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Measurement of RNA-induced PKR Activation *in vitro*

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[Abstract] Protein kinase R (PKR) is one of the key RNA-activated sensors for innate immunity. PKR is activated by pathogenic or aberrant RNAs such as short double-stranded RNAs or those with imperfect secondary structures, as well as a reduction in the amount and number of RNA modifications. Activation of PKR may be an underlying mechanism for the pathogenesis of human diseases. In this protocol, I describe a method for studying levels of RNA-induced PKR activation *in vitro*.
Keywords: PKR, Stress response, Innate immunity, Aberrant RNA

[Background] PKR is one of four mammalian kinases that phosphorylate eukaryotic initiation factor 2-α subunit (eIF2α) in response to stress signals. PKR is activated mainly in response to viral infection (Holcik and Sonenberg, 2005). PKR is a key component of innate immunity that recognizes and binds to pathogenic RNAs. The interaction of RNAs with PKR promotes and stabilizes its dimerization. PKR then undergoes auto-phosphorylation and subsequently phosphorylates eIF2α to shut off general translation, while activating the downstream signaling cascade including the increased translation of the ATF4 stress response transcription factor (Hinnebusch, 2005).

PKR is known to be activated by short double-stranded RNAs (Manche *et al.*, 1992; Zheng and Bevilacqua, 2004) as well as RNAs with some imperfect secondary structures such as hairpin loops (Bevilacqua *et al.*, 1998). In addition, defects in RNA biogenesis, including lower levels of m^6A modification, lead to a stress response via the activation of PKR (Nallagatla and Bevilacqua, 2008). Reduced levels of m^6A modification followed by the activation of the PKR-mediated stress response may serve as an underlying molecular etiology of human diseases such as Cornelia de Lange syndrome (Yuen *et al.*, 2016). The method described in this protocol allows us to study the stress response triggered by foreign or aberrant RNAs by examining their effect on the activation of PKR *in vitro*.

Materials and Reagents

1. 35 mm TC-treated culture dish (Corning, catalog number: 430165)
2. Razor blades
3. Protein purification column (Kimble Chase Life Science and Research Products, catalog number: 420400-1010)
4. Pipette tips (VWR, catalog number: 53508-794)
5. 50 ml tube (Corning, catalog number: 430290)

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6. VWR 10 ml serological pipet (VWR, catalog number: 89130-888)
7. Parafilm M (Bemis, catalog number: PM996)
8. Immobilon-P polyvinylidene difluoride membrane (EMD Millipore, catalog number: IPVH00010)
9. Dialysis tube (Sigma-Aldrich, catalog number: D6191)
10. Dialysis tube clip (Sigma-Aldrich, catalog number: Z371092)
11. Dialysis tubing (Sigma-Aldrich, catalog number: D9652)
12. pGEM®-T Easy vector systems (Promega, catalog number: A1360)
13. One Shot TOP10 competent cells (Thermo Fisher Scientific, Invitrogen™, catalog number: C404003)
14. pET-28a(+) plasmid (EMD Millipore, catalog number: 69864)
15. DMEM (Thermo Fisher Scientific, Gibco™, catalog number: 11965092)
16. FBS (Thermo Fisher Scientific, Gibco™, catalog number: 10437028)
17. Penicillin-streptomycin (10,000 U/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)
18. TRIzol reagent (Thermo Fisher Scientific, Ambion™, catalog number: 15596026)
19. Chloroform, biotechnology grade (VWR, catalog number: 97064-678)
20. 2-propanol, biotechnology grade (VWR, catalog number: 97065-048)
21. Ethanol, absolute (Sigma-Aldrich, catalog number: E7023)
22. DEPC-treated water (Thermo Fisher Scientific, Invitrogen™, catalog number: 750023)
23. 5x iScript cDNA Synthesis Kit (Bio-Rad Laboratories, catalog number: 1708890)
24. Nuclease-free water (New England Biolabs, catalog number: B1500)
25. PCR primers (IDT)
26. Phusion high-fidelity DNA polymerase (New England Biolabs, catalog number: M0530)
27. dNTP (New England Biolabs, catalog number: N0447)
28. *Taq* DNA polymerase (New England Biolabs, catalog number: M0273)
29. Magnesium chloride ($MgCl_2$) solution (New England Biolabs, catalog number: B9021)
30. dATP (100 mM) (New England Biolabs, catalog number: N0440S)
31. T4 DNA ligase (New England Biolabs, catalog number: M0202)
32. X-Gal (β-galactoside) (Promega, catalog number: V3941)
33. SOC medium (Thermo Fisher Scientific, Invitrogen™, catalog number: 15544034)
34. LB plate (Thermo Fisher Scientific, Gibco™, catalog number: 10855021)
35. Ampicillin (Sigma-Aldrich, catalog number: A9393)
36. NEBuffer 3.1 (New England Biolabs, catalog number: B7203S)
37. *Sal*I restriction enzyme (New England Biolabs, catalog number: R0138)
38. *Not*I restriction enzyme (New England Biolabs, catalog number: R0189)
39. Qiagen QIAquick Gel Extraction Kit (QIAGEN, catalog number: 28706)
40. T4 DNA ligase buffer (New England Biolabs, catalog number: B0202S)
41. IPTG (Sigma-Aldrich, catalog number: I6758)
42. Liquid nitrogen (Midwest Liquid Nitrogen Service)

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43. Lysozyme (Sigma-Aldrich, catalog number: L6876)
44. RNase A (Sigma-Aldrich, catalog number: R4642)
45. DNase I (New England Biolabs, catalog number: M0303)
46. LDS loading buffer (4x) (Thermo Fisher Scientific, Noves™, catalog number: NP0007)
47. Ni-NTA agarose (QIAGEN, catalog number: 30230)
48. Qubit Protein Assay (Thermo Fisher Scientific, Molecular Probes™, catalog number: Q33211)
49. λ-PPase (New England Biolabs, catalog number: P0753)
50. Phosphatase reaction buffer (New England Biolabs, catalog number: B6022S)
51. Sodium orthovanadate (Sigma-Aldrich, catalog number: S6508)
52. Poly I:C (Sigma-Aldrich, catalog number: P1530)
53. NuPAGE 4-12% Bis-Tris protein gel (Thermo Fisher Scientific, Invitrogen™, catalog number: NP0322BOX)
54. Silver staining kit (Thermo Fisher Scientific, Noves™, catalog number: LC6100)
55. 1% bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A9418)
56. PKR primary antibody (Santa Cruz Biotechnology, catalog number: sc-6282)
57. Phosphorylated-PKR primary antibody (Abcam, catalog number: ab32036)
58. Peroxidase-conjugated secondary antibody (Thermo Fisher Scientific, Invitrogen™, catalog number: 31460)
59. ECL reagents (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 32132)
60. Sodium phosphate monobasic (NaH_2PO_4) (Sigma-Aldrich, catalog number: S8282)
61. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
62. Imidazole (Sigma-Aldrich, catalog number: I5513)
63. PMSF (Sigma-Aldrich, catalog number: P7626)
64. Protease inhibitor (Roche Diagnostics, catalog number: 11697498001)
65. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: S8045)
66. Tris (pH 7.6) (VWR, catalog number: 97061-260)
67. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9333)
68. Magnesium chloride ($MgCl_2$) powder (Sigma-Aldrich, catalog number: M8266)
69. Glycerol (VWR, BDH®, catalog number: BDH1172-1LP)
70. EDTA (Sigma-Aldrich, catalog number: E6758)
71. DTT (Sigma-Aldrich, catalog number: D0632)
72. HEPES (pH 7.5) (Sigma-Aldrich, catalog number: H3375)
73. ATP (New England Biolabs, catalog number: P0756)
74. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalog number: P5655)
75. Tween-20 (Sigma-Aldrich, catalog number: P9416)
76. *E. coli* lysis buffer (see Recipes)
77. Protein purification wash buffer (see Recipes)
78. Protein purification elution buffer (see Recipes)
79. Protein storage buffer (see Recipes)

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80. Phosphatase reaction buffer (see Recipes)
81. PKR activation buffer (see Recipes)
82. TE buffer (10x) (see Recipes)
83. Tween-20 phosphate buffered saline (pH 7.2) (see Recipes)

Equipment

1. Cell culture incubator (Panasonic Biomedical, model: MCO-230AIC)
2. Pipette (VWR, catalog number: 89079-970)
3. Centrifuge (Eppendorf, model: 5424)
4. Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Invitrogen™, catalog number: Q33216)
5. PCR machine (Bio-Rad Laboratories, model: S1000™ Thermal Cycler, catalog number: 1852148)
6. Vortex (VWR, catalog number: 97043-562)
7. Sonicator (Branson, model: Digital Sonifier 450 or from Fisher Scientific, catalog number: 15-338-553)
8. 600 ml beaker (VWR, catalog number: 10754-956)
9. Typhoon scanner (GE Healthcare, model: Amersham Molecular Dynamics Typhoon 9410 or from GMI, catalog number: 8149-30-9410)

Procedure

A. Human PKR gene cloning
   1. RNA isolation and cDNA library generation
      a. Grow the cells in a 35 mm dish with 10 ml DMEM with 10% FBS and 1% penicillin-streptomycin in a 37 °C incubator with 5% CO_2 until 80% confluence.
      b. Remove the media from the culture dish.
      c. Add 1 ml TRIzol reagent directly to the cells in the culture dish.
      d. Pipette the cells up and down for 5 times in the culture dish to lyse the cells.
      e. Incubate the sample at room temperature for 5 min to allow for complete nuclear lysis.
      f. Add 0.2 ml of chloroform and share the tube vigorously by hand for 15 sec.
      g. Incubate for 2 min at room temperature.
      h. Centrifuge the sample at 12,000 *x g* for 15 min at 4 °C.
      i. Remove the aqueous supernatant by angling the tube at 45° to avoid contacting the interphase or the organic layer.
      j. Transfer the aqueous phase into a new tube and add 0.5 ml of 100% isopropanol.
      k. Incubate at room temperature for 10 min.
      l. Centrifuge at 12,000 *x g* for 10 min at 4 °C.
         Note: RNA should be visible as a white pellet.

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m. Remove the supernatant carefully from the tube without touching the RNA pellet.
n. Wash the pellet with 1 ml of 75% ethanol pre-chilled at 4 °C.
o. Vortex the sample briefly, followed by centrifugation at 7,600 *x g* for 5 min at 4 °C.
p. Remove the ethanol carefully and air dry the RNA pellet for 15 min on ice.
q. Re-suspend the RNA pellet in 100 µl DEPC-treated water and incubate at 37 °C for 10 min.
r. Measure the RNA concentration using a Qubit 3.0 Fluorometer, NanoDrop spectrophotometer or Bioanalyzer.
s. Prepare the 5x iScript cDNA Synthesis reaction mix on ice for reverse transcription.

5x iScript reaction mix	4 µl
iScript reverse transcriptase	1 µl
RNA (100 fg to 1 µg total RNA)	x µl
Nuclease-free water	to 20 µl

t. Incubate the reaction mix on a PCR machine with the following program:
5 min at 25 °C
30 min at 42 °C
5 min at 85 °C
Hold at 4 °C
u. Store the cDNA in aliquots at -20 °C. Alternatively, store the cDNA at 4 °C if used repeatedly in days. Avoid repeated freezing and thawing cycles.

2. PCR amplification and subcloning into pET-28a+ plasmid

Other bacterial expression plasmids may be used. Different restriction enzymes may be used in other plasmids depending on the cloning sites.

a. Amplify the human PKR gene with the following primers:
Forward: 5' TAGTCGACATGGCTGGTGATCTTTCAGC 3'
Reverse: 5' GTCAGCCTAACATGTGTGTCGTTCATTTTTCTC 3'
Notes:
i. The forward primer contains a SalI restriction site, as underlined, before the start codon.
ii. Only a single band will be obtained.

b. Prepare a PCR reaction mix on ice as follow

5x Phusion buffer	4 µl
25 mM dNTP	0.2 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
cDNA	1 µl
Phusion polymerase	0.25 µl
Nuclease-free water	12.55 µl
Total	20 µl

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c. Incubate the reaction mix on a PCR machine with the following program:

10 sec at 98 °C		
10 sec at 98 °C	}	30 cycles
2 min at 60 °C		
10 min at 72 °C		
Hold at 4 °C		

d. Incubate the following reaction mix to produce the product with an overhang

10x *Taq* buffer	1 µl
$MgCl_2$ (25 mM)	0.6 µl
dATP (10 mM)	0.2 µl
PCR product	7 µl
Taq polymerase	0.5 µl
Nuclease-free water	0.7µl
Total	10 µl

e. Prepare the following reaction mix for subcloning the PKR gene into the pGEM-T Easy vector.

2x buffer	5 µl
pGEM-T Easy vector	1 µl
PCR product	3 µl
T4 ligase	1 µl
Total	10 µl

f. Incubate the ligation reaction mix at 4 °C overnight.

3. Transformation into *E. coli*
   a. Thaw One Shot TOP 10 chemically competent cells on ice for 3 min.
   b. Add 5 µl ligation product to the cells.
   c. Incubate the tube on ice for 30 min.
   d. Place the tube at room temperature for 10 min.
   e. Mix 50 µl β-galactoside (X-Gal) (50 mg/ml) to 150 µl SOC medium and add the mixture to the LB plate with ampicillin.
   f. Spread the transformed cells on the LB plate with ampicillin.
   g. Incubate the plate at 37 °C overnight.
   h. Pick the white colonies to check for successful ligation of PKR gene.
4. Digestion and ligation of PKR gene into pET-28a+
   a. Set up *Sal*I and *Not*I digestion reactions for pET-28a+ and PKR

pET-28a+	30 µl
NEBuffer 3.1	5 µl
*Sal*I	1 µl
*Not*I	1 µl
Nuclease-free water	13 µl
Total	50 µl

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pGEMT-PKR	10 µl
NEBuffer 3.1	5 µl
*Sal*I	1 µl
*Not*I	1 µl
Nuclease-free water	33 µl
Total	50 µl

b. Run the double digestion products pET-28a+ and PKR separately on a 1% agarose gel.
Note: Look for the bands for pET-28a and PKR at 5,369 bp and 1,656 bp, respectively.
c. Carefully excise the bands from the gel using clean razor blades.
d. Perform gel extraction using the QIAGEN Gel Extraction Kit.
e. Weigh the gel slice in a tube and add 3 volumes buffer QG to 1 volume gel.
f. Incubate at 50 °C for 10 min.
g. Add 1 gel volume isopropanol to the sample and vortex.
h. Transfer the sample to the QIAquick column and centrifuge for 1 min at 18,000 *x g*.
i. Add 750 ml buffer PE to the QIAquick column and centrifuge for 1 min at 18,000 *x g*.
j. Add 50 µl buffer EB to the center of the QIAquick membrane to elute the DNA.
k. Set up a ligation reaction to subclone PKR gene into the pET-28a plasmid

10x T4 DNA ligase buffer	1 µl
pET-28a	1 µl
PKR	4 µl
T4 DNA ligase	1 µl
Nuclease-free water	3 µl
Total	10 µl

l. Incubate at 16 °C overnight and transform 2 µl of the reaction into 25 µl competent cells.

B. PKR protein purification from *E. coli*
1. Grow the cells in LB broth at 37 °C until OD_{600} reaches 0.4-0.6.
2. Induce with 40 µl of a 100 mM IPTG (final concentration 400 µM) for 3 to 5 h at 37 °C.
3. Harvest the cells and proceed immediately to cell lysis or freeze the cells in liquid nitrogen followed by storing them at -80 °C.
4. Resuspend the cells in lysis buffer at 2-5 ml per gram wet weight.
5. Add lysozyme (1 mg/ml) to the lysis buffer with the cells and incubate on ice for 30 min.
6. Sonicate on ice using a sonicator equipped with a microtip.
Note: Use five 15 sec bursts at 30x with a 45 sec cooling period between each burst.
7. (Optional) If the lysate is very viscous, add RNase A (10 µg/ml) and DNase I (5 µg/ml) and incubate on ice for 10-15 min.
8. Centrifuge lysate at 10,000 *x g* for 20-30 min at 4 °C to pellet the cellular debris and keep the supernatant.
9. Add 5 µl 2x LDS-PAGE sample buffer to 5 µl supernatant and store at -20 °C for LDS-PAGE analysis.

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10. Pipette 10 ml of Ni-NTA slurry to a 50 ml tube and briefly centrifuge. Remove supernatant and add 20 ml of lysis buffer. Mix gently by inverting. Repeat centrifugation step and remove the supernatant.
    Note: Adjust the amount of Ni-NTA used depending on the yield of the target protein.
11. Add cleared lysate to this equilibrated matrix and mix gently by shaking at 4 °C for 1 h.
12. Load 10 ml lysate-Ni-NTA mixture into a column.
13. Remove bottom cap and collect the flow-through. Save and store for Western blotting.
14. Wash 10 times with wash buffer. Collect wash factions for Western blotting.
15. Elute protein 4 times with 1 ml elution buffer.
16. Perform dialysis in storage buffer overnight at 4 °C.
17. Wet the dialysis tube with distilled water and close the tube with a clip near one end.
18. Load the sample into the dialysis tube and clip another end.
19. Immerse the knotted tube in storage buffer in a 500 ml beaker overnight.
20. Measure protein concentration by Qubit Protein Assay and check the purity of protein (Figure 1).

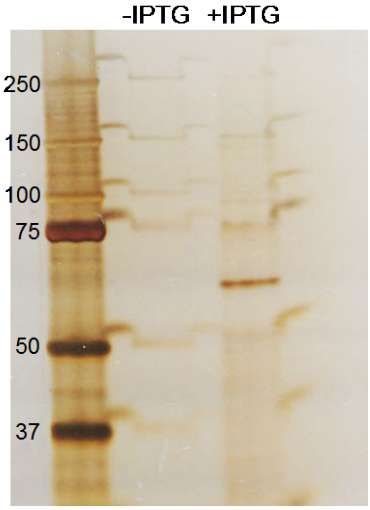

Figure 1. Silver staining of purified PKR protein. The purified PKR protein (65 kDa) was visualized by silver staining. IPTG was added to induce the protein expression in *E. coli*. Lysate from cells without IPTG induction was used as a negative control.

C. PKR dephosphorylation and activation

Human PKR protein is activated by the RNAs from *E. coli* upon cell lysis; therefore, protein dephosphorylation is necessary to obtain the non-phosphorylated form of PKR before activation assays.

1. Dephosphorylation of PKR
   a. Incubate 2 μg of PKR with 400 U λ-PPase (1 μl) in 10 μl phosphatase reaction buffer for 1 h at 30 °C.
   b. Aliquot the dephosphorylated PKR into 10 tubes each containing 0.2 μg PKR protein
   c. Add freshly prepared 2 mM sodium orthovanadate to inhibit the λ-PPase.

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2. *In vitro* PKR activation assay
   a. Prepare 10 µg/ml Poly I:C from stock (as a positive control) (10 mg/ml).
   b. Make the reaction mixture on ice.

0.1-0.2 µg PKR	1 µl
dsRNA/Poly I:C	x µl
Activation buffer	to 20 µl

   c. Incubate dephosphorylated PKR with 10 ng Poly I:C or dsRNA in PKR activation buffer at 30 °C for 0.5-2 h.
   d. Add 1x LDS loading buffer with 5% β-mercaptoethanol to stop the reaction.
   e. Perform Western blotting to determine the PKR activation by probing for level p-PKR against total PKR.
   f. Electrophorese the samples through a NuPAGE 4-12% Bis-Tris protein gel.
   g. Transfer the resolved proteins onto an immobilon-P polyvinylidene difluoride (PVDF) membrane at 100 V for 90 min at 4 °C.
   h. Block the membrane with 1% bovine serum albumin in 0.5% Tween-20 phosphate buffered saline (PBST) for 60 min.
   i. Incubate the membrane with primary antibody overnight at 4 °C.
   j. Wash the membrane three times with PBST for 5 min each.
   k. Add horseradish peroxidase-conjugated secondary antibody at a dilution of 1:3,000 for 1 h at room temperature.
   l. Wash the membrane three times with PBST for 5 min each.
   m. Add enhanced chemiluminescence detection system (ECL reagents).
   n. Scan the membrane with a Typhoon scanner (Figure 2).

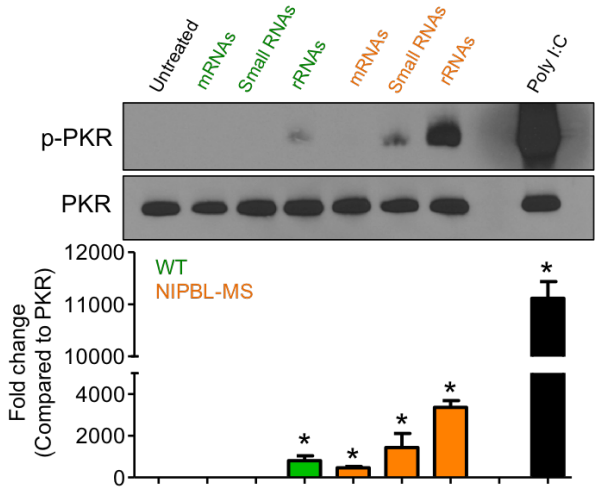

Figure 2. Representative results of PKR activation *in vitro*. PKR activation assay was used to study different types of RNAs (mRNAs, small RNAs and rRNAs) isolated from WT and *NIPBL*-MS (missense) human lymphoblastoid cell lines (LCLs). Both ncRNAs and rRNAs isolated from *NIPBL*-MS LCLs are capable of activating recombinant PKR *in vitro*; 10 ng Poly I:C was used

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as a positive control for PKR activation. *P < 0.001 compared to untreated control. (Please refer to Yuen *et al.*, 2016 for details)

Data analysis

All experiments were repeated independently at least in triplicate, and the data are presented as mean ± SD. Statistical significance was determined using the Student's *t*-test. A P value of < 0.05 was considered to be statistically significant. The experiments described in this protocol has been performed and their data have been published in Yuen *et al.*, 2016, NIPBL controls RNA biogenesis to prevent activation of the stress kinase PKR. *Cell Rep* 14(1): 93-102, which can be accessed by: http://www.cell.com/cell-reports/fulltext/S2211-1247(15)01425-4.

Recipes

1. *E. coli* lysis buffer (1 L)
   50 mM NaH_2PO_4
   300 mM NaCl
   10 mM imidazole
   1 mM PMSF
   10 mM protease inhibitor
   Adjust pH to 8.0 using NaOH
2. Protein purification wash buffer (1 L)
   50 mM NaH_2PO_4
   300 mM NaCl
   20 mM imidazole
   1 mM PMSF
   10 mM protease inhibitor
   Adjust pH to 8.0 using NaOH
3. Protein purification elution buffer (1 L)
   50 mM NaH_2PO_4
   300 mM NaCl
   250 mM imidazole
   1 mM PMSF
   10 mM protease inhibitor
   Adjust pH to 8.0 using NaOH
4. Protein storage buffer
   10 mM Tris (pH 7.6)
   50 mM KCl
   2 mM $MgCl_2$

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10% glycerol
7 mM β-mercaptoethanol

5. Phosphatase reaction buffer
   50 mM Tris-HCl, pH 7.5
   2 mM $MgCl_2$
   0.1 mM EDTA
   5 mM DTT
6. PKR activation buffer
   20 mM HEPES (pH 7.5)
   4 mM $MgCl_2$
   100 mM KCl
   1 mM ATP
7. TE buffer (10x)
   100 mM Tris-Cl
   10 mM EDTA (pH 8.0)
8. Tween-20 phosphate buffered saline (pH 7.2) (1 L)
   8 g of NaCl
   0.2 g of KCl
   1.44 g of Na_2HPO_4
   0.24 g of KH_2PO_4
   2 ml of Tween-20

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