

**Phospho-Epidermal Growth Factor Receptor
(pTyr¹⁰⁶⁸) ELISA, Human**Product Number **CS0150**
Storage Temperature 2-8 °C**Product Information****TECHNICAL BULLETIN****Product Description**

Phospho-Epidermal Growth Factor Receptor (pTyr¹⁰⁶⁸) ELISA, Human is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for quantitative determination of Epidermal Growth Factor Receptor (EGFR) phosphorylated at tyrosine 1068 in cell lysates. A monoclonal antibody specific for full length EGFR (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate provided. EGFR (pTyr¹⁰⁶⁸) standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the EGFR antigen binds to the immobilized (capture) antibody. After incubation, the excess reagents are washed away and an antibody, specific for EGFR phosphorylated at tyrosine 1068 is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized EGFR. After removal of excess detection antibody, a horseradish peroxidase-labeled anti-rabbit IgG (Anti-Rabbit IgG-HRP) is added. It binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove all the excess Anti-Rabbit IgG-HRP, substrate solution is added, which is acted upon by the bound enzyme to produce yellow color. The intensity of this colored product is directly proportional to the concentration of EGFR (pTyr¹⁰⁶⁸) present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of EGFR (pTyr¹⁰⁶⁸). Sigma Phospho-EGFR (pTyr¹⁰⁶⁸) ELISA is designed to detect and quantify the levels of human EGFR phosphorylated at tyrosine 1068 in cell lysates. It does not detect non-phosphorylated EGFR protein or the EGFR phosphorylated at other tyrosine residues such as pTyr⁸⁴⁵, pTyr¹⁰⁸⁶ and pTyr¹¹⁷³. The researcher may normalize his results using a Sigma ELISA kit for human full length EGFR (Prod. No. CS0080), that detects EGFR independent of phosphorylation status.

The epidermal growth factor receptor (EGFR belongs to the family of receptor tyrosine kinases (RTKs), which

regulate cell growth, survival, proliferation, and differentiation. EGFR, also known as ErbB1, is most related to the other members of the EGFR family of RTK's including HER2/ErbB2/neu, HER3/ErbB3, and HER4/ErbB4. EGFR at full length is a 170 kDa type I transmembrane glycoprotein that consists of an extracellular ligand-binding domain, a single hydrophobic transmembrane region, and an intracellular segment harboring the highly conserved, tyrosine kinase domain. Several deletions in the extra- and intracellular domain of the EGFR have been found in a number of tumors. For example, EGFR VIII is a 145 kDa protein with a deletion of exons 2-7 in EGFR mRNA. A 110 kDa truncated EGFR without cytoplasmic domain is observed in the culture supernatant from A431 cells, a human epidermoid carcinoma cell line. EGFR is activated by the binding of ligands such as EGF, transforming growth factor α (TGF α), amphiregulin, β -cellulin, heparin binding EGF-like growth factor (HB-EGF) and epiregulin. The binding causes EGFR homo- and heterodimerization and rapid activation of its intrinsic tyrosine kinase followed by autophosphorylation of multiple tyrosine residues in the cytoplasmic domain. The phosphorylation of tyrosine residues in the COOH-terminal tail of the molecule serves as binding sites for cytosolic signaling proteins containing Src homology 2 (SH2) domains. Several sites of *in vivo* phosphorylation have been identified in the EGFR including pTyr⁸⁴⁵, pTyr⁹⁹², pTyr¹⁰⁶⁸, pTyr¹⁰⁸⁶, and pTyr¹¹⁷³. These sites bind and activate a variety of downstream signaling proteins which contain SH2 domains, including growth factor receptor-binding protein 2 (Grb2), Src homology and collagen domain protein (Shc) and phospholipase C- γ (PLC- γ). Binding of these or other signaling proteins to the receptor and/or their phosphorylation results in transmission of subsequent signaling events that culminate in DNA synthesis and cell division. Elevated expression and/or amplification of the EGFR have been found in breast, bladder, glioma, colon, lung, squamous cell, head and neck, ovarian, and pancreatic cancers. Selective compounds have been developed that target either the extracellular ligand-binding domain of EGFR or the

intracellular tyrosine kinase region, resulting in interference with the signaling pathways that modulate mitogenic and other cancer promoting responses. These potential anticancer agents include a number of monoclonal antibodies, EGFR, immunotoxin, and small molecule tyrosine kinase inhibitors.

Reagents

- **Phospho-Epidermal Growth Factor Receptor (pTyr¹⁰⁶⁸) Standard, Human, 2 vials, Product No. P 6995** – Lyophilized from full length EGFR extract of A431, autophosphorylated. Refer to vial label for quantity and reconstitution volume.
- **Standard Diluent Buffer, 25 mL, Product No. S 3943** – Contains BSA and sodium azide.
- **Monoclonal Anti-Epidermal Growth Factor Receptor Antibody-coated 96-well plate, 1 plate Product No. E 2280** – A plate using break-apart strips coated with monoclonal antibody specific for full length EGFR (regardless of phosphorylation state).
- **Anti-Phospho Epidermal Growth Factor Receptor (pTyr¹⁰⁶⁸) 11 mL, Product No. P 6745** – A detection antibody, specific for EGFR phosphorylated on tyrosine 1068. Ready to use.
- **Anti-Rabbit IgG-HRP Concentrate, 100X, Product No. R 0153** – contains 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No. H 8912** – contains thymol and BSA. Ready to use.
- **Wash Buffer Concentrate 25X, 100 mL, Product No. W 2639** – *See Reagent Preparation for handling, dilution and storage instructions.*
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** – Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P4870**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings, 450 nm.
- Calibrated adjustable precision pipettes for volumes between 5 µl and 1,000 µl
- Cell extraction buffer (see recommended extraction procedure)
- Deionized or distilled water.

- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Graph paper: linear, log-log, or semi-log, as desired
- Glass or plastic 1.0-1.5 ml tubes for diluting and aliquoting standard
- Absorbent paper towels to blot the plate
- Calibrated beakers and graduated cylinders in various sizes
- Vortex mixer.

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples of choice – extracts of cell lysates
- Samples should be frozen if not analyzed shortly after collection.
- Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.

Cell Extraction Buffer

- 10 mM Tris, pH 7.4
- 100 mM NaCl
- 1 mM EDTA
- 1 mM EGTA
- 1 mM NaF
- 20 mM Na₄P₂O₇
- 2 mM Na₃VO₄
- 1% Triton X-100
- 10% Glycerol
- 0.1% SDS
- 0.5% Deoxycholate
- 1 mM PMSF (stock is 0.3 M in DMSO)

PMSF is very unstable and must be added prior to use, even if added previously.

Protease inhibitor cocktail (Product. No. P 2714). Add 250 µL of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 2-8 °C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at -20 °C.

Thaw on ice. Add the protease inhibitors just before use.

Procedure for Extraction of Proteins from Cells

The recommended Cell Extraction Buffer and procedure are optimized to achieve effective protein phosphorylation. Researchers may use the procedures that work best in their hands. They will have to assay their lysates for the satisfactory extraction and/or phosphorylation.

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at -70°C and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of EGFR (pTyr¹⁰⁶⁸). For example, 10^8 A431 cells grown in RPMI plus 10% FBS and treated with 20 ng/mL of EGF can be extracted in 1 mL of Extraction Buffer.
5. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 2-8 $^{\circ}\text{C}$.
6. Aliquot the clear lysate to clean microcentrifuge tubes.

Before assay:

Extracted cell lysate samples containing EGFR (pTyr¹⁰⁶⁸) protein should be diluted with Standard Diluent Buffer at least 1:10. This dilution is necessary to reduce the matrix effect of the cell lysate buffer. Example: 0.1-1 μl of the clarified cell extract diluted to a volume of 100 μl /well in Standard Diluent Buffer is sufficient for the detection of EGFR (pTyr¹⁰⁶⁸).

Reagent Preparation

Phospho-EGFR (pTyr¹⁰⁶⁸) Standard

The standard is full length EGFR purified from A431 cells that was allowed to autophosphorylate in the presence of 2 mM ATP and 1X Autophosphorylation Buffer (15 mM HEPES, 6 mM MnCl_2 , 15 mM MgCl_2).

1. Reconstitute one vial of Standard with Standard Diluent Buffer according to label directions.
2. Mix gently and wait 10 minutes to ensure complete reconstitution.
3. **Label as 100 Units/mL EGFR (pTyr¹⁰⁶⁸)**
4. Prepare serial standard dilutions as follows:

Tube #	Standard Diluent Buffer	Standard from tube #:	Final EGFR (pTyr ¹⁰⁶⁸) concentration Units/mL
1	Reconstitute according to label instructions		100 U/mL
2	0.25 mL	0.25 mL (1)	50
3	0.25 mL	0.25 mL (2)	25
4	0.25 mL	0.25 mL (3)	12.5
5	0.25 mL	0.25 mL (4)	6.25
6	0.25 mL	0.25 mL (5)	3.12
7	0.25 mL	0.25 mL (6)	1.6
8	0.5 mL	-	0

Mix thoroughly between steps.

5. Use within 1 hour of reconstitution

1 unit of standard is equivalent to the amount of EGFR (pTyr¹⁰⁶⁸) derived from 42 pg of EGFR allowed to autophosphorylate. Subsequent lots of standard will be normalized to this lot of material to allow consistency of EGFR (pTyr¹⁰⁶⁸) quantitation

Anti-Rabbit IgG-HRP Concentrate (100X), contains 50% glycerol and is very viscous. To ensure accurate dilution follow instructions below:

1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Mix: 10 μl IgG-HRP concentrate +1 ml HRP Diluent (sufficient for one 8-well strip, prepare more as needed)
4. **Label as Anti-Rabbit IgG-HRP Working Solution.**
5. Return the unused Anti-IgG-HRP concentrate to the refrigerator.

Wash Buffer

1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25X + 24 volumes of deionized water
3. **Label as Working Wash Buffer.**
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability

All components of this kit are stable at 2 to 8 $^{\circ}\text{C}$. Any unused reconstituted standard should be discarded or frozen at 70°C . Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit expiration date. To obtain C of A, go to www.sigma-aldrich.com

Procedure




Precautions

- 20-30 minutes before use equilibrate kit and all reagents to room temperature.
- Use only the coated 96 well capture plate provided with the kit.
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2-8 °C to maintain plate integrity.
- When not in use, all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4th wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.

Assay Procedure

- 1) Incubate 100 µL of Standards and Samples (diluted >1:10) for 2 hours at RT. (Optional: Incubate overnight at 4°C)
 -  aspirate and wash 4x
- 2) Incubate 100 µL of Detection Antibody for 2 hours at RT.
 -  aspirate and wash 4x
- 3) Incubate 100 µL of Anti-Rabbit IgG-HRP for 2 hours at RT.
 -  aspirate and wash 5x
- 4) Incubate 100 µL of stabilized Chromogen for 30 minutes at RT (*in the dark*).
- 5) Add 100 µL of Stop Solution and read at 450nm.

Total Time 4 hours

EGFR (pTyr¹⁰⁶⁸) ELISA Assay Summary

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilutions wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch and reseal.

1st incubation

- a Add 100 µL Standard Diluent to zero wells.
- b Add 100 µL standards, samples or controls to the appropriate wells. *Samples in Cell Extraction Buffer must be diluted at least 1:10 (1:25 or 1:50 were found to be optimal) in Standard Diluent Buffer. The dilutions should be optimized for each assay.*
- c Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature. *Alternatively, plate may be incubated overnight at 2-8 °C.*
- d Wash wells 4 times following washing instructions.
- e After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer

2nd incubation

- a Add 100 µL Anti-Human EGFR (pTyr¹⁰⁶⁸) detection antibody to all wells (except chromogen blanks).
- b Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.

- c Wash wells for a total of 4 times following washing instructions.
- d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3rd incubation

- a Add 100 μ L Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
- b Cover with Plate Cover and incubate 30 minutes at room temperature.
- c Wash wells for a total of 4 times following washing instructions.
- d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation

- a Add 100 μ L of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
- b Do not cover the plate
- c Incubate approximately 30 minutes at room temperature in the dark (place plate in a drawer or cabinet).
- d Note: If your multiwell plate reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.

Stop reaction

- a Add 100 μ L of Stop Solution to each well. This stops the reaction
- b Tap gently to mix. *The solution will turn yellow.*

Absorbance reading

- a Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
- b Blank the plate reader against the Chromogen Blank wells (contain Chromogen and Stop Solution).
- c Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution.

Results

The results may be calculated using any immunoassay software package. The four-parameter algorithm provides the best curve fit. If the software program is not readily available, the concentrations of EGFR (pTyr¹⁰⁶⁸) may be calculated as follows:

1. Calculate the Average Net OD (nm) for each standard dilution and samples as follows:
Average Net OD (nm) =
Average Bound OD (nm) – Average Chromogen Blank OD (nm)

2. On graph paper plot the Average Net OD (nm) of standard dilution (nm) against the concentration (Units/mL) of EGFR (pTyr¹⁰⁶⁸) for the standards. Draw the best curve through these points to construct the standard curve.
3. The EGFR (pTyr¹⁰⁶⁸) concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
4. Multiply the values obtained for the samples by dilution factor of each sample.
5. Samples producing signals higher than the 100 units/mL standard should be further diluted and assayed again.

Product Profile

Typical Results

The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

Phospho-EGFR (pTyr ¹⁰⁶⁸) Standard (U/mL)	Optical Density 450 nm
0	0.208
1.6	0.322
3.12	0.394
6.25	0.526
12.5	0.758
25	1.171
50	1.939
100	3.160

Limitations:

- Do not extrapolate the standard curve beyond the 10 ng/mL standard point.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- Although EGFR (pTyr¹⁰⁶⁸) degradation in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

Performance Characteristics

Specificity

This Phospho-EGFR (pTyr¹⁰⁶⁸) ELISA is specific for measurement of human EGFR protein phosphorylated on tyrosine 1068. As seen in Figure 1 this phospho-ELISA does not detect non-phosphorylated EGFR.

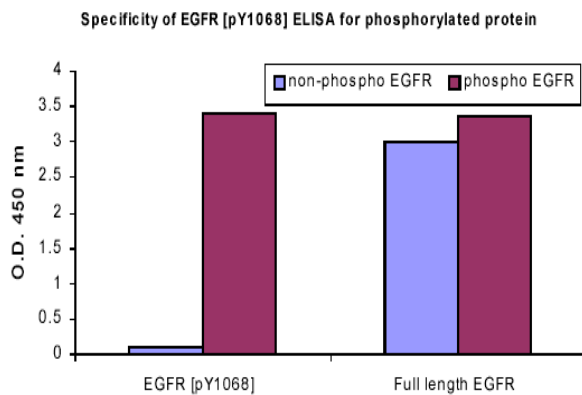


Figure 1 Specificity of Phospho-EGFR (pTyr¹⁰⁶⁸) ELISA for phosphorylated EGFR

The specificity of this assay for EGFR phosphorylated at tyrosine 1068 was confirmed by peptide competition. Phosphorylated EGFR was serially diluted and quantitated in the assay as usual except that the detection antibody was preincubated with EGFR-derived peptides at a concentration of 1 $\mu\text{g/mL}$. The data presented in Figure 2 show that only the peptide corresponding to the region surrounding tyrosine 1068, containing the phospho-tyrosine, could block the ELISA signal. The same sequence containing non-phosphorylated tyrosine at position 1068 did not block the signal.

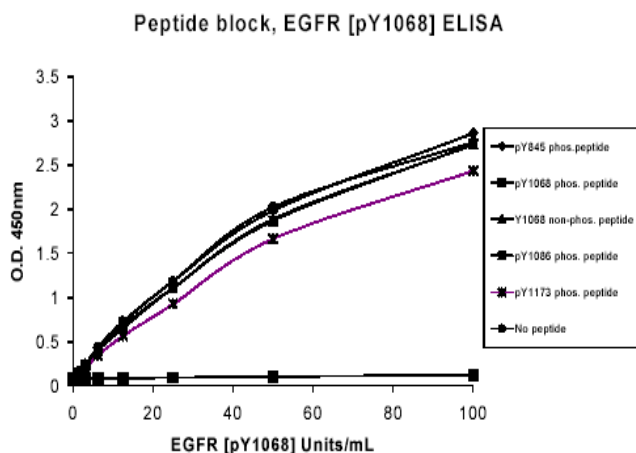


Figure 2 Peptide Blocking

Sensitivity

Sensitivity of this assay is <0.3 units/mL of human EGFR (pTyr¹⁰⁶⁸).

Sensitivity was calculated by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. The sensitivity of this ELISA was compared to immunoblotting using known quantities of EGFR (pTyr¹⁰⁶⁸). The results show that ELISA is approximately 8 times more sensitive in detecting EGFR (pTyr¹⁰⁶⁸) than immunoblotting. The bands shown in the immunoblot were developed using rabbit anti-EGFR (pTyr¹⁰⁶⁸); an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

Precision

1. Intra-Assay Precision

Samples of known EGFR (pTyr¹⁰⁶⁸) concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	39.9	12.10	2.58
Standard Deviation (SD)	1.40	0.54	0.20
% Coefficient of Variation	3.51	4.49	7.58

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	40.55	11.95	2.49
Standard Deviation (SD)	1.74	0.68	0.23
% Coefficient of Variation	4.30	5.68	7.58

Sample Recovery

The recovery of EGFR (pTyr¹⁰⁶⁸) added to Cell Extract Buffer followed by 1:10 dilution in Standard Diluent Buffer, averaged 103%.

Parallelism

Natural EGFR (pTyr¹⁰⁶⁸) from EGF treated-A431 cell lysate was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the EGFR (pTyr¹⁰⁶⁸) standard curve. Parallelism was demonstrated by the figure 3 below and indicated that the Standard accurately reflects EGFR (pTyr¹⁰⁶⁸) content in samples.

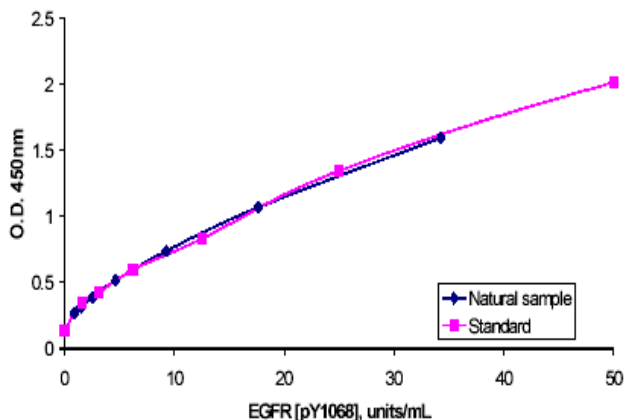


Fig. 3 Phospho-EGFR (pTyr¹⁰⁶⁸) ELISA: Parallelism

Linearity of Dilution

Extract Buffer was spiked with EGFR (pTyr¹⁰⁶⁸) and serially diluted in *Standard Diluent Buffer* over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99.

Cell Lysate			
Dilution	Measured (Units/mL)	Expected (Units/mL)	% Expected
Neat	61.68	-	-
1:2	31.11	30.58	101%
1:4	15.16	15.4	98
1:8	8.06	7.7	104%
1:16	3.92	3.9	102

References

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