Cytotoxicity LDH Assay Kit-WST

Supporting manual for antibody-dependent cell-mediated cytotoxicity assay

Notice to Users

This is a supporting manual for antibody-dependent cell-mediated cytotoxicity (ADCC) assay.
For the contents of this kit and the preparation procedure of Working Solution,
please see the technical manual originally attached to the Cytotoxicity LDH Assay Kit-WST.

For Accurate Measurement

LDH activity can be different depending on the cell type. Therefore, when cell type is changed, please confirm optimum cell number of target cells before ADCC assay.

Optimization of cell number
↓
ADCC assay

Selection of the Assay

There are two methods: homogeneous assay (Page 1) and non-homogeneous assay (Page 4), please select the assay method according to your experiments.

Homogeneous Assay

Optimization of Cell Number

1. After washing target cells with the medium, prepare cell suspension to 5 x 10^5 cells/ml in the medium.
2. Add 100 µl of the medium to each well of 96-well plates.
3. In the Row A of 96-well plate (triplicates of TMR(High Control) and TSR (Low Control)) add 100 µl of target cells prepared in Step 1. and mix by pipetting (2.5 x 10^4 cells/well). Next, take 100 µl from the Row A and add it to the Row B. Repeat the serial dilution process as indicated in Figure 1.

TMR (Target Maximum Release, High Control)
Lysis Buffer is added to the target cells – LDH released from all of the cells.

TSR (Target Spontaneous Release, Low Control)
Only medium is added to the target cells – Spontaneous LDH released from the cells.

CMB (Culture Medium Background, Background Control)
LDH activity contained in the medium

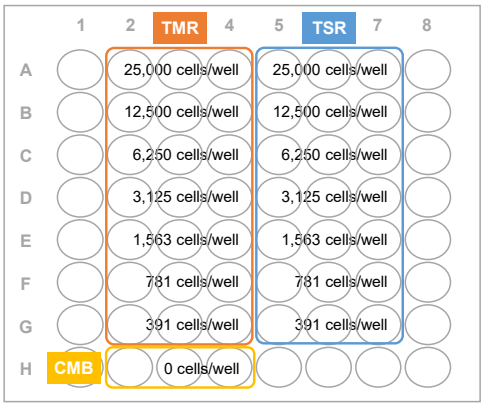


Figure 1 Plate arrangement

4. Incubate the plate at 37°C in CO_2 incubator.
   *Use the same incubation time as the ADCC assay.
5. Add 10 µl of Lysis Buffer into High Control (TMR) wells.
6. Incubate the plate at 37°C for 30 minutes in CO_2 incubator.
7. Add 100 µl of Working Solution to all of the wells. Protect the plate from light and incubate it at the room temperature for 30 minutes.
   *Duration of the color reaction should be optimized based on the target cell types.
8. Add 50 µl of Stop Solution to all of the wells.
9. Measure the absorbance at 490 nm by a microplate reader.
10. Set target cell number per well which satisfies either of below conditions

A) The difference of TMR and TSR absorbance
$(OD^{TMR} - OD^{TSR}) \geqq 2 \times OD^{CMB}$

B) Absorbance difference between TMR and TSR at the maximum

C) The absorbance of TMR (OD^{TMR}) at above 1.0 below 3.0

Preparation of Antibody Solution

Prepare 10 different concentration of antibody solution including zero concentration of antibody solution. Antibody Solution should be diluted using the medium.

ADCC Assay

Please refer to the plate arrangement (Figure 2) and the amount of solution in each well (Table 1)

Definition of the Wells

ER (Experimental Release): LDH released when all antibody solution, effector cells and target cells are mixed

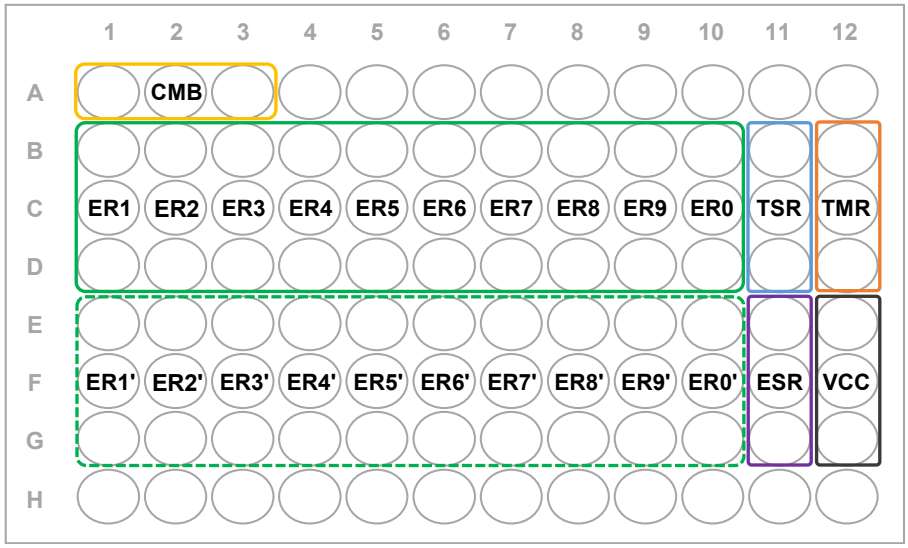
ESR (Effector Spontaneous Release): spontaneous release of LDH by effector cells

TSR (Target Spontaneous Release): spontaneous release of LDH by target cells

TMR (Target Maximum Release): LDH released from the cells after the addition of Lysis buffer to the target cells

CMB (Culture Medium Background): LDH contained in the medium

VCC (Volume Correction Control): LDH from addition of Lysis Buffer in the medium, used for volume correction



ER' wells can be used for different type of test substance.
It is recommended to add medium in the Row A and H including CMB wells.

Figure 2 Plate arrangement

Table 1 Amount of solution in each well (Homogeneous Assay)

	ER	ESR	TSR	TMR	CMB	VCC
Test Substance	25 µl	-	-	-	-	-
Medium	-	50 µl	75 µl	75 µl	100 µl	100 µl
Effector Cell Solution	50 µl	50 µl	-	-	-	-
Target Cell Solution	25 µl	-	25 µl	25 µl	-	-
Lysis Buffer	-	-	-	10 µl	-	10 µl

(Homogeneous assay)

General Protocol

1. Add each different concentration antibody solution into ER wells.
2. Add Target Cell solution into ER, TMR, and TSR wells.
3. Add Effector Cell solution into ER and ESR wells.
4. Add Medium into ESR, TSR, TMR, CMB, and VCC wells.
5. Centrifuge at 250 x *g* for 4 minutes.
6. Incubate 37°C in CO_2 incubator for an appropriate time.
7. Add Lysis buffer to TMR and VCC wells.
8. Incubate at 37°C in CO_2 incubator for 30 minutes.
9. Add 100 µl of working solution to all of the well.
10. Protect the plate from light, and incubate it at room temperature for 30 minutes.
11. Add 50 µl of stop solution to all of the wells.
12. Measure the absorbance at 490 nm.

Calculation of Cytotxicity

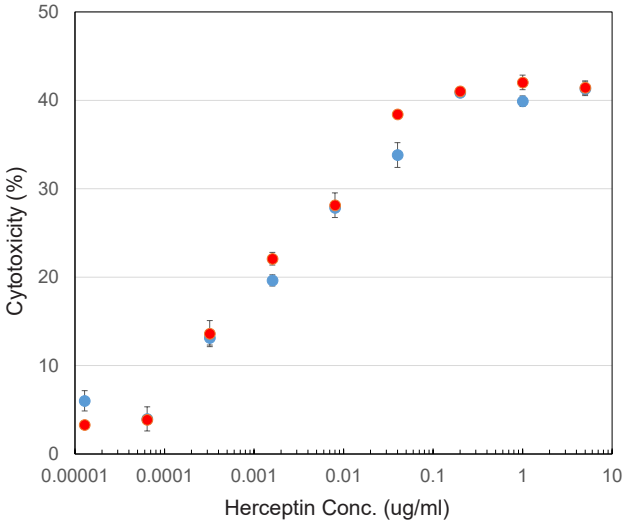
1. Subtract absorbance of CMB from absorbance of ER, ESR, TSR wells.
2. Subtract absorbance of VCC from the absorbance of TMR wells.
3. Plot the data into the following equation to obtain the cytotoxicity (%).

$$\text{Cytotoxicity}(\%) = \frac{\boldsymbol{ER} - \boldsymbol{ESR} - \boldsymbol{TSR}}{\boldsymbol{TMR} - \boldsymbol{TSR}} \times 100$$

$\boldsymbol{ER}$: $OD^{ER} - OD^{CMB}$
$\boldsymbol{ESR}$: $OD^{ESR} - OD^{CMB}$
$\boldsymbol{TSR}$: $OD^{TSR} - OD^{CMB}$
$\boldsymbol{TMR}$: $OD^{TMR} - OD^{VCC}$

Experimental Example

ADCC Assay using monolonal antibody Herceptin
Homogenous Assay



Measured two times with the same condition(Red, Blue)

Test Substance:	Herceptin
Target Cell (T Cell):	SK-BR-3 ($1x10^4$ cells/well)
Effector Cell (E Cell):	PBMC ($1x10^5$ cells/well)
Medium:	RPMI1640 (2% FBS, 1% Antibiotic-antimicotic)
Ratio of E Cell and T Cell:	10:1

Optimization of Cell Number

1. After washing target cells with the medium, prepare cell suspension to 5 x 10^5 cells/ml in the medium.
2. Add 100 µl of the medium to each well of 96-well plates.
3. In the Row A of 96-well plate (triplicates of TMR(High Control) and TSR (Low Control)) add 100 µl of target cells medium prepared in Step 1. and mix by pipetting (2.5 x 10^4 cells/well).
   Next, take 100 µl from the Row A and add it to the Row B. Repeat the serial dilution process as indicated in Figure 3.

TMR (Target Maximum Release, High Control)
Lysis Buffer is added to the target cells – LDH released from all of the cells.

TSR (Target Spontaneous Release, Low Control)
Only medium is added to the target cells – Spontaneous LDH released from the cells.

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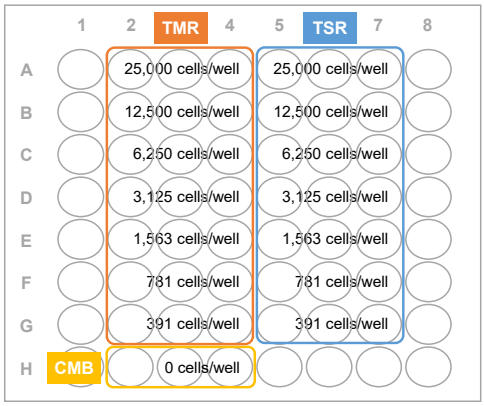

Figure 3 Plate arrangement

4. Add 100 µl medium to all of the wells.
5. Incubate the plate at 37°C in CO_2 incubator
   *Use the same incubation time as the ADCC assay.
6. Add 20 µl of Lysis Buffer into High Control (TMR) wells.
7. Incubate the plate at 37°C for 30 minutes in CO_2 incubator
8. Centrifuge at 250 x *g* for 4 minutes.
9. Remove 100 µl of supernatant from all the wells, and add it to a new 96 well plates for measurement
10. Add 100 µl of Working Solution to all of the wells. Protect the plate from light and incubate it at the room temperature for 30 minutes.
    *Duration of the color reaction should be optimized based on the target cell types.
11. Add 50 µl of Stop Solution to all of the wells.
12. Measure the absorbance at 490 nm by a microplate reader.
13. Set target cell number per well which satisfies either of below conditions

A) The difference of TMR and TSR absorbance

$$(OD^{TMR} - OD^{TSR}) \geqq 2 \times OD^{CMB}$$

B) Absorbance difference between TMR and TSR at the maximum

C) The absorbance of TMR (OD^{TMR}) at above 1.0 below 3.0

Preparation of Antibody Solution

Prepare 10 different concentration of antibody solution including zero concentration of antibody solution. Antibody Solution should be diluted using the medium.

ADCC Assay

Please refer to the plate arrangement (Figure 3) and the amount of solution in each well (Table 2)

Definition of the Wells

ER (Experimental Release): LDH released when all antibody solution, effector cells and target cells are mixed

ESR (Effector Spontaneous Release): spontaneous release of LDH by effector cells

TSR (Target Spontaneous Release): spontaneous release of LDH by target cells

TMR (Target Maximum Release): LDH released from the cells after the addition of Lysis buffer to the target cells

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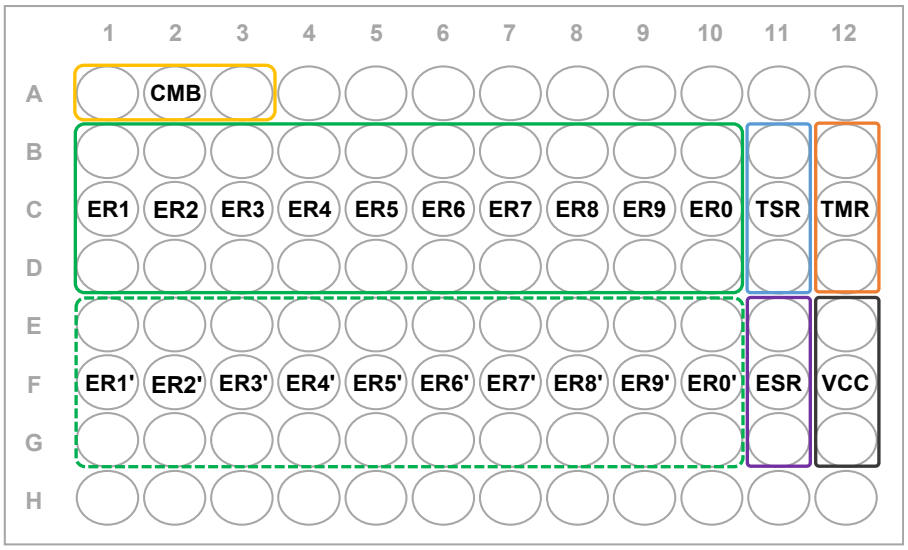


ER' wells can be used for different type of test substance.
It is recommended to add medium in the Row A and H including CMB wells.

Figure 4 Plate arrangement

Table 2 Amount of solution in each well (Non-homogeneous Assay)

	ER	ESR	TSR	TMR	CMB	VCC
Test Substance	50 µl	-	-	-	-	-
Medium	-	100 µl	150 µl	150 µl	200 µl	200 µl
Effector Cell Solution	100 µl	100 µl	-	-	-	-
Target Cell Solution	50 µl	-	50 µl	50 µl	-	-
Lysis Buffer	-	-	-	20 µl	-	20 µl

General Protocol

1. Add each different concentration antibody solution into ER wells.
2. Add Target Cell solution into ER, TMR, and TSR wells.
3. Add Effector Cell solution into ER and ESR wells.
4. Add Medium into ESR, TSR, TMR, CMB, and VCC wells.
5. Centrifuge at 250 x *g* for 4 minutes.
6. Incubate at 37°C in CO_2 incubator for an appropriate time.
7. Add Lysis buffer to TMR and VCC wells.
8. Incubate at 37°C in CO_2 incubator for 30 minutes.
9. Centrifuge at 250 x *g* for 4 minutes.
10. Remove 100 µl from each well and add it to a new 96-well plate.
11. Add 100 µl of working solution to all of the well.
12. Protect the plate from light, and incubate it at room temperature for 30 minutes.
13. Add 50 µl of stop solution to all of the wells.
14. Measure the absorbance at 490 nm.

(Non-Homogeneous Assay)

Calculation of Cytotoxicity

1. Subtract absorbance of CMB from absorbance of ER, ESR, TSR wells.
2. Subtract absorbance of VCC from the absorbance of TMR wells.
3. Plot the data into the following equation to obtain the cytotoxicity (%).

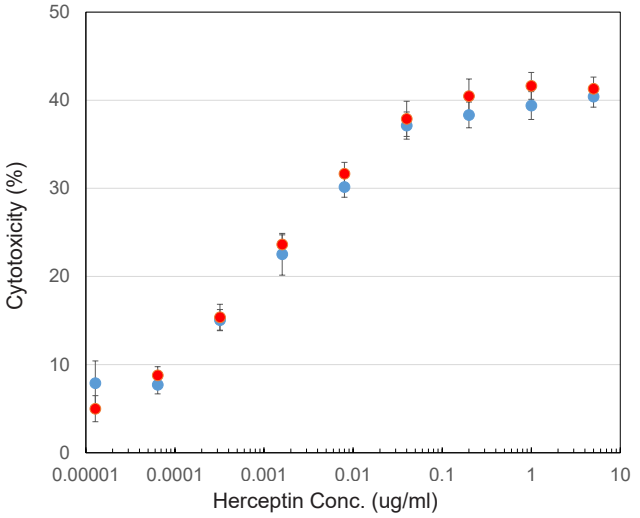
$$\text{Cytotoxicity (\%)} = \frac{ER - ESR - TSR}{TMR - TSR} \times 100$$

ER: $OD^{ER} - OD^{CMB}$
ESR: $OD^{ESR} - OD^{CMB}$
TSR: $OD^{TSR} - OD^{CMB}$
TMR: $OD^{TMR} - OD^{VCC}$

Experimental Example

ADCC Assay using monolonal antibody Herceptin

Non-Homogeneous Assay



* Measured two times with the same condition(Red, Blue)

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Effector Cell (E Cell):	PBMC ($1x10^5$ cells/well)
Medium:	RPMI1640 (2% FBS, 1% Antibiotic-antimicotic)
Ratio of E Cell and T Cell:	10:1

If you need more information, please contact Dojindo technical service.

Dojindo Molecular Technologies, Inc.
30 West Gude Dr., Suite 260, Rockville, MD 20850, USA
Toll free: 1-877-987-2667 Phone: 301-987-2667 Fax: 301-987-2687
E-mail: info@dojindo.com Web: www.dojindo.com