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R&D SYSTEMS

ELISA

HT PARP *in vivo* Pharmacodynamic ELISA Kit II

Catalog Number 4520C-096-K

High throughput chemiluminescent ELISA to quantify poly-ADP-ribose (PAR) in tissues and cultured cells.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

In response to DNA damage, poly-(ADP-ribose) polymerase-1 (PARP-1), which is the main isoform of the PARP family, is rapidly activated by DNA strand breaks that occur during exposure to environmental toxins, cancer therapy, inflammation, ischemia-reperfusion and neurodegeneration (1). Once activated, NAD^+ is consumed for the synthesis of the highly negatively-charged polymer poly-ADP-ribose (PAR), which is found on target nuclear proteins including PARP-1. These highly branched polymers are in turn rapidly degraded by poly-(ADP-ribose) glycohydrolase (PARG). As a consequence of PARP activation, extensive DNA damage can lead to the depletion of NAD^+ in the cell, and lead to cell death. PARP-1 is regarded as a promising target for the development of drugs useful in various regimens of cancer therapy, inflammation, ischemia and neurodegeneration (1-3). For example, the discovery that breast cancers deficient in homologous recombination are sensitive to nontoxic PARP inhibitors, has resulted in efforts by numerous pharmaceutical companies to develop PARP-1 specific drugs.

The HT PARP *in vivo* Pharmacodynamic ELISA Kit II, which measures net PAR levels in cellular extracts, provides the ability to monitor and quantify PARP activity across individuals and within cells. This assay employs a sample processing procedure and has been used to document differences in PAR levels among tissues and cultured cells (4).

PRINCIPLE OF THE ASSAY

A polyclonal antibody specific for PAR has been pre-coated onto a microplate to capture cellular PAR and PAR attached to proteins. Incubation with a PAR Monoclonal Detection Antibody, followed by addition of a chemiluminescent HRP substrate yields relative light units (RLU) that directly correlate with the amount of cellular PAR. This assay is ideal for quantification of PAR in tissues, and cultured cells. Additional uses may include monitoring the efficacy of PARP inhibitors on cellular PAR formation and verifying enhanced cancer cell cytotoxicity arising from PARP inhibitor/anti-cancer drug combination therapy (3). Important features of the assay include:

- Chemiluminescent, non-radioactive format.
- High throughput 96 test format with pre-coated capture antibody.
- Broad linear dynamic range to 1000 pg/mL.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Variations in sample collection, processing, and storage may cause sample value differences.
- Do not mix or substitute reagents from other lots or sources.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay.
- Variation in pipetting technique, washing technique, and incubation time or temperature can cause variations in binding.
- Relative light units (RLU) may differ among luminometers. Adjust settings as recommended by the instrument manufacturer.
- Sample processing can potentially generate artifactual PAR synthesis during resuspension and incubation in Cell Lysis Buffer. Methods to mitigate this effect should be carefully considered as part of the experimental design.
  Note: *The addition of a PARP inhibitor to the Cell Lysis Buffer such as PJ 34 Hydrochloride (Tocris™, Catalog # 3255) can help mitigate artifactual PAR synthesis.*

PRECAUTION

The acute and chronic effects of overexposure to reagents in this kit are unknown. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit components at the recommended temperatures listed below. Do not use past kit expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF OPENED/ RECONSTITUTED MATERIAL	STORAGE OF UNOPENED MATERIAL
PARP Microplate	3040040	96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for PAR.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.	Store 2-8 °C.
Calibrator Diluent RD5-20	895346	21 mL	Store 2-8 °C.	
PARP Cell Lysis Reagent	4520-096-05	30 mL		
100X Magnesium Cation	4520-096-07	500 µL		
Assay Diluent RD1-117	895859	11 mL		
PAR Monoclonal Detection Antibody	3040037	12 mL		
PeroxyGlow™ A	4675-096-01	6 mL		
PeroxyGlow™ B	4675-096-02	6 mL		
Wash Buffer Concentrate, 25X	895003	21 mL		
PAR Standard, 25X	3040038	2 vials	Use a fresh vial for each assay. Discard after use.	
PAR Control, Low	3040041	2 vials		
PAR Control, High	3040044	2 vials		
DNase I, 2 Units/µL	4520-096-06	60 µL	Store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.	Store at ≤ -20 °C.
20% SDS	4520-096-12	1 mL	Store at room temperature.	
Plate Sealers	N/A	4 adhesive strips		

OTHER SUPPLIES REQUIRED

- Distilled or deionized water
- Phenylmethyl Sulfonyl Fluoride (PMSF) in ethanol (Tocris™, Catalog # 4486)
- Protease Inhibitor Cocktail I (*optional*, Tocris, Catalog # 5500)
- Trypsin for detaching adherent cells
- PJ 34 Hydrochloride (*optional*, Tocris, Catalog # 3255)
- Pipettes and pipette tips
- Multichannel pipettor 10-100 µL
- Squirt bottle, manifold dispenser, or automated microplate washer
- 96-well chemiluminescent plate reader or luminometer
- Refrigerated centrifuge with swinging bucket rotor
- Microcentrifuge
- 15 mL and 50 mL screw cap centrifuge tubes
- 0.5 mL and 1.5 mL microtubes
- 25 mL solution reservoirs
- Incubator set at 25 °C (preferred)
- Heat block capable of maintaining 100 °C

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of 1X Wash Buffer. *May turn yellow over time.*

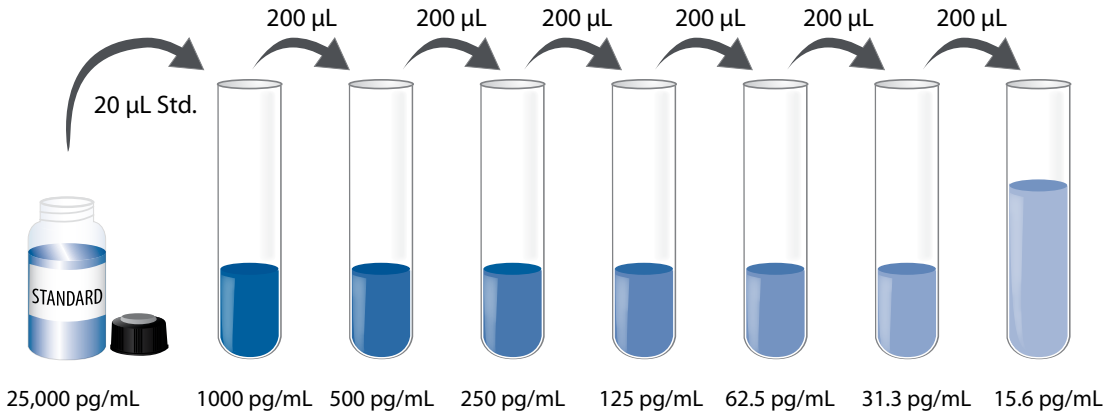
Calibrator Diluent RD5-20 (diluted 1:2) - Add 5 mL of Calibrator Diluent RD5-20 to 5 mL of deionized or distilled water to prepare 10 mL of Calibrator Diluent RD5-20 (diluted 1:2).

Assay Diluent RD1-117 (diluted 1:6) - Add 1 mL of Assay Diluent RD1-117 to 5 mL of deionized or distilled water to prepare 6 mL of Assay Diluent RD1-117 (diluted 1:6).

Substrate Solution - PeroxyGlow™ A and B should be mixed together in equal volumes immediately before use. 100 µL of the resultant mixture is required per well.

PAR Standard - **Refer to vial label for reconstitution volume.** Reconstitute the standard vial with distilled or deionized water. This reconstitution produces a stock solution of 25,000 pg/mL. Allow the standard to sit for a minimum of 5 minutes on the benchtop with gentle agitation prior to making dilutions.

Pipette 480 µL of Calibrator Diluent RD5-20 (diluted 1:2) into the 1000 pg/mL tube. Pipette 200 µL of Calibrator Diluent RD5-20 (diluted 1:2) into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-20 (diluted 1:2) serves as the zero standard (0 pg/mL).



PAR Controls - Reconstitute the controls with deionized or distilled water. Allow the controls to sit for a minimum of 5 minutes on the benchtop with gentle agitation. Mix thoroughly. Assay the controls undiluted.

Control	Lot #	Reconstitution Volume	Control Range (pg/mL)
PAR Control, Low	P490206	0.8 mL	41.6-200
PAR Control, High	P490210	0.8 mL	307-811

REAGENT PREPARATION *CONTINUED*

Cell Lysis Buffer - Immediately before use, prepare 1 mL of Cell Lysis Buffer by mixing the following and placing on ice:

Part	Catalog #	Volume
Cell Lysis Reagent	4520-096-05	980 µL
100 mM PMSF (in ethanol)	Tocris™, Catalog # 4486	10 µL
100X Protease Inhibitor Cocktail I	Tocris™, Catalog # 5500	10 µL

20% SDS - The 20% SDS may precipitate during shipping. To solubilize, warm the tube at 37 °C for 10 minutes and gently vortex periodically.

SAMPLE COLLECTION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Suspension Cells

1. Grow 2-10 x 10^6 suspension cells in complete medium in a suitable tissue culture plate or flask.
2. Transfer the cells to pre-chilled 15 mL screw cap centrifuge tubes. Count the cells and then centrifuge at 250 x g for 5 minutes at 2-8 °C. Discard the supernatant. Wash the cells with 10 mL of ice-cold 1X PBS.
3. Suspend the cell pellets in 1 mL of ice-cold 1X PBS. Transfer to 1.5 mL microcentrifuge tubes and centrifuge at 10,000 x g or top speed in a microcentrifuge for 10 seconds at 2-8 °C. Discard the supernatant.
4. Resuspend the cell pellet at a cell concentration of 1-5 x 10^7 cells/mL in Cell Lysis Buffer. Incubate the cell suspensions on ice, with periodic vortexing, for 15 minutes.
5. Add 20% SDS to a final concentration of 1%. For example, add 50 µL of 20% SDS to 950 µL of resuspended cells.
6. Incubate cell extracts at 100 °C for 5 minutes. Cool to room temperature.
7. Add 0.01 volume of 100X Magnesium Cation and 2 µL of DNase I (2 Units/µL). Vortex briefly and incubate at 37 °C for 90 minutes. This step degrades cellular DNA and reduces the viscosity of the extract.
8. Centrifuge at 10,000 x g for 10 minutes at room temperature to remove cellular debris. Save the supernatant.
9. (Optional) Measure the protein concentration of the extract with a BCA protein assay.
10. Assay immediately for PAR or aliquot the extracts and store at ≤ -70 °C.

SAMPLE COLLECTION *CONTINUED*

Adherent Cells

1. Grow 2-10 x 10^6 adherent cells in a suitable tissue culture 60 mm dish or 6 well plate in complete medium until 75% confluent.
2. Remove the medium and gently wash the cells with 5 mL of warm (37 °C) 1X PBS. Carefully pipette out the PBS. Repeat this step one more time.
3. Add 300 µL of cold Cell Lysis Buffer to each well of a 6 well plate, or 500 µL to a 60 mm dish. Place the dish or plate on ice and immediately scrape the cells with a cell scraper to detach the cells. Incubate the cell suspensions on ice, with periodic scraping, for 15 minutes.
4. Transfer the cell suspensions to 1.5 mL tubes. Add 20% SDS to a final SDS concentration of 1%. For example, add 50 µL of 20% SDS to 950 µL of resuspended cells.
5. Incubate cell extract at 100 °C for 5 minutes. Cool to room temperature.
6. Add 0.01 volume of 100X Magnesium Cation and 2 µL of DNase I (2 Units/µL). Vortex briefly and incubate at 37 °C for 90 minutes. This step degrades cellular DNA and reduces the viscosity of the extract.
7. Centrifuge at 10,000 x g for 10 minutes at room temperature. Save the supernatant.
8. (Optional) Measure the protein concentration of the extract with a BCA protein assay.
9. Assay immediately for PAR or aliquot the extracts and store at ≤ -70 °C.

Tissues

Note: *It is recommended that tissue samples are flash frozen in liquid nitrogen. This is important for stabilizing PAR levels.*

1. Add 0.5 mL Cell Lysis Buffer to the frozen tissue and mince completely with fine-point scissors. Vortex to mix and place on ice.
2. Disrupt the extracts by sonication on ice three times for 10 seconds each cycle. Vortex and allow to stand on ice for 15 minutes.
3. Move samples to room temperature and add 20% SDS to a final concentration of 1%. For example, add 25 µL 20% SDS into 475 µL lysate.
4. Vortex again and incubate at 100 °C for 5 minutes. Cool on ice for 1 minute.
5. Centrifuge at 10,000 x g for 2 minutes at 2-8 °C. Collect the tissue lysate supernatant for each sample.
6. Measure the protein concentration of the extract with a BCA protein assay.
7. Assay immediately for PAR concentration or aliquot the extracts and store at ≤ -70 °C.

SAMPLE PREPARATION

For cell lysates, quantitation of sample protein concentration using a total protein assay is recommended. The suggested range for total cell lysate protein added is 1-5 µg/well.

The extracts must be diluted at least 3- to 5-fold with Calibrator Diluent RD5-20 (diluted 1:2) to reduce the SDS concentration to below 0.33%.

PAR levels in suspension and adherent cell lines may be reported either as pg PAR per 10^7 cells or pg/mL.

It is suggested that tissue extracts are added in the range of 100-2000 ng/well using Calibrator Diluent RD5-20 (diluted 1:2). PAR levels in tissue extracts may be reported as pg PAR per 100 µg of protein extract. PAR levels can be variable depending on the tissue type. The appropriate tissue amount may be empirically adjusted to attain optimal sensitivity.

ASSAY PROTOCOL

Bring all assay reagents to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack and reseal.
3. Add 50 µL of Assay Diluent RD1-117 (diluted 1:6) to each well.
4. Add 50 µL of standard, control or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided for a record of standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. If using a 96-well autowasher, include a **30 second soak** between washes. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 µL of PAR Monoclonal Detection Antibody to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
7. Repeat the aspiration/wash as in step 5.
8. Immediately before use, mix equal volumes of PeroxyGlow™ A and B and add 100 µL per well. **Protect from light.** Take chemiluminescent readings within 15 minutes.

*Samples may require dilution. See the Sample Preparation section.

CALCULATION OF RESULTS

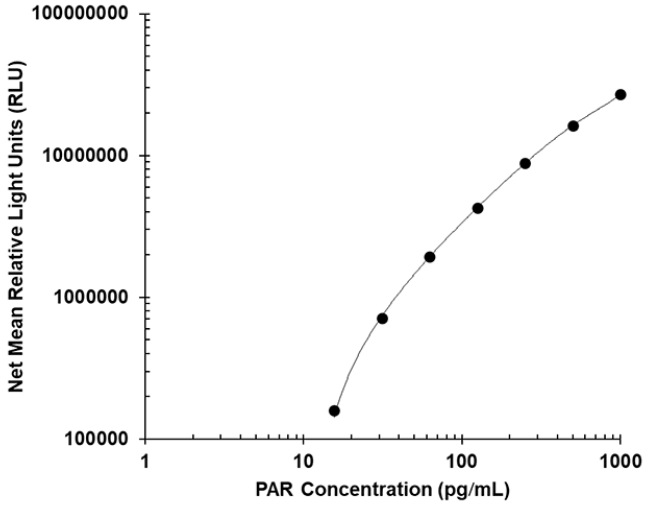
Average the duplicate readings and calculate the net mean RLU (Relative Light Units) values of the PAR standards by subtracting the background (without PAR) from the RLU values. Plot the net mean RLU as a function of PAR values (pg/mL).

Average the duplicate readings and calculate the net RLU values of the Low & High Controls or cell lysate samples by subtracting the background from the RLU values. Determine the PAR levels in each sample using a standard curve. Use a 4-PL regression line.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	RLU	Average	Corrected
0	710597 712,536	711,566	—
15.6	832,373 908,736	870,554	158,988
31.3	1,378,118 1,464,022	1,421,070	709,504
62.5	2,565,828 2,730,073	2,647,950	1,936,384
125	4,991,253 5,012,479	5,001,866	4,290,300
250	9,826,475 9,191,194	9,508,834	8,797,268
500	16,856,217 17,113,244	16,984,731	16,273,164
1000	26,918,151 28,812,624	27,865,387	27,153,821

PRECISION

Intra-Assay Precision (Precision within an assay)
Two samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Two samples of known concentration were tested in eight separate assays to assess inter-assay precision. Assays were performed by at least five technicians.

	Intra-Assay Precision		Inter-Assay Precision	
Sample	1	2	1	2
n	20	20	16	16
Mean (pg/mL)	217	843	123	570
Standard deviation	11.7	48.9	31.0	61.9
CV (%)	5.4	5.8	25.3	10.8

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of PAR were diluted with Calibrator Diluent RD5-20 (diluted 1:2) to produce samples with values within the dynamic range of the assay.

		Cell culture Lysates (n=4)
1:2	Average % of Expected	113
	Range (%)	96-129
1:4	Average % of Expected	121
	Range (%)	87-154
1:8	Average % of Expected	128
	Range (%)	87-166
1:16	Average % of Expected	127
	Range (%)	90-152

SAMPLE VALUES

WIL2-NS + 50 µM Etoposide: WIL2-NS cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were seeded at 1 x 10^5 cells/mL and incubated at 37 °C with 5% CO_2 until the viable cell density was between 0.7-1 x 10^6 cells/mL. Cells were then treated with 50 µM Etoposide (Catalog # 4684-096-06 or Catalog # 1226) for 4 hours. PARP Cell Lysis Buffer was prepared according to the product insert and the procedure for suspension cells outlined in the product insert was followed for preparing and storing samples.

WIL2-NS ± SK 575 ± Olaparib: WIL2-NS cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were seeded at 1 x 10^5 cells/mL and incubated at 37 °C with 5% CO_2 until the viable cell density was between 0.7-1 x 10^6 cells/mL. Cells were then left untreated or treated with 1 µM SK 575 (Catalog # 7583) or 1 µM Olaparib (Catalog # 7579) for 2 hours. PARP Cell Lysis Buffer was prepared according to the product insert with the addition of 10 µM PJ 34 hydrochloride (Catalog # 3255) and 10 µM PDD 00017273 (Catalog # 5952) to prevent artifactual PAR synthesis and PAR degradation. The procedure for suspension cells outlined in the product insert was then followed for preparing and storing samples.

Treatment	(pg/mL)
Untreated	206
Etoposide (50 µm)	3241
SK 575 (1 µm)	21
Olaparib (1 µm)	15

SPECIFICITY

This assay recognizes natural and synthesized PAR.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5-20 (diluted 1:2) and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in the PAR Low Control were assayed for interference. No significant cross-reactivity or interference was observed.

- NAD+
- Olaparib
- Veliparib
- Nicotinamide
- ADP-HPD

TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	SOLUTION
No relative light units (RLU) in experimental sample wells but RLU are present in wells with the PAR standard.	PAR levels in the samples are below the sensitivity of the assay.	Increase the number of "cell equivalents" or sample protein mass added to each well.
	PARG activity in the cell extract is very high.	Add a PARG inhibitor to the Cell Lysis Buffer.
		Check that specimen processing included addition of SDS and the boiling step; prepare a new specimen or add SDS and boil existing specimen.
No RLU in wells containing PAR standard.	PAR standards were not added to the wells.	Add serial dilutions of PAR standard to appropriate duplicate wells.
RLU in wells containing cell or tissue extracts too high or above that obtained for the PAR standard curve.	PAR levels in cells and tissues very high.	Extend serial dilutions of extract and check the linearity by back-calculating pg/mL PAR per cell number or per microgram of protein.
High background in wells with no PAR.	Poor washing.	Add a 30 second soak period following addition of 1X Wash Buffer.
High variability within duplicates.	Uneven distribution of reagents.	Check quality of single and multichannel pipettors.
		Practice repetitive pipetting technique.
	Incomplete solubilization and clarification of the specimen.	Check specimen for viscosity (indication of large quantity of intact DNA) and particulates. Repeat the DNase I treatment and centrifugation steps.
	Poor washing.	Add a 30 second soak period following addition of 1X Wash Buffer.
Assay Controls out of range high or low.	Uneven distribution of reagents.	Repeat assay.

REFERENCES

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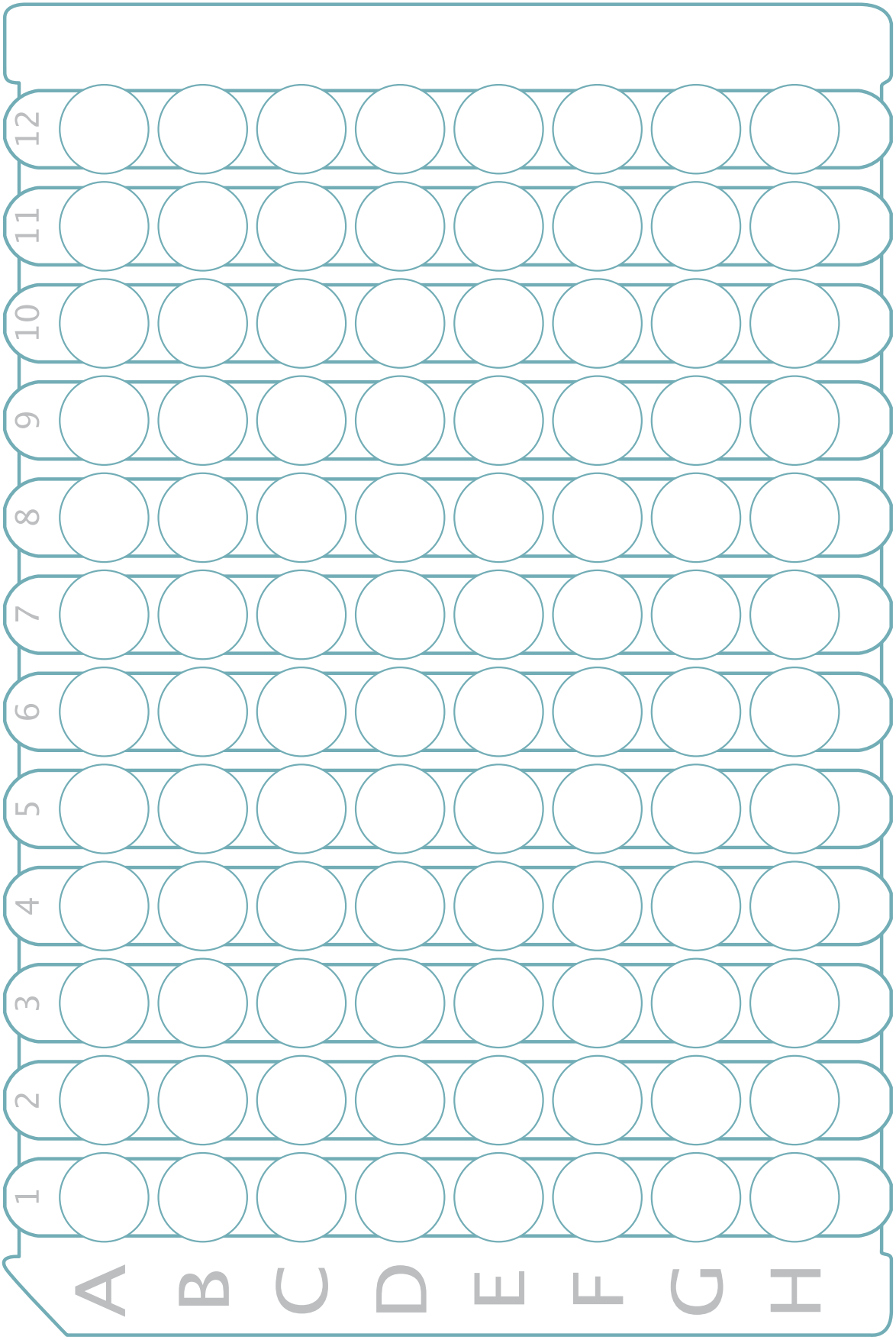
2. Thiemermann, C., J.*et al.* (1997) Proc. Natl. Acad. Sci. USA. **94**:679.
3. Curtin N.J. (2005) Exp. Rev. Mol. Med. **7**:1.
4. Kinders R.J. *et al.* (2008) Clin. Cancer Res. **14**:6877.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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