombinant benzonase
209 (smDNase, 750U per sample). Samples were spun at max. speed for 10 min and supernatant
210 was transferred into fresh tube. Sample concentration was measured using a Pierce Dilution-
211 Free BCA Protein Assay Kit (#A55864, ThermoScientific). Samples were prepared with
212 NuPAGE LDS sample buffer with final concentration of 5 mM DTT (Sigma) and loaded on 4-
213 12% Bis-Tris gel (Invitrogen). After SDS-Page the gels are transferred onto PVDF membranes
214 (Amersham) using wet transfers at 90 mA overnight on ice. The precooled transfer buffer is 1x
215 NuPAGE transfer buffer (Invitrogen), 20% ethanol in water.
216 For immunoblotting membranes were blocked in 5% non-fat dried milk before primary
217 antibodies were added. Primary Antibodies (commercial antibodies and HRP-formats of Bio-

218 Rad conjugated antibodies) were prepared in milk using concentrations found in figures. The
219 membranes were incubated overnight at 4°C before being washed in TBS-T (25 mM Tris-HCl
220 pH 7.5, 150 mM NaCl, 0.05% Tween-20) 3x10 min. Commercial antibodies (Enzo, Merck, Cell
221 Signaling Technologies) were incubated with a secondary antibody (Anti-mouse IgG HRP-
222 conjugated secondary Amersham Cat# NA931V, Anti-rabbit IgG HRP-conjugated secondary
223 Merck Cat# GENA934-1ML) for 1 hr at RT, followed by 3x10 min TBS-T wash. All membranes
224 were developed using ECL Select Western Blotting Detection Reagent (Amersham).

225 *Immunofluorescence*

226 U2OS cells were grown on sterile glass coverslips in 24-well plates. treated as indicated and
227 fixed for 20 min with Methanol at -20°C. The fixed cells were washed twice with PBS, and
228 blocked for 1hr, RT with 5% Normal-Goat-Serum in PBS supplemented with 0.3% Triton.
229 Primary antibodies (commercial antibodies and synthetic IgG formats of Bio-Rad conjugated
230 antibodies) were diluted as indicated and incubated overnight at 4°C in 1x PBS, 1% BSA and
231 0.3% Triton. Coverslips were washed in PBS 3x5 min before adding the secondary antibody
232 and DAPI, again in 1x PBS, 1% BSA and 0.3% Triton for 1 hr, RT protected from light.
233 Coverslide were washed again in PBS 3x5 min before mounted on microscopy slides using
234 Prolong Diamond Antifade Mountant (ThermoFisher). Cells were scanned for
235 immunofluorescence using a Leica SP8-X inverted laser-scanning confocal microscope.

236 **Results**

237 Generation of a serine poly-ADPr peptide

238

239 Previously, we established the PARP1/HPF1 complex[20,57,58] as an efficient and specific
240 biocatalytic tool for the rapid and scalable generation of mono-ADPr peptides, enabling the
241 development, validation and characterization of site-specific, mono-ADPr and pan-ADPr
242 antibodies[46,47]. Here, we aimed to adapt our strategy to generate modular antibodies specific
243 for poly-ADPr. Inspired by the possibility of extending the initial ADP-ribose attached to serine
244 of the peptide[40], we employed a simple protocol that does not require separation of different
245 ADP-ribosylated peptide species, reasoning that this approach would be sufficient for the
246 development of poly-ADPr antibodies. Specifically, we generated and purified the mono-ADPr
247 peptide from proteins using C18 StageTips[46] and subsequently added PARP1 without HPF1
248 to extend the mono-ADPr modification to di- and tri-ADPr. This process proved to be as
249 scalable as the previously described serine mono-ADPr peptide generation method[46]. The
250 resulting peptides were then employed in a phage display screen, leading to the identification
251 of several positive clones. From these, we selected the two most promising candidates for
252 further validation and comparison, as detailed in the following sections (AbD64138,
253 AbD64235).

254 Modular Antibodies for versatile, specific and sensitive detection of Poly-ADPr

255 Given the demonstrated value of the SpyTag system in the generation of modular
256 antibodies[46,47], we also chose to implement it for our new set of poly-ADPr antibodies. As
257 illustrated in the scheme (Fig. 1), the monovalent poly-ADPr antibody can be easily converted
258 into a bivalent format in a fast highly reliable reaction. This conversion adds an avidity effect
259 in addition to the high affinity of the antibody, further enhancing signal intensity and specificity.
260 Depending on the application, a variety of catchers can be chosen for coupling. For example,
261 an HRP-catcher can be used, resulting in an HRP-coupled bivalent poly-ADPr antibody for
262 immunoblotting. This eliminates the need for a secondary antibody, reducing costs, antibody
263 incubation time and increasing signal intensity, as demonstrated previously[47,53]. Alternatively,
264 for applications not reliant on HRP, such as immunofluorescence, a catcher fused with the
265 mouse or rabbit Fc region can be selected, creating a synthetic IgG antibody that, like a regular
266 IgG, is detectable by fluorophore-coupled secondary antibodies.

267 After coupling, we first assessed the specificity of the poly-ADPr antibodies (AbD64138,
268 AbD64235) to ensure there was no off-target binding to other forms of ADPr. To test this, we
269 took advantage of a key feature of serine ADPr: the inability of PARG to cleave the bond
270 between serine and the initial ADP-ribose, despite its ability to hydrolyze tyrosine and

271 aspartate/glutamate ADPr[21,59]. Specifically, we automodified PARP1 in the presence of HPF1,
272 resulting in nearly complete serine Poly-ADPr of PARP1. Addition of PARG to a portion of the
273 reaction completely reduced Poly-ADPr-PARP1 to mono-ADPr-PARP1[46], resulting in pure
274 mono- and poly-ADPr standards for antibody validation. As expected, our mono-ADPr
275 antibody[47] recognized exclusively mono-ADPr (Fig. 1A). Our newly developed poly-ADPr
276 antibodies did not detect any mono-ADPr, but produced an intense signal for poly-ADPr
277 PARP1. In contrast, pan-ADPr antibodies, such as D9P7Z from Cell Signaling Technology
278 (CST), detected both mono- and poly-ADPr (Fig. 1,A). We also compared our antibodies with
279 other commercially available poly-ADPr reagents, including 10H (Enzo) antibody and the
280 WWE-based poly-ADPr reagent from Merck (MABE1031)[42]. All poly-ADPr probes were shown
281 to be specific for poly-ADPr, although 10H only detected long ADPr polymers as previously
282 reported[60] (Fig. 1, A). Like MABE1031, our new antibodies were able to detect the full range
283 of poly-ADPr chains in immunoblotting. To ensure a fair comparison of signal intensities, we
284 tested the reagents at two different dilutions, starting with those recommended by the
285 manufacturers/publications for optimal performance. However, we found that using our
286 antibodies at the same concentration (1 µg/ml) led to signal bleaching before detection.
287 Consequently, we lowered the concentration by a factor of ten, which allowed for signal
288 detection without compromising the poly-ADPr detection by commercially available reagents.
289 Despite this adjustment, the sensitivity of our antibodies is so high that oversaturation occurred
290 after just one second of exposure.

291 To allow for a more accurate comparison without signal saturation, we performed a dot blot
292 using a 1:2 dilution series of poly-ADPr PARP1 (Fig. 1,B). Again, using antibody dilutions as
293 recommended by the manufacturer and a common dilution of 0.1 µg/ml, we compared the
294 signal intensities of our new tools to those of other reagents. The results showed that our new
295 antibodies provided significantly higher signal intensity compared to the MABE1031 reagent
296 and D9P7Z antibody. The 10H antibody did not produce any detectable signal at this dilution.

297 These results demonstrate a high specificity for poly-ADPr of the new antibodies, with no
298 detection of mono-ADPr, confirming that our antibodies selectively recognize poly-ADPr
299 chains.

300 <u>Sensitive detection of Poly-ADPr targets in cells</u>

301 After establishing their poly-ADPr specificity and sensitivity in *in vitro* biochemical reactions,
302 we applied the new antibodies to cell lysates to evaluate their performance on cellular poly-
303 ADPr targets and assess potential off-target binding. To induce high levels of poly-ADPr, cells
304 were treated H_2O_2 for ten minutes and additionally treated with the PARP1 inhibitor Olaparib.

305 We used optimal antibody dilutions for all probes to ensure peak performance. First, we
306 demonstrated that there was no nonspecific binding to proteins, as shown in lanes 2 and 4 of
307 each blot, where no signal was detected in olaparib-treated samples (Fig. 2,A). Second, even
308 when using ten times higher amounts of commercially available reagents, our new poly-ADPr
309 antibodies still exhibited superior signal intensity. Third, our antibodies also improved the
310 detection of poly-ADPr in unperturbed cells, where its generation has been shown to be
311 induced by unligated Okazaki fragments during DNA replication[61], even with low exposures,
312 as seen in lane 1.

313 We then tested the specificity of our antibodies in cells using immunofluorescence and the
314 synthetic IgG format. Again, we applied optimal antibody dilutions and tailored microscope
315 settings for each reagent to achieve high performance. While intensity levels are not directly
316 comparable, the focus was on the specificity of the signal and the signal-to-noise ratios. Our
317 poly-ADPr antibodies AbD64138 and AbD64235 provided a superior signal-to-noise ratio in
318 untreated versus H_2O_2-treated cells (Fig. 2,B), outperforming other poly-ADPr reagents. The
319 signal specificity was comparable to the poly-/mono-ADPr antibody (CST) and our mono-ADPr
320 reagent (AbD43647), with antibody AbD64138 performing slightly better over AbD64235.

321 In conclusion, these experiments validate the high signal-to-noise ratio of our new tools for
322 both immunoblotting and immunofluorescence, demonstrating their versatility and potential for
323 multiple applications.

324 <u>SpyTag System Enables Flexible Co-Detection of Mono- and Poly-ADPr In Cells</u>

325 Finally, to assess the potential for simultaneous use of our poly-ADPr antibodies alongside
326 our previously published mono-ADPr antibodies, we performed co-staining of both poly-ADPr
327 (AbD64138) and mono-ADPr (AbD43647) in cells (Fig. 3,B). For this, we coupled the poly-
328 ADPr antibody with a mouse Fc catcher and the mono-ADPr antibody with a rabbit Fc catcher.
329 To prevent channel bleed-through, we used two fluorophores with distinct emission peaks and
330 employed line sequencing during imaging. The results demonstrate that, following a ten-
331 minute H_2O_2 treatment, both mono- and poly-ADPr can be detected simultaneously in the
332 same cells. Notably, the staining patterns revealed distinct distributions: the mono-ADPr signal
333 appeared more homogenously distributed throughout the nucleus, while the poly-ADPr signal
334 exhibited a more punctate pattern, forming small foci.

335 The ability to co-stain both forms of ADPr enhances the depth of analysis in
336 immunofluorescence, allowing for the use of smaller sample sizes and reduced experimental
337 times. Additionally, the flexible coupling of different IgG species to either poly- or mono-ADPr

338 antibodies provides (Fig.3, B) versatility for co-staining with other targets, such as full-length
339 PARP1, facilitating broader applications in cellular analysis.

340 To summarize the results of our antibody validation, we demonstrate superior performance in
341 both signal intensity and specificity for immunoblotting and immunofluorescence applications.
342 While both of our antibodies are highly specific for poly-ADPr and exceptionally sensitive,
343 especially in immunoblotting, AbD64138 shows slightly better performance compared to
344 AbD64235.

345 **Discussion**

346 Despite decades of research on ADPr and recent technological advances[29], the availability of
347 specific and sensitive tools for studying all forms of this challenging PTM remains limited. This
348 study aimed to extend the reach of our serine ADPr technology, which enabled the
349 development of the first site-specific ADPr antibodies as well as specific and sensitive mono-
350 ADPr antibodies[21,46-49], to poly-ADPr, resulting in a highly sensitive, specific and versatile
351 modular antibody. As a technology, Serine ADPr offers several advantages inherent to the
352 nature of the physiological reaction. First, PARP1 is the most catalytically active PARP
353 enzyme, strongly activated by DNA breaks both in cells and in biochemical reactions.
354 Therefore, the modification is highly scalable, allowing reactions to be upscaled to milligram
355 quantities suitable for antibody panning. Second, HPF1 enables not only precise targeting of
356 PARP1 activity toward serine residues, but also modification of peptides rather than just
357 PARP1 automodification[20]. Third, the biological interplay between serine ADPr and canonical
358 histone marks[62] can be harnessed to program the HPF1/PARP1 complex, directing it to target
359 a specific serine using phosphorylation as an unconventional protecting group[46]. Fourth, a key
360 advantage of serine-ADPr is its chemical stability: the O-glycosidic linkage remains intact
361 across a broad range of pH conditions and withstands heat exposure up to 95°C. This stability
362 facilitates efficient handling and minimizes sample loss during experimental procedures, a
363 problem observed with other forms of ADPr, particularly aspartate/glutamate ADPr[21,63]. Fifth,
364 while the poly-ADPr eraser PARG can cleave the bonds between ADP-ribose and tyrosine,
365 aspartate and glutamate[21,59], it does not remove serine ADPr. Here, beyond peptide
366 generation for phage display and antibody screening, we harness the resistance of serine
367 ADPr to PARG to achieve nearly complete modification of PARP1 at the protein level, either
368 by poly-ADPr or, upon PARG treatment, of mono-ADPr. This enables comprehensive antibody
369 specificity testing, ensuring the selection of clones with high specificity for poly-ADPr and
370 minimal off-target binding.

371 The newly developed poly-ADPr antibodies presented in this study offer several key
372 advantages. They enable the detection of a broad range of poly-ADPr, from short to long
373 polymeric chains, as demonstrated by full signal coverage in immunoblotting analysis. The
374 modular nature of these antibodies, derived from the SpyTag technology results in strong
375 signal intensity, especially in immunoblotting, and offers versatility across multiple
376 applications, including immunofluorescence and immunoprecipitation, allowing for the
377 detection of poly-ADPr even under untreated conditions. The modular SpyTag system, as
378 illustrated previously for our mono-ADPr and site-specific antibodies[47], provides significant
379 benefits. The reaction used to couple various catchers to the monovalent antibodies is fast,

380 efficient, and highly reliable. For immunoblotting, the HRP Catcher eliminates the need for a
381 secondary antibody, significantly increasing signal intensity, reducing costs by lowering the
382 required antibody amount per experiment and shortening the overall immunoblotting
383 procedure. Additionally, the SpyTag system enables the creation of our poly-ADPr antibodies
384 as synthetic immunoglobulins, which can be used in immunofluorescence assays. This
385 flexibility allows for the selection of species-specific antibodies (e.g., mouse, rabbit, or human)
386 tailored to experimental needs, allowing co-staining with other ADPr- or protein-specific
387 antibodies.

388 Beyond their high affinity and modularity, the antibodies described in this study also offer high
389 reliability. As monoclonal recombinant antibodies, they ensure consistent quality and
390 reproducibility, unlike previously available polyclonal poly-ADPr antibodies, which were
391 subject to batch variability and eventual discontinuation. This homogeneity and consistency
392 further reinforce their utility as a long-term tool for ADPr research.

393 The most critical factor when selecting a tool to study ADPr is that its effectiveness is inherently
394 dependent on the research question being asked. Choosing the appropriate tool is essential
395 to obtaining meaningful and accurate insights. Given the high variability of ADPr arising from
396 modifications at different sites, in diverse forms, by various proteins across multiple cellular
397 signaling pathways, multiple complementary tools are required to study this PTM in an in-
398 depth manner. Broad yet ADPr-specific tools, such as the highly sensitive poly-/mono-ADPr
399 antibody from Cell Signaling Technology, allow for the general detection of ADPr. However,
400 their inability to distinguish between different ADPr forms can complicate result interpretation,
401 especially as it is emerging that mono- and poly-ADPr act as distinct signals, even within
402 pathways where they co-occur[21,47]. In this study, we aimed to develop antibodies that provide
403 both broad detection and high specificity, fully complementing our previously developed mono-
404 ADPr antibodies[46,47] to enable a clear differentiation between mono- and poly-ADPr. The tools
405 presented here contribute to expanding the available toolkit for ADPr research at this level. To
406 further investigate mono- and poly-ADPr in greater depth, additional specialized tools may be
407 necessary. For instance, site-specific mono-ADPr and poly-ADPr antibodies may enable a
408 more detailed examination of ADPr modifications, while engineered protein domains such as
409 WWE fused to an Fc chain[39,43,64], offer an alternative approach to studying certain forms of
410 poly-ADPr.

411 In conclusion, we present the first application of our technology for generating poly-ADPr-
412 specific antibodies. As with the development of site-specific and mono-ADPr antibodies, we
413 incorporated serine ADPr at nearly every phase of the antibody generation process, from

414 precisely modified peptide antigens to multiple validation steps and in-depth characterization.
415 Obtaining purely mono- and poly-ADP-ribosylated PARP1 was particularly useful for
416 unambiguously establishing the specificity of the new antibodies. In this process, phage
417 display and the SpyTag protein ligation system were essential for generating numerous initial
418 candidate clones and for conferring modularity to the final, validated antibodies, increasing
419 their versatility and sensitivity. We anticipate that these new tools, along with previously
420 developed reagents, will play key roles in deciphering the distinct roles of the two primary
421 forms of ADPr - its monomeric and polymeric signals - in various signaling pathways.

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Author Contributions

I.M. conceived the project. H.D. and I.M. designed research and wrote the manuscript. H.D. performed all the experiments.

Declaration of Interest

The authors I.M. and H.D. declare the following competing financial interest: Max-Planck-Innovation, which is responsible for technology transfer from Max Planck Institutes, has licensed the antibody AbD43647 to Bio-Rad Laboratories, which markets it for research purposes. Additionally, a similar agreement is in preparation for the commercialization of AbD64138.

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Figures

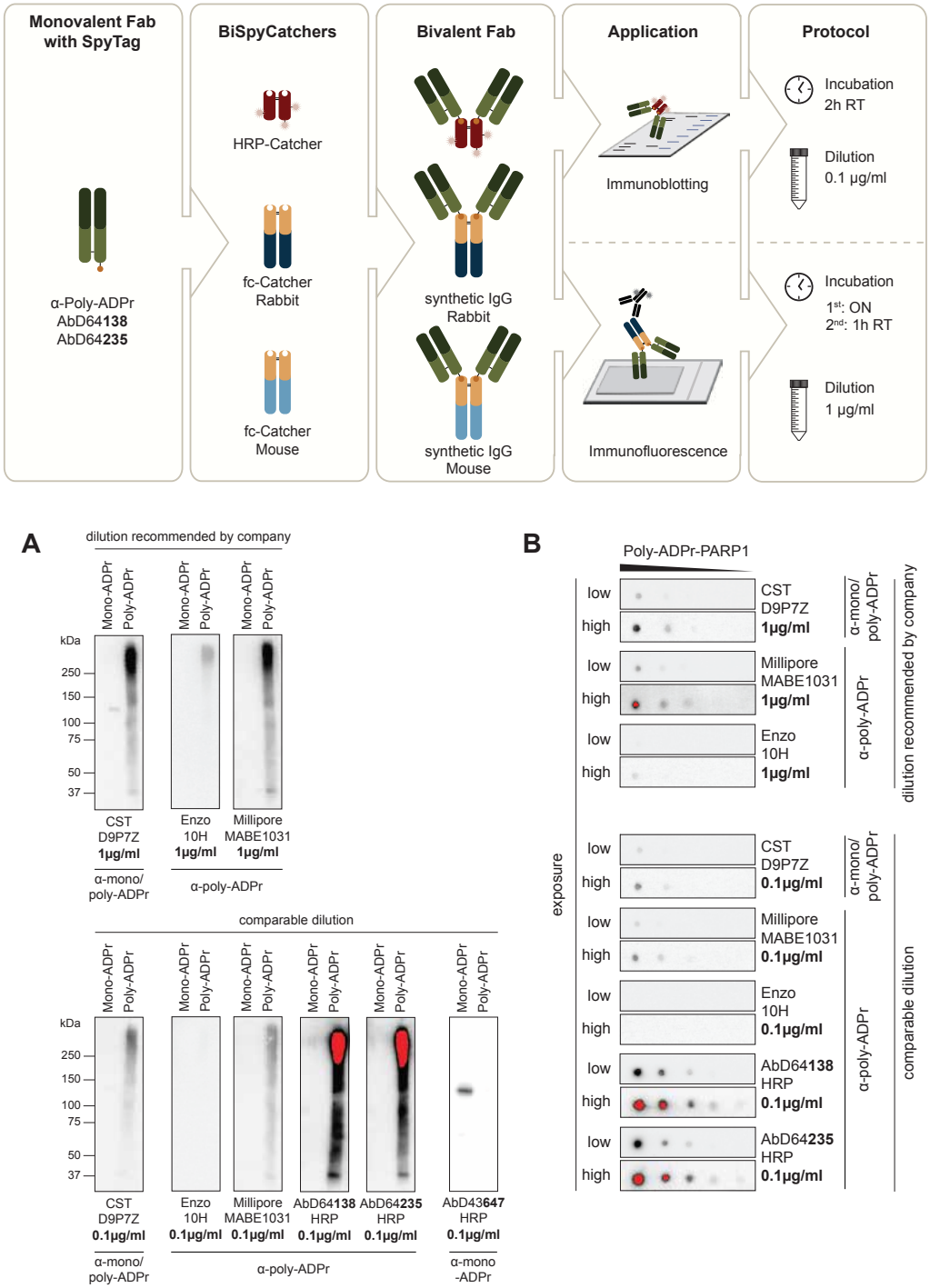

Figure 1: Modular Antibodies for versatile, specific and sensitive detection of Poly-ADPr

Scheme describing the coupling process of monovalent SpyTagged antibody, using various BiSpyCatchers to create a variety of formats for multiple approaches.
A: *In Vitro* modified recombinant PARP1, treated w/o PARG to create Poly-/ and Mono-ADPr PARP1 immunoblotted with new anti-Poly-ADPr antibodies in comparison to other available antibodies and reagents. Red represents oversaturated signal. **B:** *In Vitro* modified recombinant PARP1 dot-blotted in dilution series and incubated with new anti-Poly-ADPr antibodies in comparison to competitors. Red represents oversaturated signal.

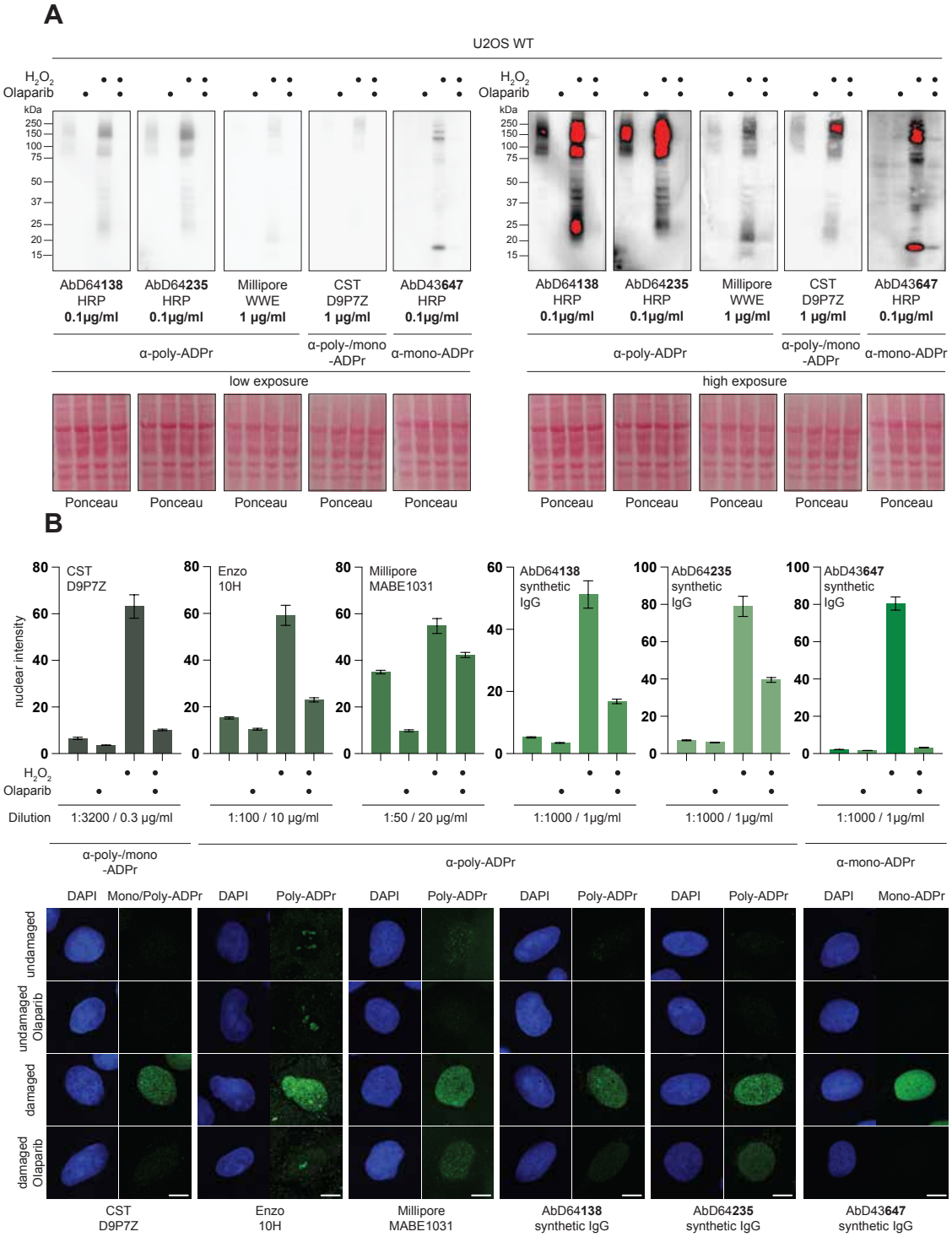

Figure 2: New highly sensitive Poly-ADPr-specific Antibodies

A: Immunoblotting of U2OS cells treated or not with H_2O_2, w/o Olaparib, lysed in SDS buffer and proceeded with immunoblotting. Antibodies and Reagents used in indicated concentration recommended by manufacturer or publication. HRP-format was used for new anti-poly-ADPr antibodies. Red represents oversaturated signal **B:** Immunofluorescence images of U2OS cells treated or not with H_2O_2, w/o Olaparib followed by methanol fixation. Antibodies and Reagents used in indicated concentration recommended by manufacturer or publication. Synthetic IgG format was used for new anti-poly-ADPr antibodies. Error bar represents SEM. Scale bar represents 10 µm.

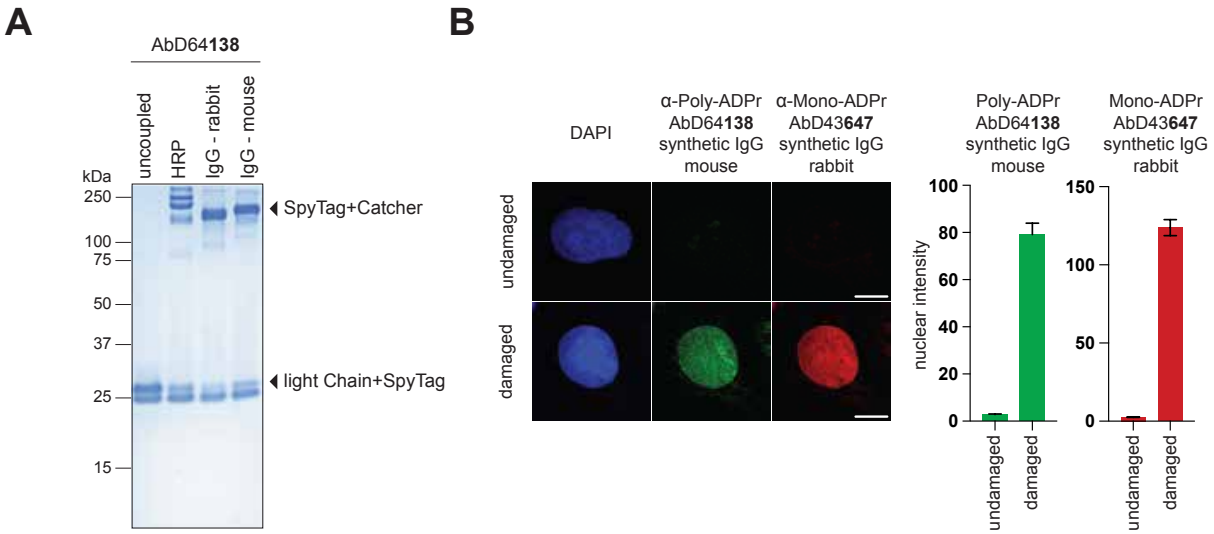

Figure 3: Versatile SpyTag System Enables Flexible Co-Detection of Mono- and Poly-ADPr In Cells

A: Coomassie Staining of the same AbD64138 antibody in monovalent unconjugated format (first lane) and bivalent conjugates formats (lane 2-4). **B:** Immunofluorescence co-staining of mono- and poly-ADPr in the same cell using synthetic mouse/rabbit IgG antibody formats.